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PKM1 is involved in resistance to anti-cancer drugs

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ABSTRACT

Resistance to chemotherapy is a crucial problem in the clinical situation. To overcome this issue, many mechanisms of chemoresistance have been elucidated so far. However, this problem still has not been solved completely. In this study, we investigated the mechanism of chemoresistance from the view of cancer metabolism-related genes, especially focusing on the expression profile of pyruvate kinase muscle (PKM) isoforms, which are rate-limiting enzymes in cancer-specific metabolism (Warburg effect). Herein, we showed that PKM1, which promotes oxidative phosphorylation (OXPHOS), was commonly up-regulated in various chemoresistant cells. To clarify the functions of PKM1 in chemoresistance, we investigated effects of PKM1 expression in DLD-1 parental, 5-FU-resistant and oxaliplatin-resistant DLD-1 cells. The overexpression of PKM1 resulted in resistance of the parental cells to 5-FU and oxaliplatin. Moreover, gene-silencing of PKM1 induced apoptosis in these cells including the resistant cells by causing a decrease in the mitochondrial membrane potential. Furthermore, combination therapy using 5-FU or oxaliplatin with siR-PKM1 was also effective against the resistant cells. Our findings should lead to the development of new agents that can cancel the chemoresistance from the view of cancer energy metabolism.

1. Introduction

Chemotherapeutics are pivotal for the treatment of advanced cancers. For example, fluorouracil (5-FU) and oxaliplatin are central anti-cancer drugs for adjuvant therapy [1] and for treatment of recurrence and metastasis of solid tumors such as colon cancers [2]. On the other hand, a crucial clinical problem is that cancer cells can adapt to chemotherapeutic stress for survival purposes. Therefore, most clinical cases ultimately result in treatment failure. Many mechanisms of chemoresistance have been reported, such as increased drug efflux, dysregulated expression of apoptosis-related genes, enhanced inactivation of drugs, DNA damage repair, mutation of survival-related genes, and deregulation of growth factor signaling pathways [3]. However, this problem of chemoresistance in clinical medicine still remains to be solved. To overcome this problem, we have established several chemoresistant cell lines and have investigated various mechanisms of chemoresistance in these cells [4–6].

Recently, it was reported that reprogramming of metabolic pathways is one of the important mechanism of chemoresistance [7]. Importantly, tumor cells use glycolysis for energy production even when an adequate amount of oxygen is present. This phenomenon is termed the Warburg effect [8]. It is known that pyruvate kinase muscle (PKM), which is a rate-limiting glycolytic enzyme, regulates this Warburg effect [9]. PKM has 2 isoforms, PKM1 and PKM2. *PKM1* contains exon 9 and lacks exon 10, whereas *PKM2* contains exon 10 and lacks exon 9 [10]. PKM2 is exclusively expressed in embryonic, proliferating, and cancer cells, and promotes the Warburg effect [11]. On the other hand, PKM1 is expressed in several normal differentiated tissues such as brain and

Abbreviations: ADM, Adriamycin; ATP, Adenosine triphosphate; DMSO, Dimethyl sulfoxide; H_2O_2, Hydrogen peroxide; IC_{50}, Half maximal (50%) inhibitory concentration; IFC, Immunofluorescence; miR, microRNA; ORF, Open reading frame; OXPHOS, Oxidative phosphorylation; PKM, Pyruvate kinase muscle; PTBP1, Polypyrimidine tract-binding protein 1; PTX, Paclitaxel; ROS, Reactive oxygen species; siRNA, Short-interfering RNA; 5-FU, 5-fluorouracil.

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muscle, and promotes oxidative phosphorylation (OXPHOS) [9,12]. However, the role of PKM isoforms in chemoresistant cells is largely unknown. Therefore, in this study, we investigated the expression profile of PKM isoforms in chemoresistant cells and found that PKM1 was commonly up-regulated in various chemoresistant cells. Then we showed functions of PKM1 in these cells.

2. Materials and methods

2.1. Cell lines and cell culture

DLD-1 and HT-29 were used as human colon cancer cell lines. MKN-45 and NUGC-3 were used as human gastric cancer cell lines. PC-3 was used as human prostate cancer cell line. K562 was used as human chronic myelogenous leukemia cell line. 5-FU-resistant cells were generated from DLD-1, HT-29, MKN-45, and NUGC-3 cells. Also, oxaliplatin-resistant cells were generated from DLD-1 cells. Paclitaxel (PTX)-resistant cells were generated from PC-3 cells. Adriamycin (ADM)-resistant cells were generated from K562 cells. The detailed information on the characteristics of cells and the culture conditions employed is described in Supplementary data. We referred to these drug-resistant cells as DLD-1/5-FUR, DLD-1/OxR, HT-29/5-FUR, MKN-45/F2R, NUGC-3/5-FUR, PC-3/PTXR, and K562/ADMR in this study.

2.2. Agents

5-FU, PTX, rotenone (OXPHOS complex I inhibitor), and antimycin A_1 (OXPHOS complex III inhibitor) were purchased from Sigma–Aldrich Co. Oxaliplatin was purchased from Wako Pure Chemical Industries, Ltd. Each agent was dissolved in dimethyl sulfoxide (DMSO). The effects of 5-FU and oxaliplatin were assessed at 48 h after the start of treatment. Rotenone and antimycin A_1 were added at 36 h after cell seeding, and their effects were assessed 12 h later.

2.3. Transfection experiments

The detailed protocols are described in Supplementary data. The effects manifested by the introduction of siRNAs into the cells were assessed at 48 h after the transfection. In the combination experiments using siR-PKM1 and 5-FU or oxaliplatin, DLD-1/5-FUR or DLD-1/OxR cells were treated with 5-FU or oxaliplatin, respectively, 24 h after the transfection with siR-PKM1; and the effects were evaluated 48 h later.

2.4. Western blotting

Protein extraction and Western blotting experiments were performed as described in Supplementary data. Primary antibodies used were as follow: anti-PARP and anti-cleaved caspase 9, (Cell Signaling Technology); anti-PKM1 and anti-PKM2 (Novus Biologicals), and anti-β-actin antibody (Sigma–Aldrich Co.). HRP-conjugated goat anti-rabbit and horse anti-mouse IgG (Cell Signaling Technology) were used as secondary antibodies. β-actin was used as an internal control.

2.5. Nuclear and cytoplasmic protein extraction

Nuclear and cytoplasmic proteins were extracted by using a CelLytic™ NuCLEAR™ Extraction Kit (Sigma–Aldrich Co.), according to the manufacturer's protocol. Histone H3 (Cell signaling Technology) was used as a control for nuclear protein; and β-actin, as a control for cytoplasmic protein. In addition, β-actin was also used as an indicator for contamination of the nuclear fraction by cytoplasmic protein.

2.6. Overexpression experiments

The detailed protocols are described in Supplementary data. The primers for ORF-PKM1 were the following: ORF-PKM1-sense, 5′-CCGCGATCGCCATGTCGAAGCCCCATAGTG-3'; and ORF-PKM1-antisense, 5′-CCGTTTAAACTCACGGCACAGGAACAACAC-3'.

2.7. Hoechst33342 staining

The detailed protocols are described in Supplementary data. The number of apoptotic cells among 300 (siR-PKM1) or 500 (combination with siR-PKM1 and chemoagent) cells was counted.

2.8. Mitochondria membrane potential

Cells were stained with Mito Tracker® Orange (Molecular Probes). The detailed protocols are described in Supplementary data.

2.9. ATP assay

ATP production was measured with an ATP Determination Kit (Invitrogen) according to the manufacturer's instructions. ATP production was normalized to cell numbers.

2.10. Immunocytochemistry

Cells were seeded into the wells of a Lab-Tek II Chamber Slide System (Thermo Fisher Scientific Inc.,). After 72 h, the cells were immunostained with anti-PKM1 antibody according to the immunofluorescence protocol of Cell Signaling Technology. The nuclei were stained with Hoechet33342, and for actin labeling the cells were incubated with the fluorescent F-actin probe Rhodamine Phalloidin (Cytoskeleton, Denver, CO). The cells were observed with a BIOREVO fluorescence microscope (Keyence).

2.11. Cell-cycle analysis

The detailed protocols are described in Supplementary data. In the case of steady-state analysis, we assessed the cells at 72 h after the seeding. In the case of transfection, the cells were examined at 48 h after the transfection.

2.12. Statistics

Each examination was performed in triplicate. Statistical significances of differences were evaluated by performing the two-sided Student's *t*-test. The values were presented as the mean ± standard deviation. A P value < 0.05 was considered to be statistically significant.

3. Results

3.1. PKM1 was commonly up-regulated in chemoresistant cells compared with its expression in their parental cells

We examined the expression profiles of PKM isoforms in various chemosensitive cell lines and in their chemoresistant cell lines. Interestingly, PKM1 was commonly up-regulated in all chemoresistant cell lines tested (Fig. 1A). On the other hand, the expression level of PKM2 was almost unchanged in all chemoresistant cell lines tested compared with that in the parental (sensitive) cell lines (Fig. 1A). In all cases, the PKM1 ratio was significantly increased in