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Full paper

Ribonucleotide reductase is an effective target to overcome gemcitabine resistance in gemcitabine-resistant pancreatic cancer cells with dual resistant factors

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ABSTRACT

Gemcitabine is widely used for pancreatic, lung, and bladder cancer. However, drug resistance against gemcitabine is a large obstacle to effective chemotherapy. Nucleoside transporters, nucleoside and nucleotide metabolic enzymes, and efflux transporters have been reported to be involved in gemcitabine resistance. Although most of the resistant factors are supposed to be related to each other, it is unclear how one factor can affect the other one. In this study, we established gemcitabine-resistant pancreatic cancer cell lines. Gemcitabine resistance in these cells is caused by two major processes: a decrease in gemcitabine uptake and overexpression of ribonucleotide reductase large subunit (RRM1). Knockdown of RRM1, but not the overexpression of concentrative nucleoside transporter 1 (CNT1), could completely overcome the gemcitabine resistance. RRM1 knockdown in gemcitabine-resistant cells could increase the intracellular accumulation of gemcitabine by increasing the nucleoside transporter expression. Furthermore, a synergistic effect was observed between hydroxyurea, a ribonucleotide reductase (RR) inhibitor, and gemcitabine on the gemcitabine-resistant cells. Here we indicate that RR is one of the most promising targets to overcome gemcitabine resistance in gemcitabine-resistant cells with dual resistant factors.

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1. Introduction

Pancreatic cancer is one of the most difficult malignancies to treat successfully. Only 15%–20% of patients are eligible for a potentially curative resection at the diagnosis. Even if surgical resection is performed, the recurrence rate is high and the survival rate after surgery is poor (1). Therefore, effective chemotherapy is indispensable to improve the prognosis of patients with pancreatic cancer.

Gemcitabine (2′,2′-difluoro deoxycytidine) is used for the treatment of not only pancreatic cancer but also lung and bladder cancers. However, the occurrence of drug-resistant cells greatly hinders successful cancer therapy. Gemcitabine is a unique antimetabolite in that its metabolites dFdCDP and dFdCTP can inhibit ribonucleotide reductase (RR) by binding to ribonucleotide reductase large subunit (RRM1) and can terminate DNA elongation processes by incorporating into DNA, respectively (2).

Multiple factors, including the attenuation of nucleoside transporters, the expression change of gemcitabine-activating or -degradation enzymes and target molecules, and the expression of efflux transporters, have been reported to cause gemcitabine resistance (3–14).

However, the relationship between each factor is not clear, although several of these factors are thought to influence each other in gemcitabine metabolism. This relationship needs to be elucidated to find a good strategy to overcome gemcitabine resistance. Therefore, we established gemcitabine-resistant cell lines from a pancreatic cancer cell line, MIA PaCa-2, and systematically examined the mechanisms underlying the gemcitabine resistance of the cells.

2. Materials and methods

2.1. Cells

The human pancreatic cancer cell line MIA PaCa-2 was provided by the Riken BioResource Center, Japan. MIA PaCa-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui Seiyaku, Tokyo, Japan), containing 10% fetal calf serum (FCS; PAA Laboratories, Pasching, Austria), at 37 °C in an atmosphere containing 5% CO_2.

MGEM6 cells were transfected with pDNA3.1-hCNT1 using electroporation (Gene Pulser, Bio Rad). The cells were selected with G418 and MGEM6/CNT1#17 cells were used for further analysis. MGEM6 and MGEM6/CNT1#17 cells were transfected with pLKO.1-shRRM1 (Sigma–Aldrich Corp., St. Louis, MO, USA) as described above. The cells were selected by puromycin and MGEM6/KD5-6 and MGEM6/CNT1#17KD5-7 cells were used for further analysis.

2.2. Plasmids

The concentrative nucleoside transporter 1 (CNT1) plasmid pDNA3.1-hCNT1is described elsewhere (15). Mission shRNA expression plasmids against RRM1 mRNA (NM_001033_2471S) were purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA).

2.3. Chemicals, anticancer agents, and antibodies

DMEM was purchased from Nissui Seiyaku (Tokyo, Japan), FCS was purchased from PAA Laboratories (Pasching, Austria), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), G418, puromycin, cytosine arabinoside (Ara-C), 2-chlorodeoxyadenosine (cladribine), *N*-methyl-D-glucamine, nitrobenzylthioinosine (NBTI), dipyridamole, and hydroxyurea (HU) were purchased from Sigma–Aldrich Corp (St. Louis, MO, USA), *p*-amidinophenyl methanesulfonyl fluoride (*p*-APMSF) was purchased from Wako (Osaka, Japan), and [^{3}H] gemcitabine was purchased from Moravek Biochemicals and Radiochemicals (Brea, CA, USA).

Gemcitabine was obtained as a gift from Ely Lilly and Co. Anti-hCNT1 was generated as described previously (16). Other antibodies were purchased from the indicated companies: hENT1 (Abgent, San Diego, CA, USA: AP1086c), hENT2 (Abcam, Cambridge, UK: ab48595), RRM1 (Cell Signaling Technology, Danvers, MA, USA: #3388), RRM2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA: sc10846), p53R2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA: sc10840), GAPDH (Cell Signaling Technology, Danvers, MA, USA) sodium/potassium-transporting ATPase α3 (Na^+/K^+ ATPase α3) (Abcam, MA, USA, XVIF9-G10).

2.4. Cell viability assay

The sensitivity to the anticancer agents was determined by the MTT assay using 96-well plates seeded 5×10^3 cells/well as described previously (17). The IC_{50} was determined as the concentration of the agents that reduced the number of cells to 50% of that of cells cultured in control medium.

2.5. Gemcitabine accumulation under normal and inhibitory conditions with nucleoside transporters

To measure the gemcitabine accumulation, subconfluent cells cultured in 12-well plates were incubated with 1 μM hot (^{3}[H]-labeled) and cold gemcitabine in the medium at 37 °C for 1 h. After removal of the media, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with 0.5 mL ice-cold PBS containing 1% Triton X-100 and 0.2% sodium dodecyl sulfate (SDS). Five milliliters scintillation solution was added to the lysate and the radioactivity was measured with a liquid scintillation counter (18).

For the prewash and the 1-hr incubation with gemcitabine under inhibitory conditions for one or more nucleoside transporter(s), we used 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered Ringer's solution for the control conditions, consisting of 135 mM NaCl, 5 mM KCl, 3.33 mM NaH_2PO_4, 0.83 mM Na_2HPO_4, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM glucose, and 5 mM HEPES (pH 7.4); Na-free buffer for the CNT1 inhibition condition, containing 140 mM *N*-methyl-D-glucamine, 5 mM HEPES, 5 mM KH_2PO_4, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 10 mM glucose (pH 7.4); Na-free buffer containing 100 nM NBTI for the CNT1 and ENT1inhibition condition; and Na-free buffer containing 10 μM dipyridamole for the CNT1, equilibrative nucleoside transporter 1 (ENT1), and ENT2 inhibition condition (19).

To determine the efflux rate of gemcitabine from the cells, subconfluent cells cultured in 12-well plates were incubated with 1 μM hot and cold gemcitabine in DMEM, containing 10% FCS, at 37 °C for 1 h, and were washed with PBS at 37 °C. After adding fresh, warm medium without gemcitabine until each indicated time, the cells were washed and solubilized. The radioactivity of the lysates was determined as described above.

2.6. Cell fractionation and immunoblotting

Cell membrane fractions were isolated as described previously (17). Total cell lysate was isolated from the cells with lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% aprotinin, and 1 mM *p*-APMSF). After lysis, the cell debris was removed by centrifugation at 14,000× *g* for 15 min at 4 °C.

Immunoblotting was performed as described previously (17) and the blotted membranes were developed using the enhanced chemiluminescence immunoblotting detection system (GE healthcare Bio-Sciences, Piscataway, NJ, USA) and were exposed to X-ray film. The chemiluminescence intensities of the bands were