Effects of Various Modes of Sonication with Low Frequency Ultrasound on *In Vitro* Survival of Human Tumor Cells

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The effect of low frequency ultrasound in the cavitation (5-15 sec exposure) and subcavitation (5-210 sec exposure) modes on *in vitro* survival of cultured human tumor cells was studied. Analysis of the dose-effect curves and mathematical estimation of the effects low-frequency ultrasonication in the subcavitation mode on cell membranes helped to choose time intervals of tumor cell sonication ensuring potentiation of the cytostatic effects.

Key Words: *tumor cells; MTT test; low frequency ultrasound*

Ultrasound of various frequencies is now widely used in medicine. It produces a wide spectrum of physiological effects from tissue destruction to stimulation of biological processes. Recent studies demonstrated the possibility of combined use of ultrasound in the cavitation mode and drug therapy [1,3,5,6] and mutual potentiation of their effects. The effect of ultrasound on tumor cells and tissues is mainly attributed to stable and inertial ultrasonic cavitation [8]. On the other hand, cavitation destroys not only tumor, but also normal cells, which prevents its clinical use as a modulator of cytostatic effects. The use of subcavitation modes of acoustic fluctuations can prevent destructive changes and absorption heating of normal adjacent tissues [4,7].

We studied *in vitro* effects of low frequency ultrasound (LFUS) in the cavitation and subcavitation modes (nonlinear effects) on human tumor cells in order to chose the mode of ultrasonic exposure not causing cell death, with the aim of further trials of these modes for modulating the effect of cytostatic drugs.

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MATERIALS AND METHODS

The study was carried out on cultured human cells: mammary adenocarcinoma MCF-7, ovarian carcinoma SCOV-3, and tumor cells isolated from ascitic fluid of 6 patients with stage III-IV ovarian cancer (before treatment and at different stages of drug therapy). The cells were isolated from ascitic fluid by 10-min centrifugation at 1000 rpm in the presence of 10 U/ml heparin. The precipitate was resuspended in RPMI-1640 medium suplemented with 10% embryonic calf serum, 2 mM glutamine, and 10 mg/ml gentamicin. Erythrocytes were lysed with a special buffer (Sigma). The percentage of viable cells was evaluated by trypan blue exclusion (Sigma). Tumor cells were exposed to LFUS in penicillin flasks in 10 ml incubation medium. The concentrations of ovarian cancer cells was 5×10^5/ml, and that of SCOV-3 and MCF-7 was 5×10^4/ml. Ultrasound was generated by an URSK-5N-17 device (26.5 kHz frequency). After ultrasonication in a cavitation (35.5±5 μ amplitude, 5-15 sec exposure) and subcavitation modes (6±1 μ amplitude, 5-210 sec exposure) tumor cells were immediately transferred to 96-well flat bottom plates (Costar) in 200-μl triplets. Intact tumor cells were used as the control. The plates

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were incubated for 1-72 h at 37°C and 5% CO_2. The effect of LFUS was evaluated by its influence on redox activity in ovarian cancer cells in the MTT test [9] using 3-4,5-[dimethylthiasole-2-yl]-2,5-diphenyl tetrasoleum bromide (Sigma). Optical density was measured on an MSS-340 spectrophotometer at 540 nm. Samples without cell suspension served as the control. The effect of LFUS was evaluated by inhibition of redox processed in tumor cells, *i. e.* by the decrease in optical density of formasane formed by the cells. This parameter (expressed in %) reflects the percentage of cell death during the exposure [2].

Acute cytotoxicity of various modes of ultrasonication was evaluated by the percentage of dead cells 1 h after LFUS exposure and the cytostatic effect 24-72 h after incubation.

RESULTS

Ultrasonication in the cavitation mode produced an acute cytotoxic effect directly proportional to the duration of LFUS exposure (Table 1). The effect peaked as soon as 1 h after the exposure and then did not increase during subsequent 72-h incubation. Hence, the effect of LFUS on tumor cells in the cavitation mode is destructive, which means that it can hardly be used as a modulator of the effect of cytostatic drugs.

The use of the subcavitation mode was not associated with acute cytotoxicity: the mean survival 1 h after ultrasonication was 74% for SCOV-3 cells (5-20-sec exposure) and 81% for MCF-7 cells (5-50 exposure) (Table 1). Cell death gradually increased with increasing the duration of treatment (Table 1). This tendency was still observed at later terms (24-72 h). In general, the cytostatic effect of LFUS manifested and increased after 30-sec sonication of SCOV-3 and more than 60-sec treatment of MCF-7 cells (Table 1).

Evaluation of the effect of LFUS on ovarian cancer cells 72 h after exposure confirmed the data obtained on cell cultures (Fig. 1). The cavitation mode was associated with cell death which increased with prolongation of LFUS exposure. After 5-sec exposure 64% cells survived, while after 15 sec only 4% were viable. Ultrasonication in the subcavitation mode for 5-30 sec had no effect on viability of ovarian cancer cells, while after 30-210-sec exposure, the percentage of cell deaths gradually increased: 30- and 210-sec exposure induced death of 8 and 92% ovarian cancer, respectively (Fig. 1). Therefore, LFUS treatment of ovarian cancer cells in the subcavitation mode helped to chose the exposure (3-30 sec) producing neither acute cytotoxic nor cytostatic effects. This mode of tumor cell treatment appears to be perspective for further studies as a method for modulating the effect cytostatic preparations.

At the next stage, we estimated the effects of LFUS in the subcavitation mode on cell membranes. Generally, the effects of LFUS are due to cavitation and acoustic currents. For detecting the relative role of these phenomena, we investigated the effects of changes in the amplitude of oscillations of the waveguide radiating tip in the 35-5 μ range. Other parameters of sonication were unchanged: 26.0 kHz ±7% frequency, 6 mm diameter of the radiating tip, and 15 mm distance from radiating tip to the nearest reflecting surface, *i. e* bottom of the vessel (Fig. 2). The density of cell suspension (1.012 g/cm^3) and its temperature (24°C) were also constant. The threshold acoustic cavitation determined by formation and clapping of cavitations bubbles was recorded at oscillation amplitudes above 10 μ, at preset fluctuation frequency, density and temperature of incubation medium. At amplitudes below 10 μ there were no acoustic noise or decreased optical permeability of the medium near the radiating surface

TABLE 1. Relationship between LFUS Exposure (sec) and Survival of SCOV-3 and MCF-7 Cells in Culture (% of Viable Cells)

Cells	Duration of culturing	Cavitation mode, sec			Subcavitation mode, sec											Number of passages
		5	10	15	5	10	15	20	30	40	50	60	90	120	150	
SCOV-3	1	54	12.5	7.5	82	74	75	66	56	45	45	37	24	12	11	2
	24	60	14	12	96	91	83	71	54	45	45	33	22	11	15	2
	48	45	7	4	85	74	60	57	41	32	32	23	13	7	3	2
	72	38	14	8	90	80	73	68	54	47	42	32	24	20	9	4
MCF-7	1	50	15	15	92	86	86	88	78	74	61	62	41	25	30	2
	24	51	21	15	87	91	87	90	79	76	65	59	44	30	29	2
	48	42	18	11	89	84	73	70	69	62	55	54	35	22	17	2
	72	49	17	15	95	90	88	84	73	72	68	66	48	38	20	5

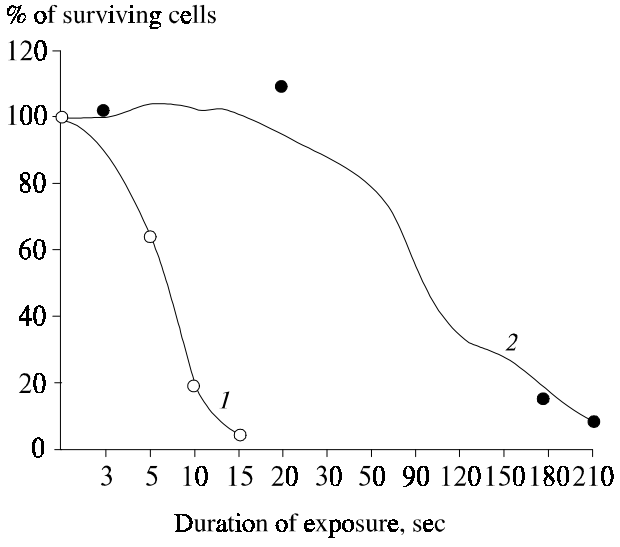

Fig. 1. Relationship between duration of low frequency ultrasonication in the cavitation (*1*) and subcavitation (*2*) modes and survival of ovarian cancer cells.

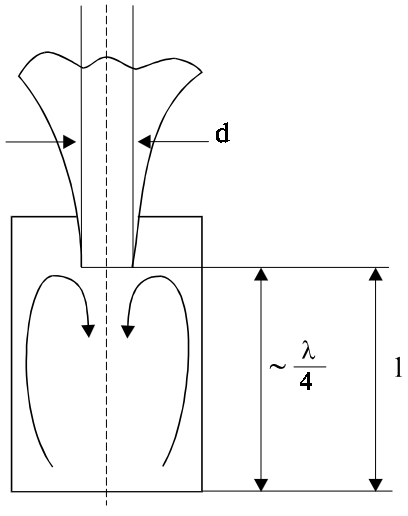

Fig. 2. Scheme of experiment in studies of the effect of fluctuations of the waveguide radiating tip (explanations in the text).

of the waveguide, which are characteristic of developed cavitation. Therefore this mode of ultrasonication can be considered as subcavitation and, hence, acoustic currents are the main factor affecting cell membrane.

For evaluating the mechanical tensions which can develop in cell membrane under the effect of acoustic currents, let us determine the maximum velocity (U_{max}) of suspension particles near the reflecting surface nearest to the source of ultrasound (vessel bottom).

$$U_{max}=\frac{\omega A^2}{d}=\frac{2\pi\times 26\times 10^3(5\times 10^{-6})^2}{3\times 10^{-3}}=1.25\times 10^{-3}\ \text{m/sec},$$

where A is amplitude of the instrument oscillations, w angular frequency, and d diameter of radiating tip of the instrument.

The process takes place in a medium nonlinear by time and coordinate, in which nonlinear phenomena develop due to absorption of ultrasonic fluctuation energy. For simplifying our estimations, let us admit that the discrepancy between phases of oscillation rate and intermittent pressure is not yet great when the distance from the radiating surface of the instrument is relatively little, and therefore it can be neglected. Then the following equation is just:

$$P_{max}/U_{max}=\rho c(t),$$

where r is the density of sonicated medium, c sound velocity depending on the duration of treatment t, and P_{max} pressure in the medium.

For evaluating the sound velocity in experimental medium, we can use the sound velocity value in distilled water, with a relevant amendment. At the beginning of ultrasonic treatment $rc(0)=1.012\times 1595=1614$ kg/m$^2\times$sec. Due to autofocussing of acoustic waves in mixed liquid medium during treatment for up to 60 sec, wave resistance increases by 150-170%. In this case the intermittent pressure will be:

$$P_{max}=U_{max}\times\rho c(0)\times 1.6=1.25\times 10^{-3}\times 1614\times 1.6=3.23\ \text{Pa}.$$

Using the pressure value, we can evaluate the level of mechanical tensions created by acoustic currents in the external membrane of the cell if the ratio of its thickness to cell radium $\delta/R=0.01$:

$$\delta=\frac{P_{max}\times S}{\delta\sqrt{R\times d}}=\frac{3.23\times 4\pi\times(8.0\times 10^{-5})^2}{3\times 10^{-7}\times\sqrt{8\times 10^{-5}\times 8\times 10^{-7}}}\approx 40.56\ \text{kPa}$$

where S is the area of external cell membrane surface.

Continuous radiation of ultrasound in a mixture longer than specified for each of the studied cell strains and human ovarian cancer cells will cause further development of nonlinear acoustic effects, which will result in accelerated mechanical destruction of cell structures.

The estimated values of mechanical tensions are much lower than the threshold mechanical strength of membrane (150-200 kPa). On the other hand, this the subcavitation mode of ultrasonic exposure can be perspective for increasing the permeability of tumor cells for cytostatics.

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