Influence of Terahertz Radiation with a Frequency 0.05 ÷ 1.7 THz on Mitochondrial Membrane Potential of Tumor Cells

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Abstract— In order to ensure safety in the use of terahertz radiation for medical problems, impact assessment of broadband terahertz radiation in the frequency range of 0.05–1.7 THz was made to investigate functional activity of tumor cells in vitro. According to the results of flow cytometry we can speculate that pulsed terahertz radiation with a power density of up to near 10 mW/cm^2 and duration of 1 minute does not change functional activity of tumor cells.

1. INTRODUCTION

In recent years, application of sources of terahertz (THz) radiation is rapidly growing. It is estimated that it can be used in medical diagnosis of skin diseases and skin cancer [1, 2]. However, the divergent data on the effects of THz radiation are questioning the safety of its use. To assess the effects of THz radiation at the cellular level, the mitochondrial membrane potential and the permeability of the cell membrane of certain cells of transplanted crops were investigated after exposure by flow cytometry.

Let us consider the works devoted to the change in membrane potential of cell and membrane permeability when exposed to THz radiation. Thus, radiation at of 2.5 THz results in damage of membrane morphology and intracellular structures, and the fall in the membrane potential of neurons [3], and radiation at a frequency of about 2.31 THz can cause reversible damage of the barrier properties of neuron' membrane [4]. However, other researchers have proved, that using the same object under the radiation of 0.60 and 0.75 THz, all the changes are solely due to the temperature increase [5].

Effect of increasing the permeability of the cell membrane was obtained when exposed to THz radiation with the frequency of 2.52 THz and the power density of 227 mW/cm^2 for over 12 seconds and the decrease was observed when decreasing the duration of exposure [6].

Lipid bilayer demonstrated an increase in membrane permeability when using a pulsed source (0.13 THz, 7 Hz, 7.7 mW/cm^2 for 2 minutes) and lack of any effects from continuous radiation source [7].

Investigators observed both blocking and increased neuronal activity upon the exposure to radiation of 0.06 THz for 1 min under low power density (0.07, 0.28, 0.56 and 0.74 m/cm^2) [11]. These authors have also demonstrated that radiation frequency of about 0.05 THz (15 mW/cm^2, 2 min) induces the opening of ion channels during the exposure, and their sealing 3 minutes after the exposure [8].

Thus, these studies indicate that the terahertz radiation increases the permeability of the membrane and results in changes in membrane potential. However, most of these data were obtained using continuous radiation sources, and the issue concerning the impact of broadband pulse THz radiation remains open. And the relevance of our ongoing work is this is due to this fact.

2. EXPERIMENTAL SETUP

Experimental setup. In the layout of the apparatus, the radiation of femtosecond laser with an Yb : KYW active medium (wavelength 1040 nm, pulse width 120 fs, pulse-repetition rate 75 MHz, mean power 1 W) was modulated by a mechanical modulator and was then fed to an InAs crystal located in a special system with magnetic induction $B = 2.2$ T. THz radiation generated in the InAs crystal was collimated by an off-axis parabolic mirror to a beam splitter plate. The femtosecond radiation reflected from the crystal was absorbed in a fluoroplastic filter. Next, the part of the

radiation transmitted through the beam splitter plate was focused by a lens onto a GC-1P optoacoustic detector, another part of the radiation was directed by parabolic mirror toward the object. The loss of THz radiation on the plate in which the cells are held was 20%. The generated THz radiation had a frequency band of 0.05–1.70 THz with a maximum signal at 0.50 THz, and the THz pulse was 2.5 ps wide, exposure time was 1 min. The irradiation area was 3.14 cm^2. The power density was 9.55, 0.63 and 0.03 $\mu W/cm^2$. Three series of experiments were carried out for each cell culture. The irradiation was done three times in each series at each power. As a control, wells in the plate that were not subjected to the action of the irradiation were used for each of the powers.

Objects. Cell cultures of the following lines were chosen as objects of study: adherent cultures A-549 (carcinoma cells of human lung), BT-20 (adenocarcinoma cells of the mammary gland); suspension cultures U937 (a cell line of leukosis of human histocytes), HL-60 (cells of human promyeloid leukemia).

Evaluation of mitochondrial membrane potential and cellular membrane permeability with flow cytometry. Assay principle. This method is based on using two fluorescent dyes-3,3'-dihexyl carbocyanine iodide ($DiOC_6(3)$) and propidium iodide (PI). $DiOC_6(3)$ belongs to the group of cationic lipophilic dyes, which have been named in literature as "mitochondrial probes", as they are used to study the mitochondrial membrane potential of cells [9]. Due to its lipophilic properties $DiOC_6(3)$ is able to freely penetrate through the lipid bilayer cell membranes (surface membrane of a cell, as well as the inner and outer mitochondrial membrane) and, thanks to the cationic properties, this dye accumulates in the areas with a high concentration of protons, i.e., under the inner membrane of mitochondria. This effect is accompanied by changes in fluorescence intensity of cells in the green subspectrum, and that is recorded during the flow cytometer analysis [10]. If the concentration of protons is reduced, as is the case during the initial stages of the physiological cell death-apoptosis, then the dye will accumulate inside them less effectively, and, as a result, the intensity of its fluorescence will be reduced. Hence, it is possible to distinguish the living cells with effectively functioning mitochondria (and, consequently, of high fluorescence intensity) from dead or dying cells with damaged mitochondrial function. As a result, these cells possess reduced fluorescence intensity. If mitochondrial depolarization is treated as an "early" event when at apoptosis start, the integrity damage of the membrane surface (i.e., its fragmentation) is usually typical for the cells at the terminal stages of their death. In this regard, to identify the different stages of apoptosis, aside from $DiOC_6(3)$, the cells are further stained with PI-dye capable of interacting with nucleic acids of cells. PI can't diffuse through bilayer lipid membranes and, consequently, can't bind with cellular DNA. However, as far as the fragmentation of cytoplasmic and nuclear membranes takes place, the dye enters the cell and interacts with RNA and DNA. This interaction results in accumulation of the dye in cytoplasm and nucleus, and the cell acquires fluorescence in the red subspectrum.

Thus, the living cells will have a bright fluorescence in the channel intended for detecting $DiOC_6(3)$, but won't accumulate propidium iodide (phenotype $DiOC_6(3)^{bright}PI^-$). Cells in the early stages of apoptosis (reduced mitochondrial potential, but plasma membrane still keeps its integrity and impermeability to propidium iodide) will be of phenotype $DiOC_6(3)^{dim\text{-}to\text{-}neg}PI^-$). However, the cells in the late stage of apoptosis or the ones that have already died (necrosis), will not efficiently accumulate $DiOC_6(3)$, but will be stained with P-$DiOC_6$ phenotype $(3)^{dim\text{-}to\text{-}neg}PI^+$.

Staining procedure. To assess the mitochondrial membrane potential the 100 µl of cell suspension ($2–3 \times 10^6$ cells/ml) was added with 20-fold working solution $DiOC_6(3)$ ("Invitrogen", USA) to yield a final dye concentration of 20 nM [11]. The working solution was prepared extempete, adding 10 µl of stock solution (stock −1 mg/ml of dimethylsulfoxide (DMSO), pipetted by 10 µl and stored before use at −20°C), 4900 µl of PBS After adding the dye, the samples were thoroughly mixed and incubated at 37°C under 5% CO_2 atmosphere in a dark place for 20 min. Upon completing the incubation, the samples were washed with PBS containing 2% FBS (8 min at 300 g). Then, the supernatant was removed and the cell pellet was transferred to 100 µl of fresh PBS. The resulting cell suspension was added with 10 µl of propidium iodide solution ("Sigma-Aldrich", USA) to get a final concentration of PI of 1 ug/ml. Further, the samples were incubated for 10 min at room temperature, protected from light. Upon completion of incubation, the samples were added with 200 µl of PBS, and the cytometric measurement was performed. At least 50,000 single cells were analyzed for each sample. To distinguish single cells from sticking together (aggregates), and subsequently discriminate aggregates from the assay, the following combinations of signals on forward (a value proportional to the cell size) and side (a value characterizing the cell structure) scattering-peak intensity against the intensity of the integral signals of FS or SS, as well as the flight time against

the intensity of the integral signals of FS or SS. Analysis of results was performed using Kaluza software ("Beckman Coulter", USA).

3. RESULTS

To analyze the results of flow cytometry the histograms of fluorescence intensity of $DIOC_6(3)$ have been plotted, the increase in fluorescence intensity of which depends on mitochondrial membrane potential. Fig. 1 demonstrates one typical example of such histogram for each cell culture used

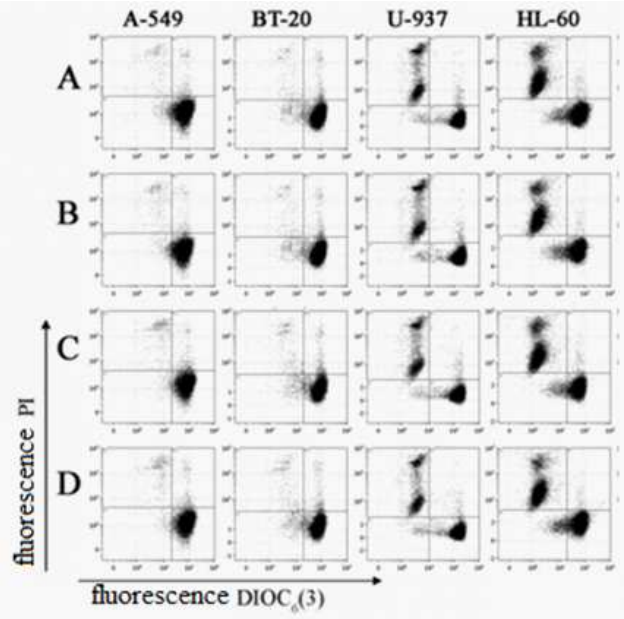

Figure 1: Examination of mitochondrial membrane potential using fluorescent dye $DIOC_6(3)$. X-direction: $DIOC_6$ fluorescence intensity (3); Y-direction: propidium iodide fluorescence intensity. Left to right — different cell lines. Downwards are the examples of histograms of cell distribution in control wells and in radiation-exposed wells with the power of 0.1; 2 and 30 mW (series of histograms A, B, C and D, respectively).

Table 1: The evaluation results the mitochondrial membrane potential and the permeability of the cell membrane using flow cytometry ($X \pm s$, $n \geq 4$ for each culture).

Cell culture	Radiation power, mW	Living cells, %	Early stage of apoptosis, %	Late stage of apoptosis/necrosis, %
A-549	Control	95.9 ± 0.5	1.2 ± 0.7	2.8 ± 0.3
	0.1	96.2 ± 0.3	1.2 ± 0.4	2.6 ± 0.3
	2	96.3 ± 0.4	0.7 ± 0.2	3.0 ± 0.3
	30	96.4 ± 0.4	0.7 ± 0.2	2.9 ± 0.2
BT-20	Control	93.3 ± 0.2	2.6 ± 0.5	4.1 ± 0.4
	0.1	93.1 ± 0.2	2.5 ± 0.4	4.4 ± 0.6
	2	93.3 ± 0.3	2.4 ± 0.5	4.3 ± 0.3
	30	93.2 ± 0.3	2.7 ± 0.5	4.1 ± 0.2
HL-60	Control	65.2 ± 7.7	4.1 ± 1.2	30.7 ± 8.4
	0.1	66.4 ± 7.7	4.3 ± 1.4	29.3 ± 8.7
	2	66.6 ± 8.5	3.9 ± 1.3	29.6 ± 9.5
	30	67.7 ± 7.3	4.0 ± 1.2	28.4 ± 8.1
U-937	Control	56.3 ± 8.6	2.4 ± 0.3	41.3 ± 6.5
	0.1	55.9 ± 6.4	2.5 ± 0.3	41.6 ± 6.5
	2	56.4 ± 6.7	2.8 ± 0.3	40.8 ± 6.5
	30	58.1 ± 6.5	2.3 ± 0.3	39.7 ± 6.6

during the experiment.

The obtained histogram data have been processed and presented in Table 1 showing the percentage of living cells and cells that are at the early and late stages of apoptosis.

4. CONCLUSION

Thus, on various tumor cell cultures such as adherent cultures (carcinoma cells of the human lung, adenocarcinoma cells of the mammary gland) and suspension cultures (a cell line of leukosis of human histocytes, and cells of human promyeloid leukemia) using flow cytometry it is shown that the action of pulsed broadband terahertz radiation in the frequency range of 0.1–1.7 THz with the power of 30, 2, and 0.1 μW has no significant effect on the change in the functional activity of cells mitochondria, as well as does not damage the integrity of their lipid bilayer surface membranes.

However, the lack of effect may be due to the fact that the cells are in an aqueous medium, which is absorbed greatly in terahertz spectral range. Despite the fact that this assumption does not explain the absence of any effect on adhesive culture monolayer cells such as A-549 and BT-20 which are tightly adjacent to the substrate surface. Based on this fact, additional researches with cell cultures are needed, involving various duration and power of irradiation.

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