Ultrasound Enhanced Drug Toxicity on Chinese Hamster Ovary Cells in Vitro

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ABSTRACT

Chinese hamster ovary cells (HA1) were exposed to therapeutic ultrasound (f = 2.025 MHz) in the presence of various drugs at temperatures of 37–43°C. The space averaged intensities used were 0.5–2 W/cm². The survival of these cells was subsequently tested using the clonogenic assay. Marked enhancement by ultrasound of the cytotoxicity of Adriamycin and amphotericin B was observed. For Adriamycin, the potentiation was dependent upon the intensity of sonication (exposure duration being 30 min). At 0.5 W/cm², there was enhancement of cytotoxicity above 41°C. At 1 W/cm², there was a 3-order increase in cytotoxicity at 37°C. Thus an increase in intensity resulted in a decrease in “threshold” temperature.

The effect with Adriamycin could be explained in part by an increase in net uptake of drug into the cells. Further, ultrasound was observed to increase the sensitivity of cells to Adriamycin. For amphotericin B, the enhancement was observed only at exposure durations >30 min and at 43°C. There was no enhancement observed for cisplatin and etoposide. From these results, it appears that ultrasound potentiates the cytotoxicity of drugs the mode of action of which (at least in part) involves the plasma membrane.

INTRODUCTION

The effectiveness of chemotherapy in eradicating solid tumors is limited in part by the ability of tumor cells to take up drugs selectively, or ultimately by the cell’s efficiency to actively exclude such drugs. There are many reports that show that temperature elevation beyond 37°C increases effectiveness of many anticancer drugs (1). This increase varies smoothly with temperature as with the nitrosoureas or shows a marked threshold near 43°C (at least at high drug doses) as with Adr or bleomycin (1). The hyperthermia enhanced drug cytotoxicity observed in tissue culture studies has also been reported for in vivo conditions. Thus, Hahn and Li (2) have shown that those drugs that show enhanced drug cytotoxicity in vitro tend to be the ones that are also most effective in vivo at elevated temperatures.

There is mounting evidence to suggest that the sites of action of Adr may be both the membrane and DNA. Thus, Tritton and Yee (3) have shown that when Adr is bound to a matrix such that it cannot enter the intracellular milieu, it still possesses cytotoxic properties in vitro. Neidle (4) showed that on structural grounds, Adr is expected to intercalate with reasonably high affinity to double stranded nucleic acids. Rusconi and DiMarco (5) demonstrated, based on fluorescence measurements, that drug accumulates in the nuclei of treated cells. Hahn and Li (2) have implicated the cell membrane as the target for the hyperthermia enhancement of drug cytotoxicity of some agents (e.g., amphotericin B) but not others (e.g., 1,3-bis(2-chloroethyl)-1-nitrosourea or cisplatin). Certainly, amphotericin B (6, 7) interacts with the membrane to produce its effects. However, cisplatin apparently interacts only with the DNA (8) while VP16 is an inhibitor of topoisomerase II (9).

Ultrasound is sometimes used in the clinic to achieve hyperthermia but is not unique in this aspect. Most equipment utilizes microwaves or lower frequency electromagnetic waves. (As is common practice in ultrasonics, we shall henceforth use the term therapeutic ultrasound to distinguish it from diagnostic ultrasound where the time averaged intensity is about 3 orders of magnitude less.) However, in addition to its ability to heat biological tissue, ultrasound is known to produce biological effects via one or a combination of three nonthermal mechanisms, cavitation, acoustic microstreaming, and radiation pressure (10). These mechanisms are not shared by the other modalities. In this paper we define any nonthermal component of ultrasound cytotoxicity as cell killing over and above that seen when temperature is raised by a hot water bath. The concept of a nonthermally mediated enhancement of drug cytotoxicity by ultrasound is not unreasonable when one considers that cell membrane alterations have been implicated in hyperthermia enhancement of drug cytotoxicity (2) and that ultrasound is known to interact with the membrane to produce biological effects that are not thermally mediated [for review see the report of Williams (10)].

An action of ultrasound upon the cell membrane could affect the rate of penetration of cytotoxic drugs (i.e., a phonophoretic effect). To date, there do not appear to be any published data on any possible phonophoretic effect of ultrasound at different temperatures. Kremkau et al. (11) have shown that the rate of cell killing by some anticancer drugs (melphalan, 1,3-bis(2-chloroethyl)-1-nitrosourea) is increased by simultaneous exposure of the cells to 1 MHz therapeutic ultrasound. They also report a negative effect for busulfan, methotrexate, and vincristine. However, Kremkau et al. (11) could not distinguish between thermal and nonthermal mechanisms of ultrasound action since in their experiments the temperature was not carefully controlled during the irradiation. Thus, temperature elevations of 4–5°C occurred in suspensions exposed to ultrasound. Kremkau et al. further report that nonultrasound production of equal temperature rises in identical cell drug suspensions did not result in cell killing as substantial as that induced by ultrasound.

In this study, we report on the enhancement of cytotoxicity of Adr and amphotericin B but not of cisplatin or VP16. Such enhancement will be shown to be dependent on the exposure duration, intensity of ultrasound, and the temperature of the medium and in part due to an increased net uptake of drug. Further, we suggest that the cytotoxicity of anticancer agents that act on the cell membrane (either in part or in full) is enhanced by a nonthermal effect of ultrasound.

MATERIALS AND METHODS

Cells and Media. Chinese hamster ovary cells (HA1) were grown in spinner culture in Eagle’s MEM F14 containing 7.5% Nusmer (Collaborative Research) and 2.5% fetal bovine serum. Aliquots were removed and centrifuged at 500 × g for 5 min, and the cells were resuspended in serum free medium at a cell density of 10^6/ml. Following experimentation, the cells were spun and washed once in Eagle’s MEM containing serum before plating to determine surviving fractions (taking into account plating efficiency).

Ultrasound Dosimetry. Field plots of the transducers were obtained by activating the transducer at its resonant frequency (f = 2.025 MHz) while it was positioned in a large degassed water tank. A piezoelectric hydrophone was held at a distance of 15 cm in front of the transducer.
The hydrophone was moved in an x, y, or z direction using a micro-manipulator. The position of the transducer was controlled by an IBM AT computer. The signal from the hydrophone was amplified, digitized, and fed into the computer. The data were used to obtain a 2-dimensional intensity map at a plane 15 cm in front of the transducer. The same procedure was repeated at a distance of 16 cm in front of the transducer. These two positions represented the field at the front and the back of the sample holder when this was positioned 15 cm in front of the transducer (see below). Power measurement for the transducer was performed using a modified version of the radiation pressure technique (12). Essentially, the transducer was placed in a beaker containing degassed water. The position of the transducer was such that the beam was directed downwards toward an absorber placed at the bottom of the beaker. The whole jig was positioned over an electronic balance. An amplified sinusoidal wave at the resonant frequency was applied across the transducer. The weight change registered on the balance was recorded. A weight change of 68 mg represents a power output of 1 W. A plot of the power output as a function of the square of the applied sinusoidal voltage revealed a straight line.

Ultrasound Exposure. Fig. 1 shows a schematic diagram of the setup used in exposing HA1 cells in vitro to ultrasound. The cells in suspension (with or without drug) were placed in a hollow “mallet shaped” sample holder. The holder was then positioned in the near field of the ultrasound beam. The sample holder was moved in an elliptical fashion from above with a side to side amplitude of 5–6 mm at a rate of 30/min to reduce settling of cells. The bath temperature can be varied (using the immersed heater) with an accuracy of ±0.1 °C (SD). Exposure was performed at intensities ranging between 0.5 and 2.0 W/cm². The exposure duration was 30–60 min.

Drugs. Four drugs were used in this series of experiments. Adr (Adria Laboratories), amphotericin B (Fungizone; Squibb), cisplatin (Platinol; Bristol Laboratories) and Vp16 (VePesid; Bristol). The drugs were dissolved in sterile saline. This was aliquoted and stored at −20°C until required. Frozen aliquots were discarded after 3 weeks. On the day of the experiment, an aliquot was thawed and dissolved in Eagle’s F14 at a concentration of 1 or 10 µg/ml.

Effect of Ultrasound upon Sensitivity of Cells to Adr Cytotoxicity. HA1 cells were suspended in Eagle’s MEM F14 without serum at a density of 10⁶ cells/ml. Using the sample holder shown in Fig. 1, these cells were either exposed to ultrasound at 1 W/cm² or sham exposed at 41°C for 30 min. Adr (1 µg/ml) was added to the suspension and this was incubated at 41°C for a further 30 min. The cells were then washed once and plated to determine surviving fractions or lysed to measure fluorescence. The latter was performed on a Perkin-Elmer fluorescence spectrophotometer (model LS 3). The excitation wavelength was varied between 300 and 580 nm and the emitted signal was measured at 600 nm. The peak for Adr was 488 nm.

Fig. 1. Schematic diagram of equipment used in exposing cells in vitro to ultrasound. Cells in suspension (with or without drug) were placed in the hollow mallet-shaped sample holder (inset). The holder had a volume of 25 cm³ and a thickness of 1 cm. The holder was positioned in the near field of the ultrasound beam and moved in an elliptical fashion from above with a side to side amplitude of 5–6 mm at a rate of 30/min to reduce settling of cells. The bath temperature can be varied (using the immersed heater) with an accuracy of ±0.1°C (SD). Ultrasound from the transducer traverses the medium and is absorbed at the end of its path thereby setting up a traveling wave.

RESULTS

Effect of Ultrasound on Adr Cytotoxicity. Fig. 2 shows a plot of surviving fraction as a function of temperature when cells were exposed to 1 W/cm² (space averaged intensity) ultrasound in the presence or absence of drug for 30 min. The control curve represents the surviving fraction of cells incubated at the indicated temperature for 30 min but not exposed to ultrasound. Ultrasound on its own caused approximately an order of magnitude decrease in the survival of the cells at all temperatures tested. Adr (1 µg/ml) caused a temperature dependent decrease in survival (30 min exposure). The combined effect of ultrasound and Adr was an enhancement of cell kill at all temperatures. The enhancement was 3 orders of magnitude more than the effect of Adr at the same temperature (even at 37°C). Because the surviving fractions after the combined treatment were so low, we could not determine whether there was a temperature dependence of the enhancement of Adr cytotoxicity by ultrasound at 1 W/cm².

Fig. 3 shows the survival of HA1 cells when the intensity was decreased to 0.5 W/cm². At this space averaged intensity, we still observed enhancement, but the threshold temperature for potentiation was increased from 37°C to 41°C.

Effect of Ultrasound upon Adr Sensitivity. Table 1 compares the resulting surviving fraction of HA1 cells when these were either sonicated or sham sonicated at 41°C for 30 min prior to incubation with Adr at 41°C for a further 30 min. If we subtract the decrease in the surviving fraction due to sonication, then the resulting sensitization to Adr cytotoxicity by ultrasound may account for the remaining 1.5 orders of magnitude increase in cell killing.

There was no significant difference in the fluorescence spectrum of cells that were preexposed to ultrasound at 41°C or...
Fig. 3. Survival of HA1 cells as a function of bath temperature when these were exposed to ultrasound at 0.5 W/cm². Every other condition was similar to the situation in Fig. 2. Note that the enhancement of Adr cytotoxicity was observed at 41°C and above. Control (C), ultrasound alone (x), Adr and sham ultrasound (.), combination (—).

Table 1 Effect of Adriamycin on HA1 cells preexposed to 1 W/cm² unmodulated ultrasound

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Surviving fraction (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.8 x 10^-4 ± 2.0 x 10^-4</td>
</tr>
<tr>
<td>Exposure to ultrasound at 41°C</td>
<td>1.0 x 10^-4 ± 2.0 x 10^-4</td>
</tr>
<tr>
<td>Preheated at 41°C + Adr at 41°C</td>
<td>1.7 x 10^-4 ± 1.4 x 10^-4</td>
</tr>
<tr>
<td>Presonicated at 41°C + Adr at 41°C</td>
<td>1.8 x 10^-4 ± 1.3 x 10^-4</td>
</tr>
</tbody>
</table>

sham exposed prior to incubation in Adr at 41°C for 30 min (plot not shown). This suggests that there was no increase in the net uptake of Adr as a result of presonication. However, fluorescence (at 488 nm) was decreased from the cells that were heated or sonicated in the presence of Adr. Thus, presonication or preheating caused a decrease in the net uptake of Adr. Hence, with presonication we show an increase in Adr cytotoxicity despite a decrease in the net uptake of drug.

Effect of Ultrasound on Amphotericin B Cytotoxicity. Fig. 4 shows that a 30-min exposure of HA1 cells to ultrasound in combination with amphotericin B did not result in enhanced killing at any temperature tested (37–43°C). Fig. 5 shows the results when the ultrasound exposure was extended beyond 30 min. The enhanced cytotoxicity shown here was obtained even though the drug concentration used was decreased 10-fold from the situation in Fig. 4. Also the water bath temperature used here was 43°C. No enhancement was obtained when the temperature was 41°C or below. Further an intensity of 0.5 W/cm² did not result in amphotericin B enhanced cytotoxicity at 43°C for exposures of up to 30 min. In contrast to the situation with Adr, preexposure of HA1 cells to ultrasound at 1 W/cm² did not increase sensitivity to amphotericin B even though such cells were incubated with the drug at 43°C for 60 min.

Effect of Ultrasound on Cisplatin Cytotoxicity. Fig. 6 shows a lack of potentiation by ultrasound of the cytotoxicity of cisplatin (1 µg/ml). A similar negative effect was obtained when the exposure duration was increased to 60 min at 43°C using 1 W/cm².

Effect of Ultrasound on VP16 Cytotoxicity. The surviving fraction resulting from a 30-min exposure of HA1 cells in the presence of VP16 was not significantly different from that observed when the cells were sonicated at 1 W/cm² for 30 min in the presence of this drug at temperatures ranging between 37°C and 43°C.
DISCUSSION

Ultrasound enhanced cytotoxicity of Adr was observed even at 0.5 W/cm² (41°C and 43°C). At 1 W/cm² the potentiation was seen at 37°C. Thus, an increase of 0.5 W/cm² resulted in a decrease in the threshold temperature at which potentiation was observed. The reason for such a decrease is open to conjecture and we can offer no explanation.

There are at least four possible mechanisms for the ultrasound enhancement of Adr cytotoxicity: (a) ultrasound may increase the uptake of Adr; (b) ultrasound may alter the sensitivity of the cells to Adr; (c) ultrasound may alter the Adr molecule to a more potent form and (d) ultrasound may affect the distribution of Adr within the cells. Saad and Hahn (13) showed that sonication of HAI cells in the presence of Adr at 41°C resulted in an increase in the net uptake of drug. Now we have shown that ultrasound also increases the sensitivity of cells to Adr. This increase in sensitivity was not accompanied by an increase in the net uptake of drug when compared to preheated cells. Further, fluorescence was decreased in both presonicated and preheated cells from that of cells where heating or sonication was done in the presence of Adr. This is in agreement with the work of Rice and Hahn (14) who showed that preheating HAI cells in vitro at 45°C for 10 min caused a decrease in the amount of Adr taken up if the drug was administered after heating. This, however, is in contrast to the work of Luk and Tannock (15) who showed that cellular Adr content is directly related to surviving fraction. At present we have no explanation for the sensitization of cells to Adr cytotoxicity by ultrasound.

Ultrasound did not have a direct effect upon Adr. The drug in solution was sonicated for 30 min at 1 W/cm² and 41°C. Cells were suspended in this solution at 41°C for 30 min. The surviving fraction was not increased or decreased when compared to that obtained from cells suspended in a solution at similar temperatures containing sham sonicated Adr. Thus, the potentiation of Adr cytotoxicity by ultrasound can be explained largely by (a) an increase in net drug uptake and (b) an increase in the sensitization of cells to Adr.

The effect of ultrasound upon amphotericin B cytotoxicity was less marked than that of Adr. Thus, for amphotericin B, the effect was observed at 1 W/cm² and for exposure durations of 45 min or longer. The threshold temperature for observance of the potentiation was 43°C. Probably this is a reflection of the pattern of potentiation of amphotericin B cytotoxicity seen with heat alone (1). Thus, there is marked enhancement of cytotoxicity at 43°C (Fig. 5). Presonication of cells prior to incubation in amphotericin B did not increase cytotoxicity to the drug. The mechanism of enhancement of cytotoxicity of Adr by ultrasound may be different from that of amphotericin B. Since we did not obtain any enhancement for cisplatin and VP 16 (which are known to interact with nuclear material to produce cytocidal effects), we are left with observations that point to an effect of ultrasound upon the membrane. Further, this is consistent with other findings suggesting that at the ultrasound intensities used here, the effect of sonication is mainly on the cell membrane (10).

In summary: (a) therapeutic ultrasound potentiates the cytotoxic action of Adr against HAI cells. This effect was intensity dependent. For 0.5 W/cm², the potentiation was observed at 41°C and above. At 1 W/cm², the enhancement was observed at 37°C; (b) ultrasound enhances the cytotoxicity of amphotericin B. Such enhancement was also dependent upon the intensity and requires longer exposures than for Adr. Enhancement was not observed below 43°C; (c) there was no potentiation observed for cisplatin and VP 16. Further work in progress has shown that the enhancement of Adr cytotoxicity is also observed in vivo.

REFERENCES

Ultrasound Enhanced Drug Toxicity on Chinese Hamster Ovary Cells  *in Vitro*

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