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Original Articles

Protection of stromal cell-derived factor 2 by heat shock protein 72 prevents oxaliplatin-induced cell death in oxaliplatin-resistant human gastric cancer cells

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ABSTRACT

Heat shock protein 72 (Hsp72) is a molecular chaperone that assists in the folding of nascent polypeptides and in the refolding of denatured proteins. In many cancers, Hsp72 is constitutively expressed at elevated levels, which can result in enhanced stress tolerance. Similarly, following treatment with anticancer drugs, Hsp72 binds to denatured proteins that may be essential for survival. We therefore hypothesized that Hsp72 client proteins may play a crucial role in drug resistance. Here, we aimed to identify proteins that are critical for oxaliplatin (OXA) resistance by analyzing human gastric cancer cell lines, as well as OXA-resistant cells via a mass spectrometry-based proteomic approach combined with affinity purification using anti-Hsp72 antibodies. Stromal cell-derived factor 2 (SDF-2) was identified as an Hsp72 client protein unique to OCUM-2M/OXA cells. SDF-2 was overexpressed in OXA-resistant cells and SDF-2 silencing promoted the apoptotic effects of OXA. Furthermore, Hsp72 prevented SDF-2 degradation in a chaperone activity-dependent manner. Together, our data demonstrate that Hsp72 protected SDF-2 to avoid OXA-induced cell death. We propose that inhibition of SDF-2 may comprise a novel therapeutic strategy to counteract OXA-resistant cancers.

Introduction

Chemotherapy is a key treatment strategy for patients with advanced and metastatic cancers [1]. However, the development of acquired drug resistance, which is believed to cause treatment failure in over 90% of patients with metastatic cancer, has limited the clinical usefulness of chemotherapeutic drugs [2]. Thus, overcoming drug resistance would substantially improve survival rates and outcomes in patients with cancer. Oxaliplatin (OXA) is a third generation platinum analogue that is one of the first line drugs in gastric cancer treatment. Notably, OXA exhibits a different spectrum of activity than cisplatin (CDDP), the most commonly used platinum drug, which may be due, at least in part, to the slightly distinct form of intrastrand cross-link produced by this compound [3]. However, as seen with other drugs, cancer cells eventually develop OXA resistance. While the mechanisms of CDDP resistance have been well defined [4], the precise mechanisms underlying the development of OXA resistance remain unclear. Given the anti-cancer activity of OXA against CDDP- and carboplatin-resistant cancer cells, however, this mechanism is predicted to differ from those of CDDP and carboplatin resistance.

Heat shock protein 72 (Hsp72) is a well-known stress-inducible molecular chaperone that assists in the folding of nascent polypeptides and in the refolding of denatured proteins [5,6]. Hsp72 expression is induced by multiple stresses and is critical for stress tolerance in many cancers of various origins [7]. Cancer cells experience multiple microenvironmental stresses such as nutrient deprivation, hypoxia, and exposure to chemotherapeutic agents [8–11]. Therefore, cancer cells must develop the ability to overcome proteotoxic stress, which arises via the intracellular accumulation of misfolded proteins [12,13]. In many human cancers of various origins, Hsp72 is constitutively expressed at elevated levels, which likely aids cancer cells in tolerating or adapting to these conditions. Moreover, high levels of Hsp72 expression have been shown

Abbreviations: OXA, oxaliplatin; CDDP, cisplatin; OCUM-2M/OXA, oxaliplatin-resistant OCUM-2M; OCUM-8/OXA, oxaliplatin-resistant OCUM-8.

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to correlate with increased invasiveness, metastasis, poor prognosis, and resistance to chemotherapy [14,15]. Indeed, Hsp72 has been shown to be highly expressed in CDDP-resistant ovarian cancer cells and to promote CDDP resistance [16]. Hence, inhibition of Hsp72 expression yields increased sensitivity to chemotherapeutic agents. However, because Hsp72 inhibition can affect the general stress responses of normal cells, there is concern that such inhibition may cause severe side effects. Hsp72 is important for cellular homeostasis, and Hsp72 client proteins are likely essential for avoiding stresses induced by chemotherapeutic agents. Moreover, the types of Hsp72-interacting proteins might vary upon the development of acquired drug resistance. We therefore hypothesized that Hsp72 client proteins play a crucial role in the acquisition of drug resistance.

Here, we performed a mass spectrometry-based proteomic analysis utilizing affinity purification with anti-Hsp72 antibodies of the human gastric cancer cell line OCUM-2M, as well as oxaliplatin (OXA)-resistant OCUM-2M (OCUM-2M/OXA) cells. By comparing the Hsp72 client profiles of OCUM-2M and OCUM-2M/OXA cells, we identified a protein that is potentially critical for OXA resistance.

Materials and methods

Reagents and antibodies

OXA was obtained from Yakult (Tokyo, Japan). Other reagents and antibodies used in this study are provided in the Supplementary Methods online.

Cell lines

Two human gastric cancer cell lines, OCUM-2M and OCUM-8, and their subcell lines resistant to OXA, OCUM-2M/OXA and OCUM-8/OXA, respectively, were used in this study [17,18]. Cell lines were derived from diffuse-type human gastric cancer patients and sublines were established from parental cells by stepwise exposure to OXA.

RNA interference

Hsp72-specific siRNAs (#1; 45112, #2; S6965), SDF-2 (#1; S12647, #2; S12649) and the scrambled siRNA control (AM4636) were obtained from Invitrogen (Carlsbad, CA, USA). Cells were reverse-transfected with siRNA molecules (10 or 30 nM final concentration) using Lipofectamine RNAiMAX (Invitrogen) reagent, in accordance with the manufacturer's instructions.

RNA isolation and real-time reverse transcriptase polymerase chain reaction (qRT-PCR) analysis

The detailed protocols used for RNA isolation and qRT-PCR analysis are presented in the Supplementary Methods online.

Protein extraction and western blotting

Cells were lysed in lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% NP-40, and 0.65% CHAPS containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan)]. Sample preparation and western blot analysis were performed as previously described [19].

Isolation of Hsp72 client proteins

For mass spectrometry analyses, cells were lysed in lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, and 5 mM EDTA, 0.5% NP-40 containing 1 mM PMSF and a protease inhibitor cocktail]. Cell lysates (1 mg of protein) were precleared by incubation with inactivated NHS-sepharose beads (GE Healthcare, Little Chalfont, UK) for 30 min at room temperature, and then subjected to immunoprecipitation using NHS-sepharose beads conjugated with anti-HSP72 antibodies at room temperature for 2 h [20]. The immunoprecipitates were washed three times with wash buffer [50 mM HEPES (pH 7.5) and 150 mM NaCl], and Hsp72 client proteins were eluted with 0.2 M glycine–HCl (pH 2.0).

Mass spectrometry sample preparation

The detailed protocols used for mass spectrometry sample preparation are available in the Supplementary Methods online.

Proteomic analysis and database search

The detailed protocols used for proteomic analysis and database searches are available in the Supplementary Methods online.

Immunoprecipitation

Immunoprecipitation was performed as previously described [21]. A detailed protocol is available in the Supplementary Methods online.

Cell proliferation assays

Cell proliferation was examined using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) as described previously [19].

Inhibitor assay

OCUM-2M/OXA cells were seeded in 6-well plates at a density of 2.5×10^5 cells per well and incubated for 24 h. Cells were then treated with the Hsp72 inhibitor VER155008 (100 μM), the proteasome inhibitor lactacystin (1 μM), or DMSO for 12 h.

IncuCyte cell growth measurement assay

Cell growth was examined using an IncuCyte kinetic imaging system (Essen BioScience, Ann Arbor, MI, USA), as described previously [12]. Detailed protocols are available in the Supplementary Methods online.

Trypan blue exclusion cell viability assay

The detailed protocol for the cell viability assay is available in the Supplementary Methods online.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) apoptosis assay

Apoptosis was detected using a DeadEnd Fluorometric TUNEL System (Promega, Madison, WI, USA), based on the manufacturer's protocols. A detailed protocol is available in the Supplementary Methods online.

Results

Hsp72 is critical for OXA resistance

We first examined the expression levels of Hsp72 in OXA-resistant gastric cancer cells by qRT-PCR and western blot analyses. OCUM-2M/OXA cells exhibited higher levels of Hsp72 mRNA and protein expression than OCUM-2M cells (Fig. 1A, B). However, OCUM-8/OXA cells exhibited similar levels of Hsp72 mRNA and protein expression as OCUM-8 cells (Fig. 1C, D). Thus, to determine whether Hsp72 was critical for OXA resistance, Hsp72 expression was suppressed by siRNA in OXA-resistant cells. Compared with cells treated with the scrambled siRNA (control), those treated with the Hsp72-specific siRNA exhibited enhanced OXA sensitivity (Fig. 1E–H); the IC_{50} values of OXA for the control and Hsp72-knockdown OCUM-2M/OXA cells were 22.06 ± 2.38 and 10.90 ± 3.25 μM, respectively ($P < 0.05$) (Fig. 1E). Similar effects were observed using the other Hsp72-specific siRNA molecules (Fig. S1). Despite there being no differences in Hsp72 expression, the IC_{50} values of OXA for OCUM-8/OXA cells were 6.85 ± 0.94 and 3.67 ± 0.20 μM, respectively ($P < 0.05$) (Fig. 1G). The Hsp72 knockdown efficiencies for the experiments presented in Fig. 1E and G were verified by western blot analysis (Fig. 1F, H). Together, these results indicate that Hsp72 is critical for OXA resistance.

Identification of Hsp72 client proteins in OCUM-2M/OXA cells

To identify novel OXA resistance-related proteins, we explored Hsp72 client proteins unique to OCUM-2M/OXA cells. Initially, Hsp72 client proteins were purified from OCUM-2M and OCUM-2M/OXA cells using anti-Hsp72 antibodies and were analyzed by mass