Exp Oncol 2010
32, 1, 29–32

Experimental ONCOLOGY

PHOTOINDUCED CYTOTOXIC EFFECT OF FULLERENES C_{60} ON TRANSFORMED T-LYMPHOCYTES

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Aim: **To estimate the viability of normal and transformed T-lymphocytes after UV/Vis irradiation in the presence of pristine fullerenes C_{60}.** ***Methods:*** **Thymocytes were isolated from Wistar rats' thymus. Murine leukemia L1210 and human lymphoma Jurkat cells were used in this study. Mercury-vapor lamp was used for fullerenes C_{60} photoexcitation. Cytotoxicity was determined by MTT assay. Changes in cell morphology were monitored by phase-contrast light microscopy.** ***Results:*** **fullerenes C_{60} exhibit cytotoxic effect against transformed T-lymphocytes when combined with UV/Vis irradiation (320–600 nm). Photoinduced effect was enhanced with the increasing of irradiation time period and C_{60} concentration, cell death was registered after 24 hours incubation. Fullerenes C_{60} photocytotoxicity against normal T-lymphocytes (thymocytes) was not observed.** ***Conclusion:*** **The present study suggests that pristine fullerenes C_{60} have the potential to be an effective photosensitizer and exhibit cytotoxic effect on transformed T-cells *in vitro*.**
Key words: **fullerenes C_{60}, thymocytes, transformed T-cells, viability, photodynamic therapy.**

It has been well recognized that reactive oxygen species (ROS) including $O_2^{\cdot}$ and $^1O_2$, generated by various photosensitizers through light irradiation are responsible for cytotoxicity and this effect is applied for photodynamic tumor therapy. Although the majority of photosensitizing compounds are based on the tetrapyrrole backbone, found particularly in porphyrins, other molecular structures have been studied for medical uses [1, 2]. Recent progress in nanobiotechnology have arised interest in biomedical application of a new class of nanostructures made exclusively of carbon atoms — fullerenes C_{60}, spheroidally shaped molecules (0.72 nm in diameter), which are nontoxic and demonstrate unique physicochemical properties. Small size of lipophilic C_{60} molecule account for steric compatibility with biological molecules and promote accomodation inside hydrophobic regions of membranes [3, 4]. Due to extended π-conjugated system of molecular orbitals fullerene C_{60} absorb UV/Vis light and can generate reactive oxygen species with almost 100% quantum yield. As the study of C_{60} biological effects is restricted by difficulty to reach satisfactory concentration in water the studies were focused on chemically modified water-soluble fullerenes with numerous substitutes on the surface [2, 5]. But substantial modification of fullerene core appears to cause a pertubation of its electronic structure and hence to reduce the photodynamic potential of the molecule [6]. Therefore pristine (nonmodified) fullerenes C_{60} or fullerenes with a few addents are suggested to be perspective photosensitizers.

Nevertheless we need better understanding of C_{60} photosensitizing potential and interaction with cells of different types. The aim of this study was to evaluate the effect of pristine C_{60} on viability of normal (Wistar rats thymocytes) and transformed T-lymphocytes (murine leukemia L1210 and human lymphoma Jurkat cells) after UV/Vis irradiation.

Received: January 11, 2010.
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MATERIALS AND METHODS

Thymocytes were isolated from thymus of Wistar rats (150–180 g). Thymus was removed, passed through nylon mesh into RPMI 1640 medium, the cells were washed by centrifugation (600 g) and resuspended in RPMI 1640 medium. Human T-cell lymphoma Jurkat cell line was kindly provided by Dr. Ludmila Drobot (Palladin Institute of Biochemistry, NAS of Ukraine). L1210 leukemia cells were obtained on day 8–12th after intraperitoneal transplantation of leukemic L1210 cells to hybrid F_1 mice (DBA2xC57B$_1$/6) kept on standart diet in vivarium of the R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine.

Stable water colloidal solutions of pristine fullerenes C_{60} (10^{-4} M, purity 99.5%) were prepared at Ilmenau Iniversity (Germany) as described in [7] by C_{60} transfer from toluene to water using continuous ultrasound sonication. Both hydrated individual C_{60} molecules and C_{60} clusters (3,4–72 nm) were present in solution.

Cells (thymocytes —1–2 x 10^6/ml, human T-cell lymphoma Jurkat and murine leukemia L1210 cells — 0.5 x 10^6/ml) were preincubated for 1 h in RPMI 1640 medium with or without C_{60} fullerenes followed by illumination with mercury-vapor lamp (320–600 nm light, irradiance 200 mW/cm^2, distance 2 cm). Cells were further incubated in RPMI 1640 medium supplemented with 5% fetal bovine serum (Sigma-Aldrich, Germany), 50 μg/ml streptomycin, 100 μg/ml penicillin at 37 °C in a 5% CO_2 humidified atmosphere. The number of viable cells was counted in Goryaev's chamber upon 0.2% trypan blue staining.

Cell viability was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction assay. At indicated time points of incubation 200 μl aliquots were removed from cell suspensions into the 96-well microplates (thymocytes — 10^6/well, Jurkat and L1210 cells — 1 x 10^5/well), 20 μl of MTT solution (2.5 μg/ml) was added to each well and the plates were incubated for another 2 h. The culture medium was then replaced with 100 μl of DMSO,

diformazan formation was determined by measuring absorption at 570 nm with a plate reader (μQuant, BioTek, USA). Controls were carried out using cells without fullerenes C_{60} but irradiated as described above. A second control was also performed with cells incubated with the fullerenes without irradiation. The indices were not corrected with those given by control without irradiation because in this case cell death was always less than 5%.

Untreated and C_{60}-treated UV/Vis irradiated Jurkat cells were monitored by light microscopy (Olympus, URFLT 50).

Statistical analysis was performed using *Statistica-6.0* computer program (StatSoft Inc.). Paired Student's *t*-tests were performed. Differences values $p < 0.05$ were considered to be significant.

RESULTS

Efficiency of C_{60} photoactivation strongly depends on optical absorption properties of the molecule. The absorption spectrum of fullerenes C_{60} is highest in the UVA and blue regions but the tail of absorption does stretch into the red region of the visible spectrum [7]. To determine if irradiation in applied light diapason (320–600 nm) *per se* affect cell survival the experiments were done with cells irradiated at different time periods without fullerenes and incubated for 6 h. Fig. 1, *a* shows that cells survival after irradiation is high — the viability of both thymocytes and L1210 cells was at the level of control and that of Jurkat cells was not less than 83% when the time of irradiation was extended to 2 min. So irradiation alone was not effective in supression of cell growth.

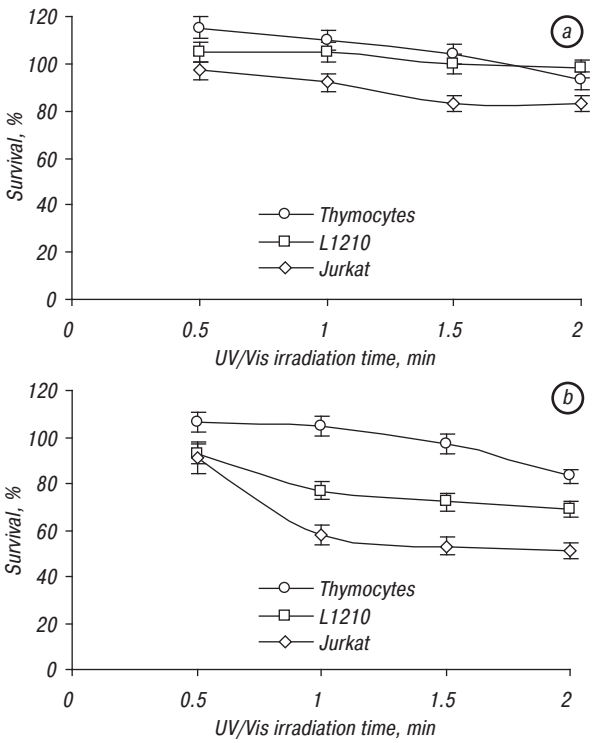

Fig. 1. Cells survival after UV/Vis irradiation for different time periods without (*a*) or with (*b*) fullerenes C_{60}

The data on viability of cells irradiated at different time periods in the presence of fullerenes C_{60} at the low concentration of 50 μM are shown on Fig. 1, *b*. No detectable activity of C_{60} against thymocytes was revealed at irradiation time period of 0.5–1.5 min and only slight decrease of viability (85%) was observed after 2 min irradiation. At the same time substantial phototoxicity of fullerenes C_{60} against transformed T-lymphocytes was detected. Viability of both L1210 and Jurkat cells was significantly reduced and the latter showed higher susceptibility to photoexcited C_{60}. The suppression effect of C_{60} was observed at the modest (1 min) exposure to UV/Vis light and became stable at more prolonged irradiation time period.

Fig. 2 shows the effect of fullerenes C_{60} in different concentrations plus 1 min irradiation on cells viability within 6 h. Again no appreciable effect on thymocytes was detected in the range of applied C_{60} concentrations. Photocytotoxic effect of fullerenes C_{60} against transformed T-lymphocytes was shown to be concentration dependent, viability of L1210 and Jurkat cells was diminished to approximately 61 and 44% accordingly when C_{60} concentration was increased up to 100 μM.

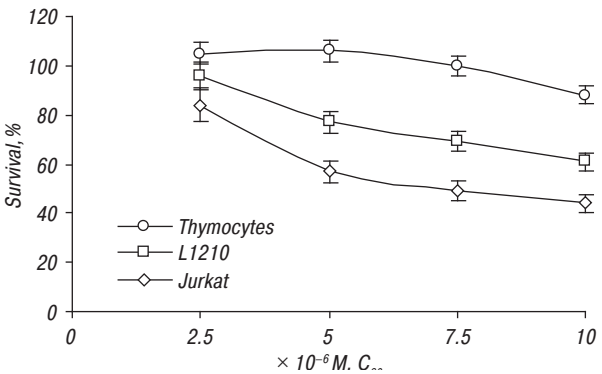

Fig. 2. Cells survival after UV/Vis irradiation in the presence of fullerenes C_{60} at different concentrations

The time course of cell death was estimated in experiments with short- and long-term incubation of transformed T-cells irradiated at the presence of 50 μM C_{60}. Fig. 3 shows that at 3 h the proliferation rate of cells irradiated with C_{60} was comparable to that of the reference cells (irradiated without C_{60}). Significant cytotoxicity against both L1210 and Jurkat cells was observed at 24 h, but Jurkat cell were more susceptible and needed 6 h for the same effect.

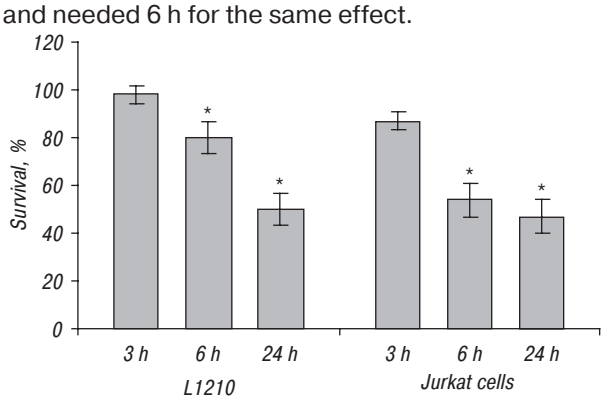

Fig. 3. Survival of cells cultured for different time after UV/Vis irradiation with fullerenes C_{60}
*$p \leqslant 0.05$ in comparison with control.

To testify cell death changes in morphology of Jurkat cells were monitored by phase-contrast microscopy

(Fig. 4). At 24 h the number of cell grown and their appearance were similar to those in control when cultured either in the presence of fullerenes C_{60} or after UV/Vis irradiation. It is apparent that by fullerenes C_{60} combined with UV/Vis irradiation the number of growing cells was pronouncedly decreased, cell fragmentation and apoptotic bodies formation were observed.

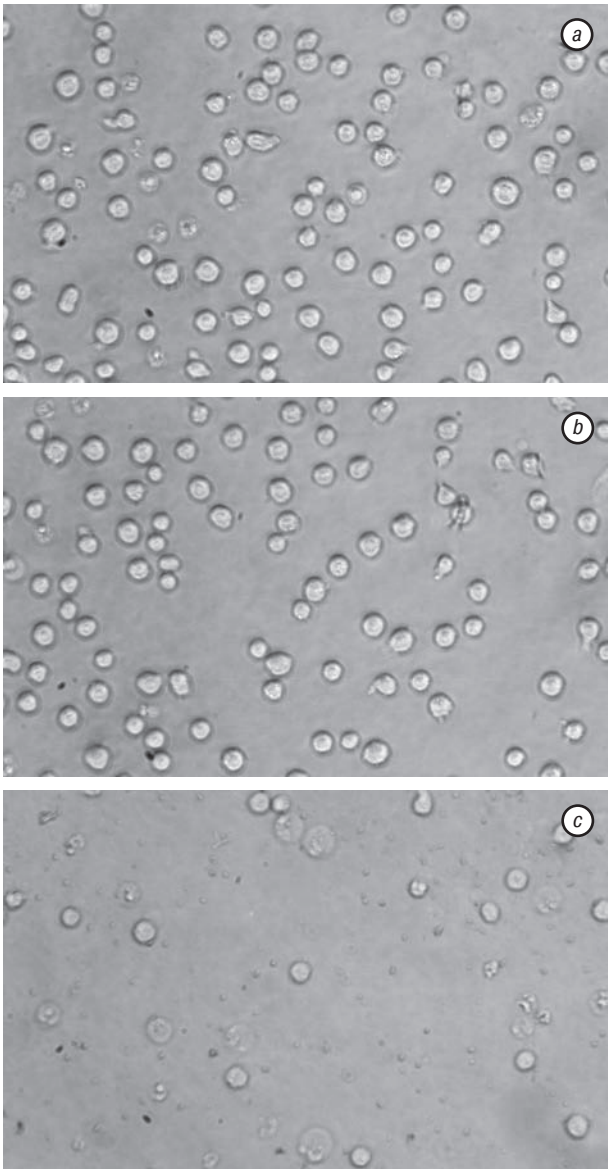

Fig. 4. Phase-contrast micrographs of Jurkat cells cultured for 24 h in the presence of fullerenes C_{60} (*a*), after UV/Vis irradiation without (*b*) or with (*c*) fullerenes C_{60}

DISCUSSION

Two mechanisms are thought to be responsible for ROS generation after C_{60} illumination. The first is energy transfer from C_{60} photoexcited triplet state to molecular oxygen with production of singlet oxygen ($^3C_{60}{}^* \rightarrow C_{60} + {}^1O_2$). The second is electron transfer from reduced fullerene triplet or radical anion to molecular oxygen forming superoxide anion radical ($^3C_{60}{}^* \rightarrow C_{60}^{\cdot-} \rightarrow C_{60} + O_2^{\cdot-}$). The second pathway of ROS generation is more effective if to consider $O_2^{\cdot-}$ dismutation to hydrogen peroxide and further formation of hydroxyl radical, the most reactive and cytotoxic of all ROS [8–10].

In previous work with the use of EPR spin trapping techniques for detection both $^1O_2$ and $O_2^{\cdot-}$ it has been shown that pristine C_{60} irradiated with white light generate ROS in water solutions and cell suspensions despite having a relatively low optical absorption at 600 nm [11, 12]. In present study we show that illuminated fullerenes C_{60} are effective in induction of L1210 and Jurkat cells death.

Our results show the selectivity of photoexcited C_{60} cytotoxic effect which is observed in transformed T-cell but not in T-cells from thymus. The explanation of this fact remains unknown but we suggest that altered behavior may have basis in C_{60} interplay with surface glycoproteins, glycosphingolipids, receptor and adhesive proteins of transformed cells plasma membrane. In addition it is supposed that O_2 in cancerous cells is not in full consumed for respiration and instead is accumulated in hydrophobic regions of membrane where is used as the substrate for energy or electrone transfer from photoexcited C_{60} and ROS production [13].

All experiments with irradiation in this study were done after 1 h incubation of cells with fullerenes C_{60}. During this period of time fullerenes should be adsorbed in the cell membrane or taken up inside the cells, because generation of ROS outside the cell will not be sufficient to produce cell death. According to our previous observation fullerenes C_{60} at concentration 10^{-6}–10^{-5} M instantly penetrate into the planar bilayer lipid membrane and enhance its ionic permeability [14]. Fullerenes have inherent photoluminiscence but it allows to detect intracellular uptake only at high C_{60} concentrations. In recent report [15] non-toxic pristine C_{60} preparation (200 μg/ml) was used and intracellular uptake of C_{60} in malignant cancer breast cells was demonstrated by fluorescence microscopy.

We suggest that transformed T-cells killing after C_{60} illumination is mediated mainly by ROS reaction with targets in cytoplasmic membrane involved in cellular signaling, but subcellular organelles can also be involved. It was shown recently [2] that C_{60} modified by single pyrrolidinium group can mediate killing of a panel of mouse cancer cells (lung and colon adenocarcinoma, reticulum cell sarcoma) at low concentration of 2 μM by induction apoptosis at 4–6 h after very modest exposure to white light, the increase of intracellular ROS probe dichlorofluoroscein fluorescence was demonstrated.

In conclusion our investigation has revealed that pristine fullerenes C_{60} exhibit cytotoxic effect against transformed T-lymphocytes when combined with UV/Vis irradiation. Photoinduced effect was enhanced with the increasing of irradiation time period and C_{60} concentration, cell death was registered after 24 h incubation. Photocytotoxic effect of fullerenes C_{60} was not detected in normal T-lymphocytes (thymocytes), the observed selectivity might be helpful for the development of complex approaches for target- treatment of leukemia. Further experiments will be done to explain the mechanisms involved in fullerenes C_{60} photoinduced cytotoxicity.

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