Suppression of heat shock protein 70 by siRNA enhances the antitumor effects of cisplatin in cultured human osteosarcoma cells

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Abstract Although advances in chemotherapy have improved the prognosis for osteosarcoma, some patients do not respond sufficiently to treatment. Heat shock protein 70 (Hsp70) is expressed at high levels in cancer cells and attenuates the therapeutic efficacy of anticancer agents, resulting in a poorer prognosis. This study investigated whether small interfering RNA (siRNA)-mediated inhibition of Hsp70 expression in an osteosarcoma cell line would enhance sensitivity to cisplatin. The expression of Hsp70 with cisplatin treatment was observed by using Western blotting and real-time reverse transcription polymerase chain reaction (RT-PCR). Changes in the IC₅₀ of cisplatin when Hsp70 was inhibited by siRNA were evaluated. Cisplatin's effectiveness in inducing apoptosis was assessed by assay of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), caspase-3 activity, and mitochondrial membrane potential. Up-regulation of Hsp70 expression was dependent on the concentration of cisplatin. Inhibition of Hsp70 expression significantly reduced the IC₅₀ of cisplatin. When cisplatin was added to osteosarcoma cells with Hsp70 expression inhibited, a significant increase in apoptosis was demonstrated in TUNEL, caspase-3, and mitochondrial membrane

potential assays. Inhibition of Hsp70 expression induced apoptosis in cultured osteosarcoma cells, indicating that Hsp70 inhibition enhanced sensitivity to cisplatin. Inhibition of Hsp70 expression may provide a new adjuvant therapy for osteosarcoma.

Keywords Heat shock protein 70 (Hsp70) · Osteosarcoma · Cisplatin · Apoptosis

Introduction

Osteosarcoma is the most common histological form of primary malignant bone tumors and occurs mainly in young people (Damron et al. 2007). Therapeutic outcomes have been currently improved by treatment that comprises surgery and pre- and postoperative chemotherapy (Ferrari et al. 2007). Before the introduction of chemotherapy, 5-year survival was less than 20%, but this percentage has greatly increased thanks to the use of chemotherapy. Additionally, before chemotherapy was introduced, surgery mainly entailed amputation of the affected limb; however, thanks to effective chemotherapy and improved surgical procedures, limb-conserving surgery is now the standard treatment (Anderson et al. 2016; Ferrari et al. 2015).

First-line chemotherapy for osteosarcoma usually comprises combination therapy with cisplatin, doxorubicin, and methotrexate. Cisplatin is a platinum agent that is widely used in many cancer chemotherapy regimens. Cisplatin acts by blocking the synthesis of DNA, thereby inducing apoptosis by cancer cells (Dasari et al. 2014). Although cisplatin is a highly effective cancer treatment, its side effects are problematic. The dose of cisplatin required to treat osteosarcoma is particularly high, and this can easily damage the kidneys, peripheral nerves, and auditory nerve. A method is therefore required for obtaining an adequate

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therapeutic effect from a lower cisplatin dose. Caffeine, for example, has been shown to block the repair of DNA damage by tumor cells and thereby enhance their sensitivity to cisplatin, and its efficacy has been demonstrated clinically (Takeda et al. 2005). Temsirolimus, a mammalian target of rapamycin (mTOR) inhibitor, and genistein, an isoflavone, have also been shown to enhance sensitivity to cisplatin (Liu et al. 2014a; Fleuren et al. 2014). However, caffeine entails problems with cardiotoxicity, and the use of mTOR inhibitors for sarcomas has yet to be approved. Genistein has also yet to be proven to be effective against sarcoma.

Heat shock proteins (Hsps) are chaperone proteins that are induced by a range of different stresses such as heat stimulus and act to protect cells against stress. The expression and action of heat shock protein 70 (Hsp70) in cancer cells vary among different types of cancers; in breast cancer, bladder cancer, and osteosarcoma, it constitutes a negative prognostic factor, whereas in esophageal cancer and renal cancer, it reportedly acts as a positive prognostic factor (Ciocca et al. 2005; Uozaki et al. 2000; Goloudina et al. 2012). Since Pocaly et al. (2007) first reported that Hsp70 expression is involved in imatinib resistance in chronic myeloid leukemia (CML), several other studies have also identified the involvement of Hsp70 expression in resistance to anticancer agents in other forms of cancer (Yang et al. 2012; Behnsawy et al. 2013).

Against this backdrop, we hypothesized that inhibition of Hsp70 expression in osteosarcoma might improve the therapeutic efficacy of an anticancer agent (cisplatin). The objective of this study was to investigate if inhibition of Hsp70 expression in cultured osteosarcoma cells by small interfering RNA (siRNA) might enhance sensitivity of the cells to cisplatin.

Materials and methods

Cell lines and culture conditions

The human osteosarcoma cells MG63 (CRL1427) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Human osteosarcoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; Equitech-Bio, Kerrville, TX, USA), 100 units/ml penicillin, and 100 mg/ml streptomycin (complete DMEM; Nacalai Tesque) at 37 °C in 5% CO₂/95% humidified air. The cells were trypsinized with trypsin/ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque) and sub-cultured.

Reagents

Cisplatin was obtained from Wako (Tokyo, Japan) and was dissolved in a saline solution to give a stock solution of 1.0 mM and stored at −80 °C.

Transfection with small interfering RNA

MG63 cells were seeded into six-well plates at a density of 2.0×10^5 cells per well. The next day, the medium was changed and the cells were transfected with validated MISSION HSPA1A siRNA (SASI_Hs01_00051449; Sigma-Aldrich, St. Louis, MO, USA) or a negative control (MISSION siRNA Universal Negative Control; SIC-001; Sigma-Aldrich), each at a final concentration of 50 nM in cationic liposome (Lipofectamine RNAiMAX Reagent; Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The plates were cultured for 48 h following which the cells were trypsinized and used in various experiments.

Western blot analysis

The cells were washed twice with PBS and then harvested and lysed in RIPA buffer (Nacalai Tesque) diluted tenfold in ultrapure water. Samples were frozen at −80 °C for 10 min, thawed at 37 °C, and then incubated on ice for 30 min. The samples were centrifuged at 20,600×g for 5 min and the supernatant was aspirated. Protein concentrations of the supernatants were measured using bicinchoninic acid (BCA) assays, and equal amounts of total proteins were loaded onto gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples containing 5 µg of protein were separated by 10% Bis-Tris Gel NuPAGE® electrophoresis using 5% MOPS SDS Running Buffer (Thermo Fisher Scientific, Waltham, MA, USA). Separated proteins were dry-blotted onto nitrocellulose iBlot®gel transfer stacks in iBlot Gel transfer devices (Thermo Fisher Scientific) for 7 min. The nitrocellulose membrane was blocked by shaking in Blocking One solution (Nacalai Tesque) for 60 min at room temperature. The blots were subsequently shaken overnight at 4 °C in this solution containing the following specific primary antibodies: anti-human Hsp70 mouse monoclonal antibody (1:1000 dilution; BioVision, Milpitas, CA, USA; catalog no. 3096–100) and mouse anti-human β-actin antibody (1:4000 dilution; Sigma-Aldrich; catalog no. A2228). β-Actin was assayed as a loading control. Blots were washed three times with TBST and shaken for 60 min at room temperature with peroxidase-conjugated anti-mouse IgG secondary antibodies (1:4000 dilution; Sigma-Aldrich; catalog no. A4416). The blots were then again washed three times with TBST, and chemiluminescence emission was visualized using Chemi-Lumi One Super (Nacalai Tesque). Protein band intensities were assessed using the ECL Select LAS500 (GE Healthcare, Buckinghamshire, England).

Total RNA extraction and real-time RT-PCR analysis

Cells were harvested and total RNA was extracted from those cells using ISOGEN (Nippon Gene, Osaka, Japan).

Extracted RNAs were reverse transcribed using a PrimeScript™ RT Master Mix (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) was performed using the Biosystem 7300 (Applied Biosystems, Carlsbad, CA, USA). Sequences of the primers used were as follows: human Hsp70 (forward primer, 5'-CGGCAAGGTGGAGATCAT-3'; reverse primer, 5'-GGTGTCTCGCGGTTCAG-3') and 18S ribosomal RNA (forward primer, 5'-ATGAGTCCACTTTAAATCCTTTAACGA-3'; reverse primer, 5'-CTTTAATATACGCTATTGGAGCTGGAA-3'). Each 25 µl reaction mixture contained 2 µl of cDNA (100 ng) and 12.5 µl of the TaqMan Gene Expression PCR Master Mix (TOYOBO, Osaka, Japan) for the target gene. The amplification protocol consisted of 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Relative changes in gene expression were normalized against the internal control gene, 18S ribosomal RNA.

Cell viability assays

Cell viability was determined using the RealTime-Glo MT Cell Viability Assay Kit (Promega, Madison, WI, USA). Cells were plated in a poly-L-lysine (PLL)-coated 96-well plate at a density of 1000 cells per well in 100 µl of medium containing 10% FBS. After 24 h, MT cell viability substrate and Nanoluc® Enzyme was added to the medium and the cells were exposed to various concentrations of cisplatin. IC₅₀ values (drug concentration causing 50% inhibition of cell viability) were determined after 48-h treatment with cisplatin. The CentroXS3 LB 960 system (Berthold Technologies, Oak Ridge, TN, USA) was used to measure light emission.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay

DNA fragmentation in apoptotic cells was assayed using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assays that were performed using the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Mannheim, Germany). After exposure to cisplatin for 48 h, the cells were trypsinized and then recovered with culture medium. The cells were washed twice in PBS and fixed for 60 min with 2% paraformaldehyde. The cells were then immersed in permeabilizing solution (0.1% sodium citrate and 0.1% Triton X-100 with distilled deionized water) for 2 min. TUNEL staining was performed according to the manufacturer's instructions. Samples were finally analyzed by flow cytometry using a FACS Vantage flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). For each

Fig. 1 Cisplatin (CDDP) enhanced the Hsp70 levels in MG63 cells. Cells were treated with CDDP for 48 h and Hsp70 protein expression was then analyzed by Western blotting. β -Actin protein was used as a loading control

sample, 10,000 events were recorded. The M1 domain was defined based on the peak of fluorescence intensity of the group of cells for which TUNEL staining was not performed. The percentage of cells in the M1 domain (considered as TUNEL-positive cells) was determined and compared between groups.

Caspase-3 activity

The activity of caspase-3, an apoptosis-related factor, was assessed using the Caspase-Glo 3/7 Assay (Promega). Cells were sub-cultured in a PLL-coated 96-well plate (1×10^4 cells/well) and were then treated with cisplatin for 24 h. After this treatment, the Caspase3/7 Reagent was added to the cells for 1 h at room temperature. A CentroXS3 LB 960 system was used to measure light emission.

Measurement of mitochondrial membrane potential

Mitochondrial membrane permeability was measured by assay of the uptake of rhodamine 123 (Rh123). The cationic fluorescent probe, Rh123, is preferentially retained in mitochondria in an amount proportional to the mitochondrial membrane potential ($\Delta\psi$). The loss of mitochondrial integrity or the opening of the permeability transition pore channel results in leakage of the probe from the mitochondria and a consequent decrease in fluorescence (Ferlini et al. 2007). Cells were exposed to cisplatin for 24 h, collected with a cell scraper, washed in PBS, and incubated at 37 °C for 15 min with 10 µM Rh123. After centrifugation at $1500 \times g$ for 5 min, the cells were analyzed using flow cytometry. Cells with reduced fluorescence intensity compared to the control group were defined as $\Delta\psi$ low cells.

Statistical analysis

All duplicated and triplicated experiments yielded almost identical results. All experimental data are expressed as mean $\pm$ standard deviation (SD). Parametric one-way analysis of variance (ANOVA) was used to test for any differences

Fig. 2 Effects of Hsp70 siRNA transfection on cisplatin induction of Hsp70 expression. MG63 cells were first transfected or not with Hsp70 or control siRNA for 48 h, following which they were treated with cisplatin (CDDP; 50 μ M) for 48 h. Hsp70 protein expression was analyzed by Western blotting (a) and Hsp70 mRNA was analyzed using real-time RT-PCR (b). β -actin protein was used as a loading control. The data are expressed as mean $\pm$ standard deviation ($n = 3$). ** $p < 0.01$

among the groups. If the result was significant, the Tukey-Kramer test was used to determine specific differences

between the groups. In all analyses, $p < 0.05$ was defined as statistically significant.

Fig. 3 Effects of treatment with Hsp70 siRNA and cisplatin on cell viability. The cells were treated with control or Hsp70 siRNA for 48 h and were then treated with cisplatin (CDDP) at the indicated concentrations for 48 h. Cell viability was determined using the RealTime-Glo MT Cell Viability Assay (a). IC₅₀ values were determined after 48-h exposure to cisplatin (b). Values for Hsp70-silenced cells were compared to those of control siRNA-administered cells treated with the same concentration of cisplatin. The data are expressed as mean $\pm$ standard deviation ($n = 5$). * $p < 0.05$; ** $p < 0.01$

Results

Up-regulated expression of Hsp70 in cisplatin treatment

To investigate up-regulation of the expression of Hsp70 by the chemical stressing of osteosarcoma cells with cisplatin treatment, MG63 cells were treated for 48 h with cisplatin that was adjusted to a concentration of 10, 20, or 50 μ M, and Hsp70 protein levels were then analyzed using Western blotting. Hsp70 protein expression levels increased as the concentration of cisplatin increased (Fig. 1).

Effects of Hsp70 siRNA transfection on cisplatin induction of Hsp70 expression

To evaluate whether Hsp70 siRNA can inhibit Hsp70 protein expression during treatment with cisplatin, MG63 cells were first transfected with Hsp70 siRNA for 48 h, following which they were treated with 50 μ M cisplatin for 48 h. The dosage of cisplatin (50 μ M) was selected as a high-dose administration example (Cummings et al. 2002; Liu et al. 2014b). The effect of Hsp70 siRNA transfection of MG63 cells on cisplatin induction of Hsp70 expression was then analyzed using Western blotting and real-time RT-PCR. Both the protein and mRNA expression of Hsp70 were clearly suppressed in the Hsp70 siRNA-transfected cells compared to that observed in control

◀ **Fig. 4** Hsp70 regulates cisplatin-induced apoptosis. Apoptosis of the indicated cells following exposure to cisplatin (CDDP; 50 μ M) was measured using the TUNEL assay and subsequent flow cytometry. The percentage of TUNEL-positive cells is indicated within the M1 domain in (a) and is quantified in (b). The highest increase in TUNEL-positive cells was seen in cells treated with both Hsp70 siRNA and cisplatin. Caspase-3 activity was examined after 24-h exposure to cisplatin (CDDP; 50 μ M). Inhibition of Hsp70 increases caspase-3 activity induced by cisplatin (c). The data are expressed as mean $\pm$ standard deviation ($n = 4$). ** $p < 0.01$. *n.s.* not significant

siRNA-transfected cells and in cisplatin-treated siRNA-transfected cells (Fig. 2a, b).

Suppression of Hsp70 expression in MG63 cells increased their sensitivity to cisplatin

To determine whether treatment with Hsp70 siRNA enhances the cytotoxic effect of cisplatin, MG63 cells were transfected with 50 nM Hsp70 siRNA and then treated with various doses of cisplatin, following which cell viability was assayed. Figure 3a shows that there was a decrease in cell viability as a function of drug concentration following treatment with cisplatin for 48 h. Hsp70 siRNA transfection enhanced the sensitivity of MG63 cells to cisplatin compared to control siRNA transfection. IC₅₀ values (drug concentration causing 50% inhibition of cell viability) for cisplatin were 36.4 ± 1.94 μ M in control siRNA-transfected cells and 23.4 ± 2.08 μ M in Hsp70 siRNA-transfected cells (Fig. 3b).

Suppression of Hsp70 expression promotes cisplatin-induced apoptosis

We next assessed the effect of Hsp70 siRNA transfection alone, cisplatin treatment alone, and combined treatment with Hsp70 siRNA and cisplatin on apoptosis of MG63 cells, using TUNEL staining. The TUNEL assay was performed after 48-h exposure of the siRNA-transfected or siRNA-non-transfected cells to 50 μ M cisplatin, and stained cells were

measured using flow cytometry. The percentage of apoptotic cells in cells transfected with Hsp70 siRNA alone did not differ in comparison with control or control siRNA-transfected cells. Cisplatin treatment significantly increased the percentage of TUNEL-positive cells compared to cells not treated with cisplatin. The highest increase in TUNEL-positive cells was observed in cells that were treated with both Hsp70 siRNA and cisplatin (Fig. 4a, b).

Analysis of caspase-3 activity, which also reflects apoptosis, in cells treated as described in Fig. 4a, b, displayed similar results of apoptosis to those obtained in the TUNEL assay (Fig. 4c).

Combined Hsp70 siRNA and cisplatin treatment affects the intrinsic pathway of apoptosis

We examined the mitochondrial membrane potential ($\Delta\psi$) of cells treated as described in Fig. 5 to check the apoptotic pathway involved in the induced apoptosis. The mitochondrial membrane potential ($\Delta\psi$) was assayed by determination of the reduction of rhodamine 123 using flow cytometry. The percentage of cells with decreased mitochondrial membrane potential increased following treatment of cells with cisplatin alone compared to cells not treated with cisplatin. However, the highest increase in the percentage of cells with a low $\Delta\psi$ was detected in cells treated with a combination of Hsp70 siRNA plus cisplatin (Fig. 5).

Discussion

Hsps are proteins that are induced in cells as a result of physical, chemical, or other forms of stress (Zhang et al. 2002). They are classified by molecular weight, and those so far identified include Hsp27, 60, 70, and 90. Although their expression and function vary, many Hsps have been reported not only to protect normal cells against stress but also to promote

Fig. 5 Combined treatment with cisplatin and Hsp70 siRNA induces depolarization of the mitochondrial membrane potential ($\Delta\psi$). After treatment with cisplatin for 48 h, the mitochondrial membrane potential, $\Delta\psi$, was assessed by flow cytometry using rhodamine 123. Data are shown as mean $\pm$ SE ($n = 3$). ** $p < 0.01$

their recovery from damage (Jaattela et al. 1992, 1993; Li et al. 1992). Studies are thus ongoing for the prevention or treatment of a variety of diseases by promoting the expression of Hsps.

The expression of Hsps has also been observed in a range of different cancer cells (Gimenez et al. 2010). Aghdassi et al. (2007) found that Hsp70 is strongly expressed in pancreatic cancer cells. Uozaki (2000) and Moon et al. (2010) found that Hsps are expressed in pathological tissue from osteosarcoma patients, with the level of expression corresponding to the degree of malignancy of the cells concerned. We also observed Hsp70 expression in the cultured MG63 osteosarcoma cells used in this study.

Hsps may also protect tumor cells. In particular, if Hsps protect tumor cells against anticancer agents, this may weaken the effectiveness of chemotherapy, posing a problem for treatment (Guttmann and Koumenis 2011). Drugs such as alkylating agents, antimetabolites, anticancer antibiotics, and platinum agents have conventionally been used as anticancer agents. Among these drugs, cisplatin is a platinum agent that is widely used in clinical practice to treat a variety of cancer cells. It has been used in the treatment of osteosarcoma since the 1970s and is one of the basic drugs for treating this cancer (Yamamoto et al. 2013). Here, we found that Hsp70 up-regulation in osteosarcoma cells corresponded to the cisplatin dose, suggesting that Hsp70 may act to protect MG63 cells against the antitumor action of cisplatin.

Cisplatin is administered in large doses in the treatment of osteosarcoma and its side effects are correspondingly severe, meaning that it may be difficult to continue the administration of the amount of cisplatin required (Rabik et al. 2007). If the up-regulation of Hsp70 expression induced by cisplatin administration could be inhibited, this might not only prevent the attenuation of antitumor effect but also enable a sufficient antitumor effect to be obtained with a lower dose. In this study, we reduced the IC_{50} of cisplatin in MG63 cells by using siRNA to specifically inhibit Hsp70 expression, showing the possibility that an equivalent antitumor effect to that of the usual dose can be obtained with low dose cisplatin. Figure 3 illustrates the results being described.

Cisplatin is known to induce cell death by activating the apoptosis pathway (Zhang et al. 2015; Seki et al. 2000), and this constitutes the main mechanism of its antitumor action. In cells that express Hsp70, however, Hsp70 expression has been shown to exert a strongly anti-apoptotic effect (Terauchi et al. 2003; Saleh et al. 2000). In this study, we used three different types of assessments to show that apoptosis is significantly increased: the changes in mitochondrial membrane potential that occur at an early stage of apoptosis; activity of caspase-3, an enzyme in the caspase family that is activated downstream; and DNA fragmentation, the final stage of cell death. Enhanced sensitivity to cisplatin may be induced by inhibiting the anti-apoptotic effect exerted by Hsp70.

In order to translate this study into clinical use, it is necessary to develop means for safely and selectively suppressing Hsp70. Treatment with siRNA has a specific inhibitory effect; however, there are problems of in vivo stability and of delivery to locations (Inoue et al. 2014; Barba et al. 2017). Hsp70 inhibitors such as VER155008 may not obtain the expected inhibiting effects in some cases because of the influence of other chaperones and co-chaperones (Asling et al. 2016). Some natural products can suppress Hsp70 and these agents are said to have a smaller adverse effect on the human body, and future clinical application can be expected (Zhao et al. 2016). The mechanism of Hsps in tumor growth has been clarified in recent years and it is important to find a new treatment for the regulation of Hsps expression (Bruning et al. 2015; Calderwood and Gong 2016).

Our results showed that inhibition of Hsp70 expression in osteosarcoma cells enhanced their sensitivity to cisplatin. This finding suggested that inhibition of Hsp70 expression might have the potential to provide a new adjuvant therapy for chemotherapy in the treatment of osteosarcoma.

This study was limited by its nature as an in vitro study of a single human osteosarcoma cell line and by the fact that it selectively knocked out only Hsp70-1 in the Hsp70 family.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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