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● *Original Contribution*

ULTRASOUND REVERSES MULTIDRUG RESISTANCE IN HUMAN CANCER CELLS BY ALTERING GENE EXPRESSION OF ABC TRANSPORTER PROTEINS AND BAX PROTEIN

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Abstract—Multidrug resistance (MDR) is the major obstacle to successful chemotherapy of human malignancies and strategies for overcoming MDR phenomena are still unavailable to clinical use. Previous results showed that ultrasound (US) exposure could make MDR cancer cells become more sensitive to anticancer drugs, and the physical parameters of US exposure could adjust the uptake and retention of rhodamine 123 in MDR cells. In this study, we investigated the mechanisms of therapeutic ultrasound as a physical approach to overcoming MDR in a multidrug resistant human hepatocarcinoma cell line (HepG2/ADM). Our results demonstrated that the percentage of P-glycoprotein$^+$ (P-gp), multidrug resistance-associated protein$^+$ (MRP) and lung resistance-related protein$^+$ (LRP) cells was 96.97% ± 2.41%, 20.84% ± 3.12% and 1.16% ± 0.59% in HepG2/ADM cells, and 62.84% ± 3.42%, 10.26% ± 1.18% and 3.05% ± 0.37% in US-exposed HepG2/ADM cells, respectively. A significant decrease in the number of P-gp$^+$ and MRP$^+$ cells was observed between US-exposed HepG2/ADM and HepG2/ADM cells ($p < 0.05$). Using RT-PCR technique, we found that US could significantly downregulate the expression of P-glycoprotein (P-gp) and (MRP) at the mRNA level in HepG2/ADM cells. Compared with the control, the percentage of apoptotic cell death was significantly increased in HepG2/ADM after ultrasound exposure. Using immunocytochemistry, the percentage of Bcl-2$^+$ and Bax$^+$ cells was 21.7% and 4.1% in the control, and 18.46% and 8.1% in the US-exposed cells, respectively. The percentage of Bax$^+$ cells was significantly higher in US-exposed HepG2/ADM cells ($p < 0.05$), suggesting that US exposure could lead to cellular apoptosis in HepG2/ADM cells. It is concluded that US exposure could reverse MDR in HepG2/ADM cells *via* decreasing P-gp and MRP levels and their mRNA expressions and increasing expression of Bax protein. It may lead to the development of a novel strategy of using a targeted, noninvasive physical approach for the induction of MDR reversal in cancer cells. (E-mail: mfengwu@yahoo.com) **© 2011 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Therapeutic ultrasound, Multidrug resistance, Neoplasm, P-glycoprotein, Multidrug resistance-associated protein, Apoptosis, Hepatocellular carcinoma, High-intensity focused ultrasound.**

INTRODUCTION

Development of resistance to chemotherapy is a major cause of treatment failure in patients suffering a variety of solid cancers (Higgins 2007). This resistance may be due not only to the original drug, or a cocktail, used, but also to a whole range of agents with different structures and cellular targets. This phenomenon is called multiple drug resistance (MDR), which can limit the effectiveness of chemotherapy and is responsible for the overall poor efficacy of drug therapies.

While there are a number of mechanisms that may contribute to the MDR phenomenon, decreased intracellular drug accumulation caused by drug efflux pumps in the cell membrane is one of the most common mechanisms responsible for MDR development in cancer cells. These membrane efflux pumps belong to the superfamily of ATP-binding cassette (ABC) transporter proteins and contribute to drug resistance *via* adenosine-triphosphate (ATP)-dependent pathways. The overexpression of the membrane efflux pumps, including P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), and lung resistance-related protein (LRP), induces MDR phenomenon (Gottesman et al. 2002; Szakacs et al. 2006). In addition, alternations in the pro- or antiapoptotic effect of B-cell lymphoma 2 gene family on tumor cells, such as B-cell leukemia protein 2 (Bcl-2) and Bax protein, have been shown to confer the drug-resistant phenotype in various types of cancer (Yang et al. 2003).

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In the past two decades, a number of strategies have been implemented to overcome cancer cell MDR. Numerous targeted molecules and pharmacologic compounds that modulate ABC transporter-mediated MDR have been identified. They have been successfully shown to inhibit MDR *in vitro* by suppressing and circumventing the mechanisms involved (Shabbits et al. 2001). However, translating this greater understanding into clinical efficacy has seldom been achieved and few of these have so far proved to be clinically useful for reversing the resistance to drugs in cancer patients (Mellor and Callaghan 2008; Persidis 1999). Additionally, physical approaches, such as hyperthermia applied in combination with either chemotherapy or radiation therapy, appeared to be very promising *in vitro* and *ex vitro* studies. But the clinical application of hyperthermia is still characterized by unsatisfactory results due to heat diffusion and inhomogeneous temperature rise in a targeted tumor. Recently, the use of nanotechnology-based drug delivery systems, such as polymer- and lipid-based nanoparticles, has been investigated to overcome MDR phenomena in solid tumors. Some preliminary animal studies have shown that these engineered nanocarriers develop the potential application to enhance drug delivery and overcome MDR by either simultaneous or sequential delivery of resistance modulators, or in combination with energy delivery to enhance the effectiveness of anticancer agents (Schluep et al. 2009; Susa et al. 2009). However, the full potential of these emerging technologies has not yet been fully realized and the toxicology of nanomaterials in humans still needs to be fully studied and evaluated (Jabr-Milane et al. 2008; Malam et al. 2009).

Ultrasound (US) is a form of mechanical energy. It has a long history of use in diagnostic medicine. Medical US has recently taken on a new life in the form of the development of a number of new therapeutic applications (Mitragotri 2005). US is being used, either by itself or in combination with drugs, for the treatment of cancer (Kennedy 2005), diabetes (Prausnitz et al. 2004), stroke (Sacco et al. 2007) and thrombosis (Polak 2004). Although the cell's outer membrane provides a barrier through which extracellular substances find it difficult to pass, US has been shown to open this membrane temporarily, thus, allowing enhanced delivery of drugs, proteins and genes into living cells. This effect is known as sonoporation and it is mediated by ultrasound cavitation (Marmottant and Hilgenfeldt 2003; Mitragotri 2005).

Our recent study has demonstrated, for the first time, that after US exposure MDR cancer cells become more sensitive to anticancer drugs (Shao et al. 2008). The physical parameters of US exposure, including US frequency and duty cycle, can adjust the uptake and retention of rhodamine 123 (Rh123) and adriamycin (ADM) in MDR cells (Shao et al. 2008; Zhai et al. 2008). Rh123, a lipophilic cationic fluorescent dye, is one of the substrates of P-gp. It is usually used as a molecular probe to study the functional activity of P-gp and also to assess the effects of P-gp modulators on reversal of MDR in cancer cells by the determination of intracellular retention of Rh123. ADM is a chemotherapeutic agent for the treatment of a wide range of cancers. These results suggest that US exposure may influence the membrane drug efflux pumps and, thus, induce an increased retention of rhodamine 123 in the MDR cancer cells after intervention, rather than during US exposure. This is different from the previous results that have shown that US can delivery drugs into cancer cells *via* sonoporation effects.

In this study, we hypothesized that US would increase uptake and retention of chemotherapeutic agents *via* inhibiting the functions of ABC transporter proteins and alter the expression of key molecular components of the apoptotic processes in MDR cancer cells. The goal of this study was to investigate the cellular mechanisms of US-mediated reversal of MDR in cancer cells, which may lead to a physical approach that would resensitize the MDR cancer cells to chemotherapy. This novel strategy uses therapeutic US as a targeted tool in combination with chemotherapeutic agents for optimal therapeutic efficacy.

MATERIALS AND METHODS

Chemicals and reagents

Adriamycin (ADM), vincristine (VCR), cisplatin, (CDDP), etoposide (VP-16), 5-fluorouracil (5-Fu), dimethyl sulphoxide (DMSO) and 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilideinner salt (MTT) were purchased from Sigma (St Louis, MO, USA). Rat monoclonal antibody MRPr1 and mouse monoclonal antibody LRP56 were obtained from Caltag Laboratories (Burlingame, CA, USA). Mouse monoclonal antibody MRK16, BCL-2 and BAX were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fluorescein isothiocynate (FITC) labeled goat anti-mouse immunoglobulin G (IgG) was obtained from Tago Immunologicals (Camarillo, CA, USA) and mouse monoclonal IgG was from Chemicon (Temecula, CA, USA). TRIzol and RT-PCR kit were supplied by Life Technologies Inc. (Rockville, MN, USA). Streptavidin–biotin–peroxidase complex (SABC) immunocytochemistry kit was manufactured by Boster Company (Wuhan, Hubei, China).

Cell lines

The human cell line *in vitro* study was approved by the Institutional Review Board (Chongqing Medical University, Chongqing, China), including the purchase