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Full Length Research Paper

Ultrasound sensitizes chemotherapy in chemoresistant ovarian cancers

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Chemotherapy resistance is still a great challenge to the management of ovarian cancers. Using $SKOV_3$/ADR or COC1/DDP subline as a model of adriamycin- or cisplatin-resistance, ultrasonic chemosensitization was investigated. The addition of noncytotoxic insonation led to a higher cell-death rate as compared with a drug alone. Ultrasound sensitized chemotherapy *via* increasing intracellular drug accumulation, enhancing drug-induced apoptosis and decreasing the threshold dose for cell apoptosis/necrosis. Ultrasound exposure enhanced cisplatin-induced DNA breakages in COC1/DDP cells but did not decrease the level of glutathione-S-transferase. Chemosensitization attributable to insonation was mostly mediated by cavitation. Ultrasonic chemotherapy had the property of a targeted treatment, in that the dose-anticancer effect and dose-toxicity curves differed from those in conventional chemotherapy. The findings indicated that ultrasound was a non-drug modality for sensitizing chemotherapy in refractory ovarian cancers.

Key words: Chemoresistance, ovarian cancer, ultrasound, sonochemotherapy, targeted therapy.

INTRODUCTION

Ovarian cancer is the leading cause of death in gynecological malignancies. Of those factors which lead to the failure of treatment, the development of chemoresistance plays a determining role. Overcoming chemoresistance can efficiently deactivate cancer cells, thereby improving the clinical outcomes.

A chemical chemosensitizer commonly disappoints oncologists due to severe toxicities to noncancerous tissues (Morjani and Madoulet, 2010; Takara et al., 2006). A non-drug modality is therefore an alternative.

We reported the use of ultrasound to sensitize chemotherapy in chemoresistant ovarian cancers in this note. Chemotherapy resistance and ultrasonic therapy were theoretically introduced, and then experimental findings from two chemoresistant human ovarian cancer cell sublines, $SKOV_3$/ADR and COC1/DDP, were briefly described and discussed.

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THEORETICAL

Chemoresistance

Platinum- and multidrug-resistance (MDR) are the two most frequent phenotypes in ovarian cancers (Arts et al., 2000). Adriamycin-resistance is a typical MDR. Mechanisms of chemotherapy resistance are briefly summarized in Table 1 (Agarwal and Kay, 2003; Itamochi et al., 2008; Piulats et al., 2009).

Many chemical chemosensitizers have been tested *in vitro* and *in vivo*. These include, but not limit to: (i) calcium channel blocker, (ii) immunosupressant, (iii) hormone agonist and (iv) antifugal agent (Morjani and Madoulet, 2010; Takara et al., 2006). Briefly, clinical outcomes disappoint oncologists. Severe toxicities to noncancerous tissues and the lack of chemosensitization *in vivo* are the main limitations.

Ultrasonic cancer therapy

Ultrasound induces thermal and nonthermal (mechanical effect and cavitation) effects in the insonated tissues. These effects occur concurrently, but do not work in the same way. A specific mechanism plays a leading role in a specific biological response (Yu et al., 2004a). Ultrasound heats tissues when the intensity is high enough with long exposure-duration. Cavitation, the formation and/or activity of gas-filled bubbles in sonicated medium, causes

Table 1. Mechanisms of platinum- and adriamycin-resistance in ovarian cancers.

Type	Mechanisms
Adriamycin resistance (MDR)	Decrease of intracellular drug level
	P-glycoprotein (P-gp)
	Multidrug resistance protein (MRP)
	Lung resistance protein (LRP)
	Malfunction of apoptosis
	BCL-2 family
	p53
	Alteration of drug target
	Topoisomerase
Platinum resistance	Decrease of intracellular drug level
	Lung resistance protein (LRP)
	Inactivation of a drug
	Glutathione-S-transferase (GST)
	Glutathione (GSH)
	Gamma-glutamyl cysteine synthetase
	Enhancement of DNA repair
	ERCC1
	XPA
	DNA-polymerase
	Defect of apoptosis
	BCL-2 family
	p53
	Change of cell cycle
	JUN
	FOS
	MDM2
	Cyclins
	Growth factor signaling
	c-erb B2
	PTEN

microstreaming and microjet (with a velocity of 1000 m/s), which create shear force thus distorting the surrounding objects. Cavitation leads to localized high temperature (10^4-10^6 K) and high pressure (10^4 atmospheres) (Paliwal and Mitragotri, 2006). Such an extreme condition leads to the production of free radicals. Bioeffects depend on both ultrasound and the property of tissues. Therapeutic ultrasound operates within the range of nonlinear acoustics, and the biological response varies considerably between tissue types and individuals. Thus, the acoustic parameter must be individually selected according to the therapeutic goal. Tissue changes comprise structural and functional profiles. Structural alterations vary from just detectable injury to immediate cell death, and cell function can be up- or down-regulated (Yu et al., 2004a).

The uses of ultrasound for cancer treatment are listed in Table 2 (ter Haar, 2007; Yu et al., 2004a, 2006). Only ultrasonic chemotherapy will be discussed in this paper. Sonochemotherpy (ultrasonic chemotherapy) is the use of ultrasound to enhance a cytotoxic agent (Yu and Zhang, 2010). This technique equipotentially kills cells with a lower dose when compared with conventional chemotherapy. Thus, sonochemotherapy improves the anticancer effect whilst decreasing toxicity. Cavitation plays the determining role in that reactive species and shear force damage cell membrane including pore formation (with a size of up to 3-5 μm) (Liang et al., 2010; Zhao et al., 2008). These permeabilize cell membrane and vessels favoring the influx of drugs into the lesion since ultrasound can be focused on a predetermined volume within the body without harming overlying/surrounding tissues. The use of ultrasound to enhance an antincancer drug has been reviewed in several published papers (Liang et al., 2010; Paliwal and Mitragotri, 2006; Yu et al., 2004a, 2006; Yu and Zhang, 2010).

EXPERIMENTAL

Cells, ultrasound exposure and statistics

Ovarian carcinoma cell sublines $SKOV_3$/ADR and COC1/DDP were used as models of adriamycin- and cisplatin-resistance, respectively. The resistant indexes, determined by the ratio of 50% inhibiting concentration, were 6.37 for $SKOV_3$/ADR and 6.50 for COC1/DDP (Yu et al., 2003; Zhou et al., 1996). Experimental data were statistically processed with the analysis of variance and *t* test.

The mode of drug followed by insonation was used. Ultrasound exposure was performed as described previously (Zhou et al.,

Table 2. List of ultrasonic therapies for cancers.

Therapeutic modality	Leading role	Dosimetry
High intensity focused ultrasound	Heat	Temperature rise (>56 ℃)
Ultrasonic hyperthermia	Heat	Temperature rise (<50 ℃)
Gene transfer/expression	Cavitation	Free radicals
		Passive cavitation
Gene expression using temperature-depended promotor	Heat	Temperature rise (promotor specific)
Prodrug therapy	Cavitation	Free radicals
Gene-directed prodrug therapy	Heat	Temperature rise (promotor specific)
Sonodynamic therapy	Cavitation	Free radicals
Ultrasonic chemotherapy (Sonochemotherapy)	Cavitation	Free radicals
		Passive cavitation

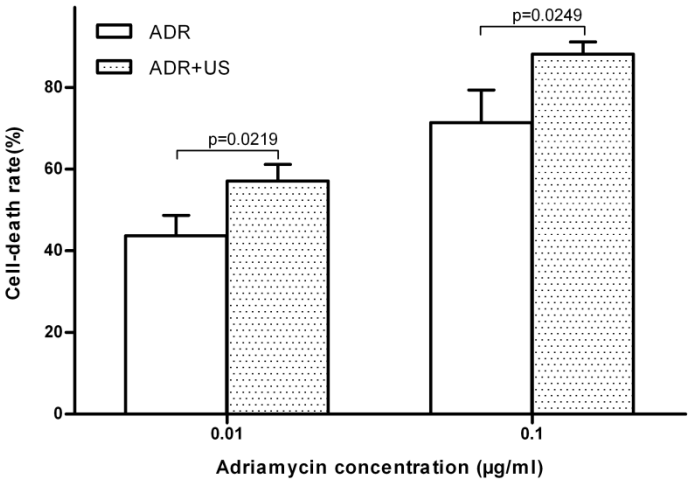

Figure 1. Cell-death rate of $SKOV_3$/ADR cells. The rate was increased in group ADR+US. ADR, cells were exposed to adriamycin; ADR+US, cells were treated with adriamycin followed by noncytotoxic insonation.

2011). The insonation parameter (frequency, intensity and exposure duration) was individually selected according to cells' response. A level which alone produced no cytotoxicity was used, which did not lead to an obvious temperature-rise (temperature in the insonated medium was <37 ℃).

Adriamycin-resistance

$SKOV_3$/ADR cells were subjected to adriamycin in group ADR, and to adriamycin followed by insonation (0.24 MHz, 5.76 W/cm^2 for 30 s) in group ADR + US. Drugs were washed away after 4 h, and cell viability was determined with a tetrazolium assay at 72nd h. A higher cell-death rate was detected in group ADR + US (Figure 1) (Yu et al., 2003).

The decrease of intracellular drug accumulation was one of the most important mechanisms of adriamycin-resistance (Itamochi et al., 2008; Takara et al., 2006). Thus, the intracellular drug level was determined with a 1.5-h-exposure assay. Intracellular drug accumulation in group ADR + US was higher than that in group ADR at 2 μg/ml adriamycin (0.2194 ± 0.0030 vs. 0.2446 ± 0.0100 μg, p = 0.0139) (Yu et al., 2003).

The overexpression of *mdr1* gene pumped out drugs decreasing the intracellular drug level, and many chemosensitizers overcame resistance *via* down-regulating the gene expression (Takara et al., 2006). *mdr1* was assayed with reverse-transcription polymerase chain reaction. The data did not show that ultrasound modulated the expression of *mdr1* in $SKOV_3$/ADR cells (0.63 ± 0.20 vs. 0.61 ± 0.17, p = 0.8965) (Figure 2).

Insufficient apoptosis resulted in chemoresistance (Agarwal and Kay, 2003). Cell apoptosis was quantitatively determined by measuring the sub-G1 peak with flow cytometry. Apoptosis rates in group ADR + US were higher than those in group ADR at 48th, 72nd and 96th h (Figure 3) (Yu et al., 2004b).

Chemoresistant tumors were established using subrenal capsule transplantation of cell-clot into female BALB/C mice. Adriamycin

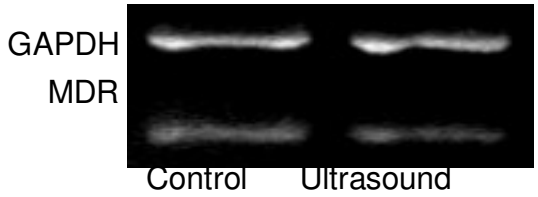

Figure 2. The expression of *mdr1* gene determined with reverse-transcription polymerase chain reaction. The expression was not modulated by insonation.

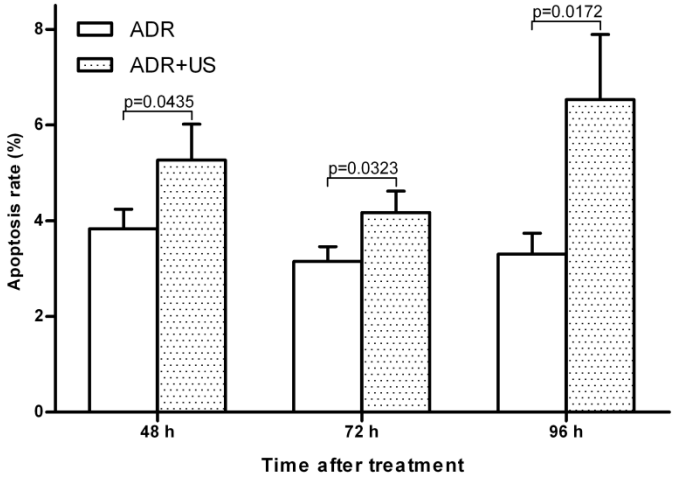

Figure 3. Apoptosis rates in $SKOV_3$/ADR cells at 48th, 72nd and 96th h. The use of ultrasound enhanced adriamycin-induced apoptosis. ADR, cells were exposed to adriamycin; ADR+US, cells were treated with adriamycin followed by noncytotoxic insonation.

were injected peritoneally (8 mg/kg). Insonation (0.24 MHz, 7.84 W/cm^2 for 10 min) was performed 15 min after administrating drug since the concentration in kidney reached the peak at that time. Treatment was performed at days 2, 3, 5 and 6, and the tumor volume was calibrated under a stereomicroscope at day 7. The volume in group ADR + US was smaller than that in group ADR, although insonation alone did not inhibit tumors (Figure 4) (Yu et al., 2004c).

Cisplatin-resistance

COC1/DDP cells were exposed to cisplatin in group DDP, and insonation (0.8 MHz, 2 W/cm^2 for 10 s) was added in group DDP + US. Chemicals were washed away after 3 h, and cell viability was assayed at 72nd h. The cell-death rate in group DDP+US was higher than that in group DDP at 5 μg/ml cisplatin (69.60 ± 2.51% vs. 63.93 ± 1.30%, p = 0.0254).

Intracellular cisplatin level was assayed with high performance liquid chromatography after 1 h exposure. Intracellular cisplatin accumulation was increased in group DDP + US when compared with group DDP (1.7320 ± 0.2140 vs. 2.3143 ± 0.0217 μg, p = 0.0201).

Cisplatin deactivated cells *via* the formation of DNA crosslink (Agarwal and Kay, 2003). DNA damages were detected with single cell gel electrophoresis. Comet tails appeared in groups DDP and DDP + US, and a longer tail occurred in group DDP + US (Figure 5) (Yu et al., 2009).

Glutathione-S-transferase (GST) inactivated cisplatin, thereby protecting cancer cells (Agarwal and Kay, 2003). One of the strategies to treat refractory lesions was to modulate the level of GST in cancer tissues. Active GST was determined with an enzyme assay. Insonation did not decrease the GST level in COC1/DDP cells (0.0168 ± 0.0051 vs. 0.0153 ± 0.0033 μmol/mg protein, p = 0.4716).

DISCUSSION

Mechanisms of chemoresistance have not been understood thoroughly so far. The resistance to a specific drug may result from several pathways and a specific pathway may be involved in the resistances to several agents; pathways overlap frequently and combined chemotherapy is the standard strategy for ovarian cancers (Rahaman et al., 2009). Thus, it is difficult to develop a chemical sensitizer. Either adriamycin- or cisplatin-resistance was overcome by insonation in this

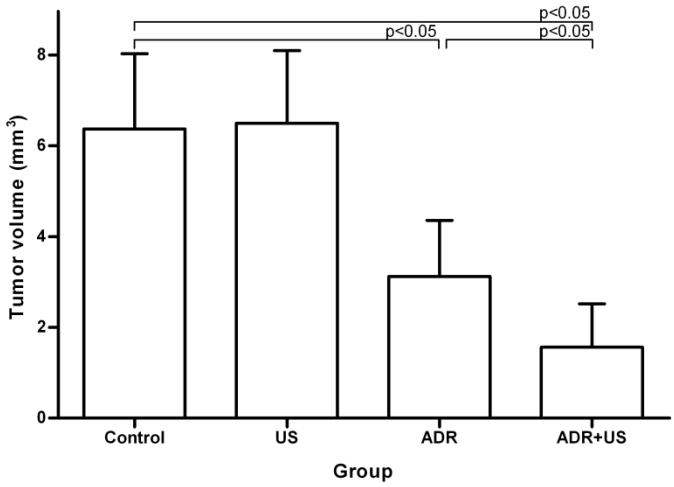

Figure 4. $SKOV_3$/ADR tumor volumes after treatment. The volume in group ADR+US was smaller than that in group ADR indicating chemosensitization *in vivo*. ADR, tumor was exposed to adriamycin; US, tumor was subjected to insonation; ADR+US, tumor was treated with the combination of adriamycin and ultrasound, and ultrasound exposure was performed after drug administration.

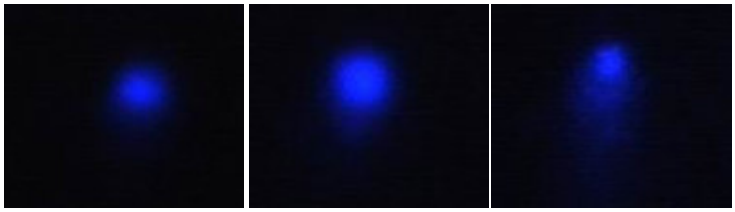

Figure 5. DNA breaks in chemoresistant cells COC1/DDP detected by single cell comet electrophoresis. No damages were detected in cells exposed to ultrasound alone (*left*). DNA damages occurred in cells subjected to cisplatin (*middle*) and cisplatin followed by insonation resulted in a longer comet tail, an indicator of DNA fragmentation (*right*).

study, indicating that ultrasound was a potential therapeutic modality for refractory ovarian cancers.

Ultrasound increased the intracellular drug levels in both $SKOV_3$/ADR and COC1/DDP cells, which was one of the mechanisms of ultrasonic chemosensitization. The therapeutic efficacy of a drug is reliant on the peak concentration and area under the concentration-time curve (Fruehauf, 2002). Both of them will be increased with increasing intracellular drug accumulation. Polymerase chain reaction did not demonstrate the down-regulation of *mdr1* gene. Cavitation permeabilizes cell membrane thus facilitating the transmembrane influx of drugs.

An anticancer agent usually deactivates cells *via* inducing apoptosis, and the malfunction of apoptosis leads to chemoresistance (Agarwal and Kay, 2003). The addition of ultrasound enhanced adriamycin-induced apoptosis in $SKOV_3$/ADR cells in this study. Apoptosis is realized *via* cytochrome C-dependent or -independent pathway. Cytosol cytochrome C was increased in insonated $SKOV_3$/ADR cells but decreased in sonicated COC1/DDP ones in our previous study (Yu et al., 2005). Ultrasound, therefore, induced cell apoptosis *via* both approaches. Cells can also be efficiently deactivated through nonapoptotic pathways, which is a strategy against refectory cancers (Finkel, 1999). Frequently, the increase of apoptosis rate was not proportional to that of cell-death rate in ultrasonic chemotherapy, indicating the involvement of nonapoptotic cell dearth (Yu et al., 2006). The role of other modes of cell death (necrosis and autophage) in ultrasonic sensitization should be investigated.

Cisplatin-resistant cancer cells commonly have an increased capacity of DNA repair, thereby decreasing

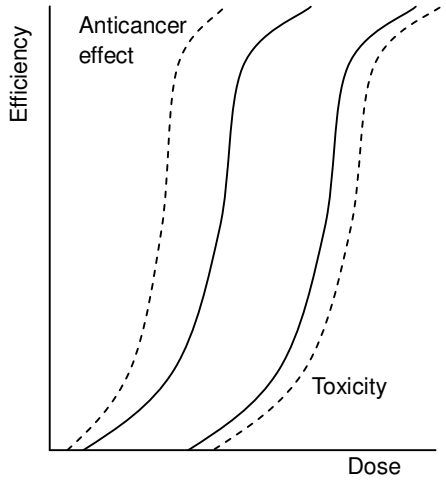

Figure 6. The dose-anticancer effect curve paralleles the dose-toxicity curve in conventional chemotherapy (*solid line*). In ultrasonic chemotherapy, the dose-anticancer effect curve was shifted left; and the dose-toxicity curve shifted right as cytotoxic drugs were released into cancer tissues directly and efficiently; toxicities to surrounding noncancerous tissues were decreased as a result of much lower drug level (*dashed line*). Sonochemotherapy was a targeted therapy, improving both the therapeutic and safe indexes.

drug induced DNA damages (Agarwal and Kay, 2003; Piulats et al., 2009). Insonation enhanced DNA breaks induced by cisplatin, where the increase of intracellular drug level played a role. DNA repair is a programmed procedure involving in many molecules. Thus, how ultrasound enhanced DNA damages should be explored. Drug inactivation diminishes the active form of a drug and GST is one of the most important toxinicides (Agarwal and Kay, 2003). GST was not impacted in this study. Effects of ultrasound on other detoxification molecules should be explored.

Our previous investigation manifested that ultrasound enhanced adriamycin despite an absence of increase of intracellular drug level. The cell-surviving curve was therefore evaluated with mathematic models, and the data showed that ultrasound was a sensitizer. Nontoxic insonation sensitized a cell making it prone to being impaired by other cytotoxic factors. Ultrasound lowered the threshold for cell necrosis/apoptosis. Some cells befell necrosis directly in ultrasonic chemotherapy, but would be deactivated *via* triggering apoptosis in conventional chemotherapy (Yu et al., 2006). The interaction between an anticancer drug and ultrasound in sonochemotherapy can be a synergism, an addition or an antagonism; cancer type and the drug are the determinants of an interaction (He et al., 2011; Yu et al., 2011). A cytotoxic agent should be carefully selected for a specific cancer, thus realizing an expected synergism.

A smaller volume of $SKOV_3$/ADR tumor suggested that ultrasonic sensitization was effective *in vivo*. How to perform ultrasonic chemotherapy for ovarian cancers should be explored. Under the guidance of medical images, ultrasound can be efficiently delivered into the lesion thereby enhancing an anticancer drug. The interval between drug administration and insonation should be optimized according to the pharmacokinetics. Ultrasound should be applied at the time when the drug level in cancer tissues is high enough and that in the adjacent tissues is below a critical value. Such a manner can improve the therapeutic effect and decrease the toxicity to noncancerous tissues.

Ultrasound has excellent tissue penetration and can be focused on a predetermined volume within the body without harming overlying tissues (ter Haar, 2001). This suggests that cytotoxic drugs can be efficiently released into a lesion when using ultrasound. Sonochemotherapy had the property of a targeted therapy, which produced stronger therapeutic effects at a given dose of drug when compared with conventional chemotherapy. The dose-effect curve paralleled the dose-toxicity one in common chemotherapy, and was shifted left in sonochemo-therapy. Anticancer drugs were released into cancer tissues efficiently when applying ultrasound; thus surrounding noncancerous tissues were exposed to a much lower level of drug. This led to a right shift of the dose-toxicity curve (Figure 6). Sonochemotherapy, there-

fore, improved both the therapeutic and safe indexes.

Free drugs were used in this study. Drug encapsulation has been recently applied for ultrasonic drug delivery (Lentacker et al., 2009; Maeda et al., 2009). In chemoresistant ovarian cancer cells A2780/ADR, the addition of ultrasound enhanced cell-killing due to adriamycin micelles (Rapoport, 2004). Capsulated drugs can be linked to an antibody or a ligand thus improving the tissue selectivity. This was another modality for chemotherapy sensitization using ultrasound (Yu et al., 2006).

In summary, ultrasound provided a non-drug means for sensitizing chemotherapy in resistant ovarian cancers. This modality had the property of targeted chemotherapy, thereby improving both the safety and therapeutic efficacy. Only limited mechanisms were explored in the present study, and further investigations were needed.

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