Cell proliferation under intense pulses of terahertz radiation

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Abstract. Terahertz (THz) waves can influence a diverse spectrum of cellular processes. In the present study, we focus on the effect of high-power broadband THz pulses on cell culture. An experimental setup for long-term irradiation of cells with THz pulses with intensities of about 32 GW/cm\textsuperscript{2} has been developed. Human skin fibroblasts were exposed to THz pulses with a field strength of about 3.5 MV/cm for 90 min. Preliminary results on the THz-induced apoptosis process in cells are presented.

1. Introduction
The use of terahertz (THz) radiation is rapidly increasing both in basic research and in various practical areas (security and military applications, medicine, etc). Nevertheless, certain concerns have been expressed about the possible health impacts associated with THz radiation. The THz-BRIDGE international project conducted in 2001–2004 was devoted to studying the interaction of THz radiation with biological systems. The official conclusion noted a possible genotoxic effect (detected in lymphocytes) of THz radiation as well as a change in the permeability of liposome membranes under certain conditions. Nevertheless, the exact conditions and parameters of the THz exposure leading to such effects remained unclear.

After the completion of the THz-BRIDGE project, quite a lot of research has been carried out on the bioeffects of THz radiation [1–4]. A wide range of processes have been investigated in an attempt to understand the mechanisms of interaction of THz radiation with cells. They include the gene expression alteration [5,6], cellular death and membrane permeability [7,8], and even deoxyribonucleic acid (DNA) double-strand breaks [9–11]. The latter is considered to be one of the most dangerous types of DNA damage, since it can lead to cancer or cell death. At the same time there are plenty of studies demonstrating no effect of THz radiation on \textit{Escherichia coli} and human corneal epithelial cells (neither mutagenic [12] nor genotoxic effect [13]), on viable human blood leukocytes (no direct DNA damage [10]), on the keratinocyte differentiation
and viability [14], and on the mesenchymal stem cells (MSC) morphology and viability [15]. For now, variations in the cell types studied and in the experimental conditions, including frequency, power levels, and duration of exposure, make it difficult to draw firm conclusions regarding the characteristics of THz radiation that induce a response in living cells. Hence questions regarding the positive and–or negative effects of THz radiation on cells are still a matter of controversy and require a further study.

2. Experimental setup

Optical rectification is known to be an efficient method for generating THz radiation. In addition to efficiency, its advantages include shot-to-shot radiation stability and high beam quality, which allows tight focusing and enables the highest field strengths to be achieved. Recently, Cr:forsterite laser system has been used to demonstrate a high conversion efficacy (up to 3%) to generate broadband THz radiation in organic crystal [16]. Such a laser system was used in our experiments. The energy and pulse width at the output of the system reached $1.2 \pm 0.05 \text{ mJ}$ and $95 \pm 5 \text{ fs at the full-width half-maximum (FWHM)}$, correspondingly. A nonlinear organic crystal, OH1 (2-[3-(4-hydroxystyryl)-5,5-dimethylcyclo-hex-2-enediylidene]malononitrile), 6 mm in diameter and $420 \pm 10 \mu\text{m}$ thick (Rainbow Photonics, Switzerland) was employed to obtain THz pulses. To cut off laser pump radiation, THz filter LPF8.8-47 (Tydex, Russia) with 70% transmission in the THz spectral range was placed behind the organic crystal. To measure the energy of THz pulses that reached $18 \pm 0.5 \mu\text{J}$, a calibrated Golay cell (optoacoustic detector GC-1D, Tydex) and a 10% silicon wafer attenuator (Tydex) were installed immediately following the OH1 crystal.

In order to subject the cells to high-intensity THz pulses, the radiation should be focused to a spot of a minimal diameter. Thus, a telescope consisting of two off-axis parabolic mirrors (with reflected focal lengths of 15 and 152.4 mm) was assembled to expand the THz beam after the OH1 crystal. A $22^\circ$ off-axis parabolic mirror (MPD229H-M01, Thorlabs) with a 23 mm hole parallel to the focused beam and a reflected focal length of 50.8 mm was used to focus the THz beam.

The pulse duration and spot size were measured using a well-known electro-optic sampling technique and knife-edge method to determine the strength of the THz field. The waveform and the spectrum of a THz pulse are demonstrated in figure 1(a). The pulse duration can
Figure 2. Experimental setup for cell irradiation. (a) Schematic: 1—source of fs laser pulses; 2, 5—iris diaphragms; 3—attenuation unit; 4—lens telescope; 6—THz crystal with filter; 7—mirror telescope (1 : 10); 8—focusing parabolic mirror; 9 and 9’—Petri dish in THz and video channels, respectively; 10—cells; 11—heating plate with a lid; 12—3D motorized translation stage; 13—microobjective; 14—video camera; 15—dried box. (b) Photo of the setup in the dried box (heating lid and Petri dish are not shown).

be derived from the Gaussian envelope approximation of the registered electric field waveform, \(\tau_{\text{THz,E}} = 0.685\ \text{ps FWHM}\). Generally, the pulse duration implies the full width at half maximum value of the intensity profile (not the field one). Thus, the presented Gaussian function should be squared; the duration of a THz pulse is then considered to be \(\tau_{\text{THz,1}} = \tau_{\text{THz,E}}/\sqrt{2} = 0.484\ \text{ps}\).

The minimum FWHM sizes of the focused THz beam were 308 and 270 \(\mu\text{m}\) in the \(X\) and \(Y\) directions, respectively. For simplicity, in the subsequent calculations the spot was considered to be round in shape with the diameter \(d_{0.5} = 290\ \mu\text{m FWHM}\); this corresponds to a value of \(d_{1/e} = d_{0.5}/\sqrt{\ln(2)} = 350\ \mu\text{m}\) at \(1/e\) level. The Rayleigh length of the THz beam was measured to be about 750 \(\mu\text{m}\). Knowing the energy, duration, and the spotsize, intensity and electric field strength of the THz pulse were estimated to be 32 GW/cm\(^2\) and 3.5 MV/cm correspondingly. Detailed information on measuring the parameters of a THz pulse and the electric field strength estimation is given elsewhere [17].

A heating plate with a lid (heating system, Ibidi) was used for long-term cell irradiation placed in a standard 35-mm Petri dish. It was mounted on a 3-dimentional motorized linear stage (8MT167-100 along \(X\) and \(Y\) axes, 8MT173-20 along \(Z\) axis, Standa), with a tilt opportunity about \(X\) and \(Y\) axes to align the dish in the horizontal plane. The cell exposure was performed by focusing the THz radiation with a parabolic mirror and directing it through the bottom of a plastic dish with a cell monolayer attached.

The entire experimental setup presented in figure 2(a) is described in detail in [18]. It was assembled in a housing filled with dry air figure 2(b). An air compressor (SB4-100.OLD15T, Remeza) and an adsorption dryer (ADS3, Ceccato) made it possible to locally reduce the relative humidity of the air, typically to 2–3%, within about 10 min, thereby minimizing THz absorption in the air.
3. Methods

3.1. Object of investigation
The cell line of human skin fibroblasts was chosen as the object of the study. Skin fibroblasts are located under the skin basal layer and are responsible for the production of the main components of the skin extracellular matrix. Along with keratinocytes, skin fibroblasts are among the first targets exposed to THz radiation. In addition, skin fibroblasts are more sensitive to apoptosis than mesenchymal stem cells (the object of our previous studies [18,19]) for certain toxic agents [20]. For the experiment, the characterized line of skin fibroblasts was selected from the cell bank of the Federal Research and Clinical Center of Federal Medical-Biological Agency.

Human skin fibroblasts were taken from a cryobank and slowly thawed in a water bath. The resulting sediment was washed from the freezing medium and resuspended in the DMEM/F12 (Dulbecco’s modified eagle medium with nutrient mixture F-12, Gibco, USA), mixed with 10% fetal bovine serum (Gibco, USA), 2 mM L-glutamine (Gibco), antibiotic-antimycotic solution (1X; Gibco, USA), then seeded on culture plastic at a density of \(1 \times 10^5\) cells/cm\(^2\) and incubated at 37 °C and 5 vol % CO\(_2\). When cells reached the confluence more than 70%, they were trypsinized (TrypLE\(^\text{TM}\) Select CTS\(^\text{TM}\), Gibco) and subsequently passaged in 35 mm culture-insert 4 well \(\mu\)-dishes (80466, ibidi) with special inserts and polymer coverslip (89% transmission [17] in THz region). Each of the four wells of the insert was seeded at a density of about \(1 \times 10^5\) cells/cm\(^2\) one day prior to the experiment. In order to maintain the acid-base balance of the medium during the irradiation, a HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) solution was added in advance.

3.2. Cell irradiation
THz treatment was performed in a single well of a 4 well culture-insert; cells in the remaining wells were considered the control samples. To keep the optimal cell temperature during irradiation, the dish was placed into the heating system (ibidi) mounted on the three-dimensional (3D) motorized stage. The treated area was about 720 \(\times\) 630 \(\mu\)m\(^2\) that was equal to the THz spotsize at the 1/e\(^2\) level. To facilitate finding the site of THz irradiation within the dish, the bottom surface was marked by four laser shots (THz filter and OH1 crystal were temporary removed) at a distance of 300 \(\mu\)m aside in each direction. The experimental system developed for cell irradiation allowed the intensity of the THz pulses to be as high as 32 GW/cm\(^2\). The resulting electric field strength reaching the cells was 3.5 MV/cm.

3.3. Cell viability assay
The study of cell survival under the exposure to THz radiation was performed using immunocytochemical analysis of proapoptotic enzymes (caspase 3) and mitochondria morphology (human Mito). The cells were fixed with a 4% buffered formalin solution containing 0.1% saponin, then incubated sequentially with primary antibodies to human Mito (Abcam, USA, 1 \(\mu\)g/ml), caspase 3 (ThermoFisher, USA, 1 \(\mu\)g/ml) and washed. After that secondary antibodies conjugated with Alexa Fluor 488 (1 : 400; Invitrogen, USA) and Alexa Fluor 633 (1 : 400; Invitrogen, USA) were added. Hoechst 33342 dye (Thermo Fisher Scientific, USA) was used to stain the cell nuclei. The immunofluorescence analysis was performed using a Celena\(^\text{R}\) Digital Imaging System (Logos Biosystems, South Korea). Human Mito, caspase 3, and Hoechst 33342 are represented by the attributed colors of green, red, and blue in figure 3, respectively.

4. Results and discussion
Apoptosis, a natural cell death, is regulated by a cascade of enzymes such as bcl2 and caspase families. Caspase 3 plays a central role in apoptosis as an effector caspase [21]. If apoptosis occurs via the mitochondrial pathway, caspase 3 may participate in the regulation of reactive oxygen species (ROS) release. Typically, mitochondria maintain the membrane potential (\(\Delta\Psi_m\))
and shuttle electrons through electron transport chains (ETCs) with minimal ROS formation, which can occur in complex III. During the stimulation of apoptosis, the loss of cytochrome-C from mitochondria occurs, which is necessary for the transfer of electrons from complex III to IV, which leads to the loss of electrons due to the formation of ETC and ROS. If the import of substrates into the ETC stops due to a loss of $\Delta \Psi_m$ or the electron transport through complex III is blocked, the production of ROS decreases. Therefore, caspase 3 is necessary to inhibit the electron transport through the ETC below $\Delta \Psi_m$ and–or to reduce the production of ROS. This indicates that caspase 3 is a key link in apoptosis and inactivation of mitochondria.

Assuming that cells are quickly repaired and restore their vital activity, it was of interest to study the effect of cell fixation time post irradiation on the fluorescence intensity of proapoptotic enzymes and mitochondrial morphology. A series of experiments were conducted to determine irradiation parameters causing metabolic abnormalities. For a given exposure time of 90 min, the effects were studied at different times after the experiment—0.5 and 24 h. To obtain statistically reliable data, each study was repeated thrice.

Figure 3. Immunofluorescence pictures of fibroblasts: experimental groups for 90 min of exposure, cell fixation performed 0.5 (a) and 24 h (b) after irradiation; parallel control groups (c) and (d); green—human Mito; red—caspase 3; blue—Hoechst 33342.
We found a slight increase in the amount of proapoptotic enzymes such as caspase 3, shown in figure 3(a, c), compared with the parallel control group in figure 3(b, d). It should be noted that the increase in caspases as a result of the THz effect on the cell does not lead to its death.

This indicates that the observed increase in the number of caspases in the experimental group indicates a violation of the oxidative balance in the cell and an increase in the release of ROS. However, the immunocytochemical analysis of cells has demonstrated the absence of changes in both the cell morphology (neither the cells nor their nuclei were destroyed), and the morphology of their mitochondria (shape, size and amount remained without significant changes). The latter finding indicates that the increase in the ROS production is not significant, i.e., not fatal to cells.

5. Conclusions
An experimental setup has been created for long-term irradiation of a monolayer of cells with intense THz pulses. The energy, duration, and spectrum of the generated THz pulses, as well as their spatial distribution, were measured in order to estimate the electric field strength. Broadband (0.2–3 THz), sub-picosecond (τTHz ≈ 0.5 ps) THz pulses with an energy of 18 µJ were focused to a spot of 290 µm FWHM in diameter and delivered through the plastic coverslip of a Petri dish, resulting in a peak intensity of up to 32 GW/cm² and the electric field strength of 3.5 MV/cm.

Human skin fibroblasts were used as a cellular model for the investigation of the bioeffects of THz radiation. Cells in the form of a monolayer were exposed to THz pulses for 90 min and divided into two experimental subgroups differed in the fixation time after the irradiation. Cells were fixed 0.5 and 24 h post-exposure in the first and the second group, respectively. It has been demonstrated that the effect of THz radiation on cultured fibroblasts consists in the activation of the cascade of proapoptotic enzymes and inhibition of the ROS action on cells, not leading to cell apoptosis.

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