

Distinct Sensitivity of Normal and Malignant Cells to Ultrasound *in Vitro*

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The effect of ultrasonic irradiation on the viability of human normal (foreskin fibroblast and amniotic fluid epithelial) and tumor (breast carcinoma, melanoma, and lung carcinoma) cell lines was studied. Cells were subjected to ultrasonic irradiation with a frequency of 20 kHz and an intensity of 0.33 W/cm² for variable periods of time. Several parameters were tested to determine the effects of ultrasonic irradiation on cell viability and cellular function. Normal cells were relatively resistant to ultrasonic irradiation, whereas malignant cells were much more sensitive. Maximum damage occurred 4 min after exposure of the malignant cells to irradiation. Cellular DNA and protein synthesis were significantly affected as a function of time of irradiation and cloning efficiency of malignant cells exposed to irradiation was greatly reduced. To generalize the consistency of the ultrasonic effect, studies on additional normal and malignant human cells of distinct origin are under way to test their sensitivity to ultrasonic irradiation. Thus, the applicability of ultrasonic irradiation as an antitumor agent may be important in the development of a new methodology in the treatment of cancer. — *Environ Health Perspect* 105(Suppl 6):1575–1578 (1997)

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Introduction

Ultrasound is a nonionizing modality of irradiation that is widely used at various frequencies and intensities for diverse purposes, including diagnostic applications, sterilization, and hygiene (1–7). Furthermore, ultrasound is being used for therapeutic purposes such as urological applications, surgical interventions, angioplasty, lithotripsy, and tooth cleaning (8–13).

Ultrasound is also being used in conjunction with hyperthermia, or photo-, radio-, and chemotherapy (sonodynamic therapy) (14–20). It is thought that the combined effect of two or more methods in treatments might render synergistic effects and result in a better therapy.

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Abbreviation used: FCS, fetal calf serum.

The use of ultrasound irradiation in cancer therapy has become an important issue, as ultrasound alone appears to have no deleterious effects. So, based on the premise that a method which selectively affects malignant cells without causing any damage to the surrounding normal tissue is safe, ultrasound could certainly be considered a treatment of choice for at least certain malignant diseases. Thus, this study investigates the effect of ultrasonic irradiation on the viability of normal and tumor cells.

Materials and Methods

Cell Cultures

Human foreskin fibroblast or amniotic fluid epithelial cells were used as normal cells. In addition, several malignant cell lines were studied: two of breast carcinoma, one bearing estrogen and progesterone receptors (I) and one lacking these receptors (II), one of melanoma and another of lung carcinoma (21).

Ultrasound Irradiation

Cell cultures were grown in flasks (TPP, Trasadingen, Switzerland) as monolayers. All the cell lines were grown and maintained in RPMI 1640 medium (Biological

Industries, Beit Haemek, Israel) supplemented with 10% fetal calf serum (FCS; Biological Industries) and antibiotics in a humidified incubator at 37°C and 5% CO₂. For the experiments, cells were plated 2 to 3 days before experimental treatments, reaching about 80% confluency on the day of the experiment. Cells were washed with phosphate-buffered saline and maintained in the same buffer for irradiation. The ultrasonic apparatus used in this study is described elsewhere (21). Cells were irradiated with a continuous wave (CW) ultrasound at a frequency of 20 kHz and intensity of 0.33 W/cm² for several periods of time (up to 4 min). Flasks were supported at a fixed spot at the center of the bath facing the transducer and immersed at a depth of 0.5 cm in the sonication liquid (degassed distilled water). Care was taken to maintain constant temperature to avoid hyperthermia.

Determination of Cell Viability and Cellular Functions

Several parameters were used to test cell vitality:

Vital Staining for the Determination of the Rate of Cell Growth. After ultrasonic irradiation, total cells were collected from each flask, sedimented by centrifugation, and subjected to the trypan blue exclusion test to determine cell viability; dead cells were stained. Nonirradiated control cells showed no detachment from the monolayer and their viability averaged >95%. Detached cells after irradiation were likely to be dead cells, and no more than 5% of the cells were viable. Thus, we concentrated on the cells that remained attached to the surfaces of the flasks, and the number of cells in control cultures were an indication of the total number of the cells in the flask (100%).

³H-Thymidine Incorporation. To determine the rate of DNA synthesis, incorporation into acid-insoluble material of ³H-thymidine (40–60 Ci/mole, Amersham International, Buckinghamshire, United Kingdom) was performed at the end of each ultrasonic irradiation. Cells were maintained in medium supplemented with ³H-thymidine for 1 hr, washed with phosphate-buffered saline, and disrupted in a hypotonic buffer plus 0.5% sodium dodecyl sulfate at room temperature. Acid-insoluble material was precipitated with cold 10% trichloroacetic acid and filtered through GF/C glass filters (Tamar,

Jerusalem, Israel). Radioactivity was determined in a scintillation beta-counter.

³H-Leucine Incorporation. To determine the rate of protein synthesis, we followed the same protocol as described above except that the cells were incubated in the presence of 120–190 Ci/mmol of ³H-leucine (Amersham).

Cloning Efficiency. For cloning efficiency (a measurement of a cell's ability to multiply) determinations after treatments, 200 live cells were plated in a 5-cm dish in the presence of a medium containing 10% FCS. After approximately 2 weeks, when clones become visible, cultures were fixed with methanol, stained with Giemsa, and colonies counted.

Results and Discussion

To determine differences in response to ultrasonic irradiation of normal and malignant cells, cells were exposed to ultrasonic irradiation for various periods of time, and several biological parameters were analyzed. The values obtained with nonirradiated control cultures are referred to as 100%. The results presented in Figure 1 demonstrate that there are major differences in the sensitivity to irradiation of normal and malignant cells. In normal cells, such as foreskin fibroblasts, the number of viable cells was unaffected even after 4 min of exposure to ultrasonic irradiation, but amniotic fluid epithelial cells were somewhat more sensitive. In contrast, malignant cells such as melanoma or breast carcinoma were highly susceptible to growth retardation by ultrasound, showing a clear decrease in the number of live cells after 4 min of irradiation.

To further substantiate this finding, we established the rates of both DNA and protein synthesis after irradiation. Again, malignant cells were much more sensitive than normal cells to ultrasonic irradiation, responding in a significantly reduced rate of thymidine (–75% inhibition after 4 min) or leucine (>90% inhibition after only 1 min) incorporation compared to control unirradiated cultures. Still, some differences were evident in the sensitivity of the various malignant cells. Thus, lung carcinoma cells were somewhat more resistant than other cancer cells to the inhibitory effect of ultrasonic irradiation on leucine incorporation (15, 43, and 74% inhibition after 1, 2, and 4 min, respectively). However, lung carcinoma cells were more sensitive than either type of normal cells, particularly after 4 min of irradiation.

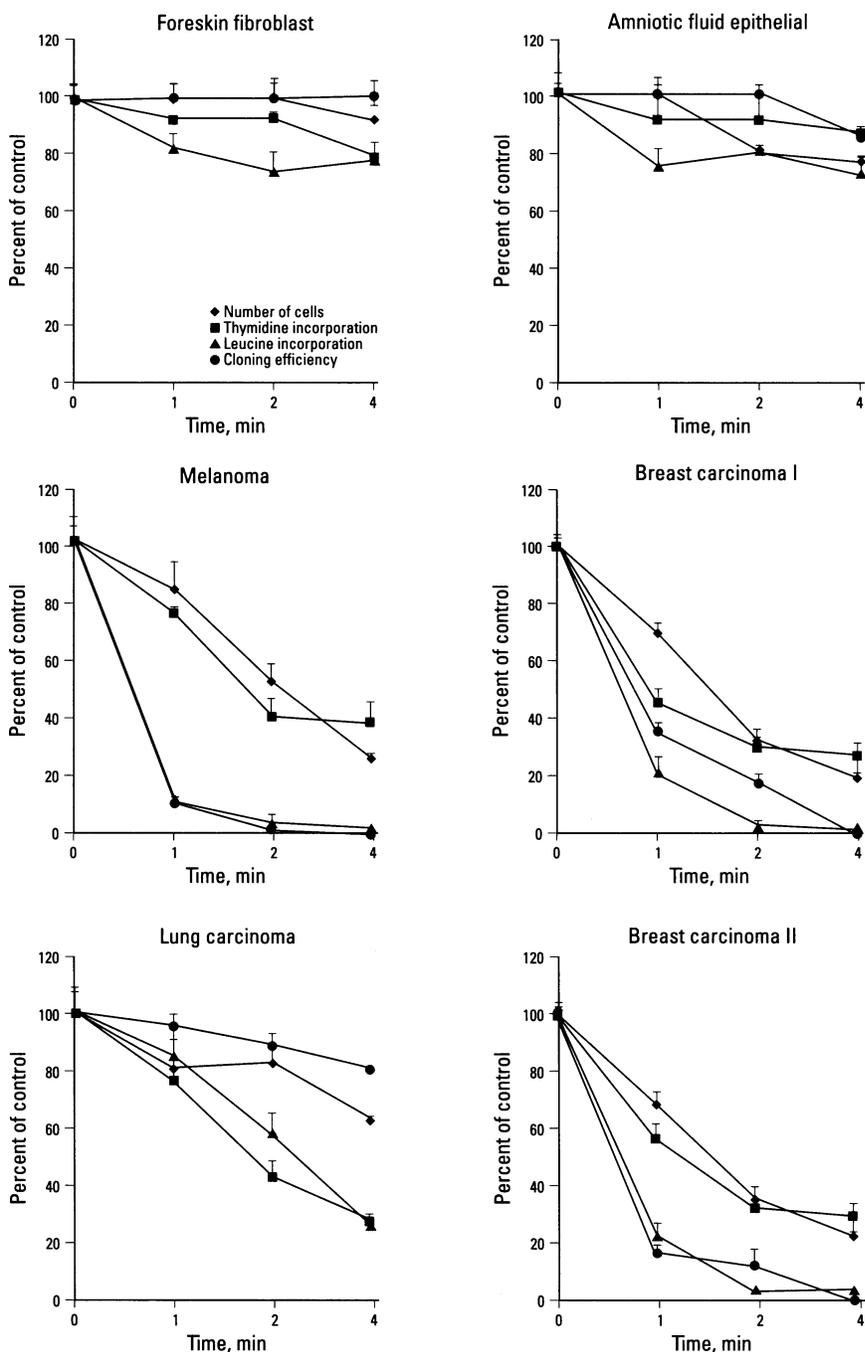


Figure 1. Ultrasonic irradiation effects on human and malignant cells. Different cell cultures were exposed to ultrasonic irradiation for several periods of time, and cells were submitted to viability staining, thymidine and leucine incorporation, and cloning efficiency determination, as described in "Materials and Methods." Each point represents the means \pm SD of 3 to 5 independent experiments. Percent of control as compared to the nonirradiated control cultures (100%) was calculated for each experiment to normalize results.

Finally, additional experiments were performed to assess whether the competence of a cell to form a colony is affected after ultrasonic irradiation. Therefore, the effect in cloning efficiency of the irradiated cells was determined. It is noteworthy that normal

cultures were resistant even after exposure to 4 min of irradiation and compared to control cells no reduction in cloning efficiency was evident. On the other hand, almost total inhibition was observed in melanoma and breast carcinoma after only

1 min of irradiation. Cells derived from lung carcinoma were less sensitive.

Summarizing all the parameters tested for cell viability and cellular functions, we concluded that two normal cell lines were significantly resistant to the effects of ultrasonic irradiation. Exposure to irradiation up to 4 min rendered 12 to 20% inhibition in cell vitality. On the other hand, malignant cells (melanoma and breast carcinoma) were much more sensitive even after 1 min of irradiation (55–65% inhibition), reaching almost 90% inhibition after 4 min of irradiation. Interestingly, cells derived from lung carcinoma were relatively more resistant to ultrasound irradiation.

To support these findings, further studies are under way with other cell lines from different tissue origins such as colon, bladder, glioma, liver, and kidney cancer cells. In general, these cell lines have shown sensitivity to ultrasound irradiation (F Lejbkovicz and S Salzberg, unpublished data).

What is the mechanism by which ultrasonic irradiation manifests cytotoxic characteristics? Biological damage induced by ultrasonic irradiation is determined by frequency and intensity applied. The process of cavitation, the oscillation of microbubbles, could be responsible for cell damage after ultrasonic irradiation (22,23). Following the cavitation process, two different bioeffects were obtained. First was a direct mechanical effect, including cell permeabilization, similar to transdermal drug delivery (24), cell lysis (25,26), or disruption of cell–cell attachment (27). Second, this process may induce formation of free radicals (28–30). Extracellular free radicals are short-lived

molecules that can attack cellular membranes (31). However, intracellular free radicals may induce DNA damage such as single-strand DNA breaks (32) found in Chinese hamster ovary cells exposed to irradiation at 1.61 MHz in a rotating tube system. It is also possible that cavitation generates other sonochemicals that may cause other bioeffects. Long-lived sonochemicals could be responsible for an indirect effect of cavitation and thus should be further explored in the presence or absence of scavengers (33,34).

In addition, cavitation could engender local hyperthermia leading to a reduction in cell survival (35,36). In our experiments this seems not to be the case because in previous experiments we followed the temperature (initial temperature, 25°C) with a thermocouple during continuous irradiation and only after 40 min did we observe an increase to about 42°C (the minimal temperature for generating the thermal effect). In the present study, the maximum irradiation for each treatment lasted 4 min, and it should be noted that an interval was interposed between each treatment.

Cancer cells may be more susceptible to ultrasound than normal cells because their morphophysiological properties are distinct. In addition, sonication may affect cells in division, and because tumor cells have a faster cell cycle expressed as an increased rate of growth, they will be less resistant to irradiation. It is important to point out that cells from solid tumors may have a more rigid membrane than that in normal cells (37), and this may contribute to their increased sensitivity to ultrasound.

According to our observations, it appears that the main outcome of low frequency

ultrasound irradiation is damage to the membrane in the form of either disruption of the cell–cell attachment or an increase in cell permeability. Perhaps cavitation induces disarrangement of the membrane lipid bilayers, which increases permeability of the membrane and leads to loss of cell viability. Further experiments are in progress on cell–cell contact including studies at the microscopic level. Yet, the occurrence of DNA damage after ultrasonic irradiation as suggested by several authors (30,32) cannot be ruled out. If incorporation of thymidine into cellular acid-insoluble material after irradiation reflects any damage to single- or double-strand DNA breaks, our results may confirm the sensitivity of malignant cells.

To expand our results, an *in vivo* system is being constructed to examine more physiological adverse effects following ultrasonic irradiation. Also frequency and intensity should be optimized for each treatment to achieve the maximum safety and obtain the most productive results.

It should be emphasized that there are wide variations in intensities and frequencies used for clinical ultrasound. This was briefly reviewed by Barnett et al. (38) who examined the most common clinical set-ups.

In conclusion, the results presented in this study indicate that certain malignant cells are highly sensitive to ultrasonic irradiation, which suggests that low-frequency ultrasound could be useful for cancer treatment. However, a more widespread, detailed, and cautious study should be conducted before any clinical applications are undertaken.

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