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Influence of femtosecond laser radiation on cells of the transplantable tumour Krebs-2

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Abstract. **The influence of femtosecond radiation of a titanium-sapphire laser on cells of the transplantable ascitic tumour Krebs-2 was studied. After *in vitro* irradiation by the pulsed fundamental-harmonic radiation with the wavelength 800 nm, pulse duration 30 fs, repetition rate 1 kHz, mean power 100 and 300 mW and exposure time 3 min, as well as by the second-harmonic radiation (40 nm, 50 fs, 120 mW), all cells were diffusely stained by the vital stain trypan blue, which may be an evidence of their death or abnormalities of membrane permeability. However, implantation of such cells to experimental animals led to formation of tumours at the transplantation site with the kinetics slightly different from the control one. In the group of mice to which the cells were inoculated after irradiation with second harmonic pulses of titanium-sapphire laser the inhibition of tumour growth was observed due to partial death of cells under the action of UV spectral components. To explain the mechanism of the observed phenomenon the possibility of pore formation (photoporation) in the cell membrane, described earlier in the papers on foreign DNA transfection into cells, is considered.**

Keywords: *titanium-sapphire laser, femtosecond radiation, Krebs-2 tumour cells, trypan blue, photoporation, in vivo kinetics of tumour growth.*

1. Introduction

From the moment of their invention lasers were an attractive instrument for acting on cell structures. In the very first experiments by M. Berns on the action of micro-focused (up to 0.5 μm) laser radiation on intracellular structures (1970) it was shown that the most vulnerable structure is the cell nucleus rather than mitochondria, ribosomes or membranes. Direct action of argon laser radiation (488 nm) or the second harmonic of an Nd:YAG laser (532 nm) on the nucleus caused multiple chromosome aberrations [1].

In 1984 it was proposed to use focused laser beams for introducing foreign DNA into cells (transfection) [2]. In the case of transient transfection it is enough to introduce DNA into the cell for a short time necessary for its expression. In this case the transfected DNA is usually not included into the nuclear genome and is not replicated, but is lost in the course of cell reproduction. Therefore, for the transfection procedure it is necessary to make the cell membrane permeable for DNA, e.g., via the formation of pores.

Different lasers were used for optical transfection, and the efficiency of radiation influence on membranes was also different [3]. In the first paper on optical transfection the authors used pulsed radiation of a nanosecond laser with the wavelength 355 nm, focused into a spot up to 0.5 μm in diameter. It was assumed that the main mechanisms of damage are the local heating and the thermoelastic shock of the cell. The transfection efficiency was 0.3%–38% of all exposed cells. A cw argon laser and the second harmonic of a nanosecond Nd:YAG laser were also used for transfection. However, the maximal efficiency of transfection (over 90%) was achieved only with femtosecond radiation of a titanium-sapphire laser.

The use of a near-IR laser with a femtosecond pulse duration for optical transfection was proposed in 2002 by Tirlapur and König [4]. Besides high efficiency of transfection this laser is characterised by principally different mechanism of the cell membrane damage, probably based on multiphoton effects and generation of low-density plasma [3].

Detailed investigation of the action of femtosecond near-IR radiation on cells has shown that when the peak power of laser radiation is high, the local damages of membrane referred as photoporation occur [5]. Under the optimal choice of the laser radiation power these damages are transient and after some time the pores become closed thanks to the mechanisms of cellular reparation. It is accepted that the reversible local damages of the cell membrane occur at the energy density ~ 0.1 μJ m^{-2} on the cell membrane [5].

In the experiments on cell transfection the titanium-sapphire laser with the wavelength 800 nm, pulse duration about 120 fs and repetition rate 80 MHz is used most often. The radiation is strongly focused on the monolayer of cells, placed on a coordinate table, by means of an objective with a high numerical aperture (NA ~ 0.85) [2, 3]. The exposure time varies from 10 to 250 ms at the mean power 50–100 mW.

To prove the existence of local cell membrane damages the methods of optical microscopy with vital stains, such as trypan blue, are used [5]. After *in vitro* treatment of Chinese

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hamster ovary cells with the femtosecond laser radiation they were stained with trypan blue. Both dead and living cells with local damages of membranes were present in the cellular array. Hence, the precise differentiation of dead and living cells was not possible. Incubation of cells with foreign DNA and its further assessment in the living cells required application of complex methods of molecular biology; moreover, in this case many additional factors appeared that led to an increasing error in measuring the transient transfection efficiency.

In the present paper the photoporation effect is for the first time reported in the cells of Krebs-2 tumour. The control of functional activity of cells after irradiation was implemented via measuring the kinetics of the tumour growth *in vivo* after their implantation to experimental animals. It was revealed that due to the optical amplification of femtosecond pulses the irradiation of cells does not require strong focusing of radiation and, therefore, simultaneous exposure of a large cellular array is possible.

Further investigation of femtosecond laser photoporation of tumour cell membranes may become a basis for developing highly efficient methods of introducing chemotherapeutic preparations into the cell.

2. Samples and methods of study

Transplantable tumour strain Krebs-2 was maintained at the Institute of Cytology and Genetics, the Siberian Branch of the Russian Academy of Sciences in the ascitic form in CC57BR/Mv linear mice. Just before irradiation the cells were extracted, placed in a test tube and diluted with saline up to the concentration of 1 million cells per 1 mL of solution. For irradiation the cells were put into a standard 96-cell tray in the volume of 200 μL. Viability of cells before the irradiation was determined by staining with 0.1% trypan blue. Counting of stained cells was performed in the Goryaev chamber. In the control probes the fraction of died cells did not exceed 3%.

The cells were irradiated with the femtosecond titanium-sapphire laser (Femtopower compact Pro). For irradiation we used the fundamental harmonic of the laser with the wavelength 800 nm, pulse duration 30 fs, repetition rate 1 kHz, and mean power 100 and 300 mW, as well as the second harmonic with the wavelength 400 nm, pulse duration 50 fs, repetition rate 1 kHz, mean power $P_{2\omega}$ = 120 mW, and pulse energy 0.12 mJ.

The optical scheme of the setup is presented in Fig. 1. The 16-mm diameter laser beam with the Gaussian intensity transverse distribution was compressed using a long-focus lens to the size of a tray cell which was equal to 6 mm. The parameters of laser radiation (mean power, pulse duration, intensity distribution in the beam) were measured directly after the folding mirror.

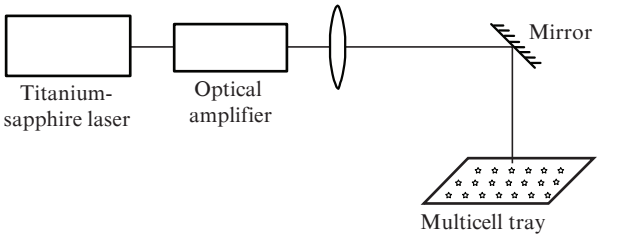

Figure 1. Schematic diagram of the experimental setup for cell irradiation.

The exposure time for each tray cell containing the tumour cells was 3 min. After irradiation a part of tumour cells was stained with trypan blue to determine the fraction of 'living' cells, and another part of cells was inoculated to experimental animals, the four-month-old male mice of the CC57BR/Mv line, received from the Laboratory of Experimental Animals of the Institute of Cytology and Genetics, the Siberian Branch of the Russian Academy of Sciences. The tumour cells were injected into the hip muscle in the doze of 100 thousand cells in the volume of 100 μL. The control group of animals was inoculated with tumour cells that passed the same procedure, including extraction, dilution and placing into the tray cells, except irradiation. Then, in the course of the tumour growth, three projections of the hip were measured with trammels in living animals to calculate the effective volume of the tumour. In each of the four groups there were five animals. Cells without irradiation were implanted to the mice of the first (control) group, cells after irradiation with fundamental harmonic having the mean power P_ω = 100 mW were implanted to the mice of the second group. Cells after fundamental harmonic irradiation with the mean power P_ω = 300 mW were implanted to the third group, and after irradiation with the second harmonic having the mean power $P_{2\omega}$ = 125 mW to the fourth group of mice.

Generally, the time between the extraction of tumour cells from the donor mouse and the implantation after irradiation did not exceed three hours.

3. Results and discussion

It was found that in the control group (without irradiation) not more than 3% of cells were stained by trypan blue, while after irradiation by the fundamental harmonic of the titanium-sapphire laser with P_ω = 300 mW or by the second harmonic with $P_{2\omega}$ = 125 mW all 100% of cells were diffusely stained. However, when these 'conditionally dead' cells were implanted to mice, the growth of a tumour was observed in all animals. The curves illustrating the tumour growth for all groups of animals are presented in Fig. 2. It is seen that the dynamics of the tumour growth in the group of mice inoculated with the cells, irradiated by the fundamental harmonic radiation with mean power values 100 and 300 mW, is practically the same as in the control group. In the general case the kinetics of the tumour growth is described by an exponential law [6]

$$F = F_0 e^{\varphi t}, \quad (1)$$

where F is the effective size (volume) of tumour; φ is the function, characterising the specific rate of the tumour growth.

It is worth noting that the growth of the Krebs-2 tumour is relatively slow. Due to this fact, the kinetic curves are plotted for the initial stage of growth, when the exponential dependence is expressed not quite clearly.

The tumours of different types are characterised by different rates of growth. Besides, the specific rates of growth even for one type of cells may differ when transplanted to different lines of animals. Thus, e.g., as far as we know, the lymphosarcoma tumour in the mice of hybrid line CDAxC57B1 without treatment grows with the specific rate 0.21±0.02 mm^3 per 24 hours (statistics of 5 animals), while in mice of the CBA line this rate is 0.46±0.06 mm^3 per 24 hours (statistics of 36 ani-

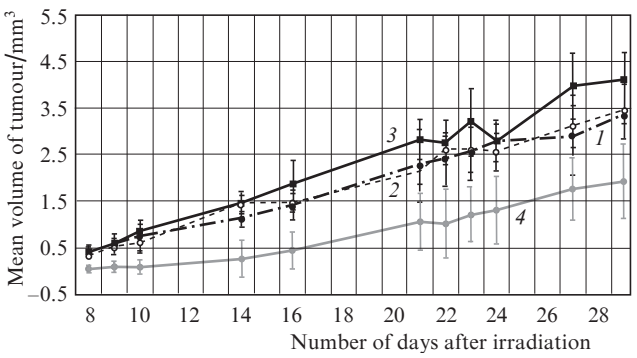

Figure 2. Kinetics of tumour growth after implantation of irradiated cells to experimental animals: (*1*) cells without irradiation; (*2*) after irradiation by the fundamental harmonic of a titanium-sapphire laser (800 nm) with the mean power $P_\omega = 100$ mW; (*3*) after irradiation by the fundamental harmonic with $P_\omega = 300$ mW; (*4*) after irradiation by the second harmonic (400 nm) with the mean power $P_{2\omega} = 125$ mW.

mals). The higher the specific rate of growth, the faster the tumour grows and the shorter the lifetime of the animal in the absence of treatment.

For quantitative estimation of the laser effect on the tumour growth one can make use of the method of equivalent exponents, proposed by N.M. Emanuel [6] and applied by us to evaluate the efficiency of combined chemotherapy and laser treatment of tumours [7].

Let the mean specific rate of growth in the time interval t_1, t_2 be

$$\overline{\varphi}(t_1, t_2) = \frac{1}{t_2 - t_1}\int_{t_1}^{t_2} \varphi(t)\,\mathrm{d}t. \qquad (2)$$

Because

$$\varphi(t) = \frac{1}{F}\frac{\mathrm{d}F}{\mathrm{d}t} = \frac{\mathrm{d}\ln F}{\mathrm{d}t}, \qquad (3)$$

then

$$\overline{\varphi}(t_1, t_2) = \frac{1}{t_2 - t_1}\int_{t_1}^{t_2} \frac{\mathrm{d}\ln F}{\mathrm{d}t}\mathrm{d}t = \frac{\ln F(t_2) - \ln F(t_1)}{t_2 - t_1}. \qquad (4)$$

Therefore, the specific growth rate averaged over the entire interval t_1, t_2 depends only on the values of F at the ends of the interval. The quantity $\overline{\varphi}(t_1, t_2)$ is equal to the argument of the 'equivalent exponential function' that coincides with the kinetic curve $F(t)$ at the ends of the considered time interval.

As a measure of the efficacy of treatment one can take the ratio of mean specific rates of tumour growth in the control group and in the experimental one:

$$\overline{\chi} = \frac{\overline{\varphi}_{\mathrm{con}}(t_1, t_2)}{\overline{\varphi}_{\mathrm{exp}}(t_1, t_2)} = \frac{t_{2\mathrm{exp}} - t_{1\mathrm{exp}}}{t_{2\mathrm{con}} - t_{1\mathrm{con}}}\frac{\ln F_{\mathrm{con}}(t_{2\mathrm{con}}) - \ln F_{\mathrm{con}}(t_{1\mathrm{con}})}{\ln F_{\mathrm{exp}}(t_{2\mathrm{exp}}) - \ln F_{\mathrm{exp}}(t_{1\mathrm{exp}})}. \qquad (5)$$

In the general case the quantity $\overline{\chi}$ depends on the choice of both time intervals ($t_{1\mathrm{con}}$, $t_{2\mathrm{con}}$ and $t_{1\mathrm{exp}}$, $t_{2\mathrm{exp}}$). When these intervals are chosen equal, their ratio in Eqn (5) is equal to unity.

Since at complete stopping of tumour growth $\varphi_{\mathrm{exp}} = 0$ and the efficiency of treatment becomes infinite, it is more convenient to use the parameter

$$\chi^* = 1 - \frac{1}{\overline{\chi}}. \qquad (6)$$

When $\chi^* = 0$ the effect is absent, positive χ^* indicates efficient inhibition of the tumour growth, and negative χ^* means that the tumour growth is stimulated rather than inhibited.

To process our data we took the time interval from 14 to 29 days. The lower time value was chosen long enough to assure the tumour appearance in all animals of the fourth group.

As a result of the experimental data processing it was shown that the exposure to femtosecond radiation having the mean power $P_\omega = 100$ mW slightly stimulates the growth of the tumour ($\chi^* = -0.06$). The exposure to fundamental harmonic radiation with the mean power $P_\omega = 300$ mW leads to insignificant inhibition of the tumour growth ($\chi^* = -0.15$), and in the case of exposure to the second harmonic the inhibition is more pronounced ($\chi^* = 0.44$). Note for comparison, that earlier, using the combined treatment with cyclophosphan and laser radiation, we obtained the parameter of treatment efficacy $\chi^* = -1.34$ [7].

Apparently, the inhibition of tumour growth is due to the death of some part of cells in the process of irradiation. For the mean power 300 mW the appropriate damages may be caused by multiphoton processes, and for the irradiation with the second harmonic of the laser (central wavelength 400 nm, half-width greater than 50 nm) the UV components are present in the spectrum, which may cause direct thermal destruction of proteins and enzymes. Obviously, the mechanisms of photoporation and photodestruction are similar and both determined by the energy flux density through the cell membranes.

4. Conclusions

From the results of the present work one can draw the following conclusions:

(i) Femtosecond radiation of a titanium-sapphire laser is able to cause photoporation of cells in the Krebs-2 tumour.

(ii) As a consequence of photoporation, the vital stain trypan blue penetrates into the cells.

(iii) The irradiated cells remain functionally active after their implantation, causing the tumour growth in experimental animals.

(iv) The analysis of kinetic curves reveals the fact that after the exposure of cells to femtosecond radiation of the fundamental harmonic of the titanium-sapphire laser with the wavelength 800 nm and the mean power 100 mW a certain activation of tumour growth is observed, while the increased mean power of the fundamental harmonic or irradiation with the second harmonic causes substantial inhibition of the tumour growth, i.e., a medicinal effect.

(v) Tumour cells, in our opinion, are a convenient model for studying the mechanisms of photoporation of cells by laser radiation.

(vi) Photoporation of cells by femtosecond laser radiation opens prospects for developing methods of introduction of different medical preparations into cells.

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