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PHOTOCYTOTOXIC EFFECT OF C_{60} FULLERENE AGAINST L1210 LEUKEMIC CELLS IS ACCOMPANIED BY ENHANCED NITRIC OXIDE PRODUCTION AND p38 MAPK ACTIVATION

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Aim: **To estimate the combined action of C_{60} fullerene and light irradiation on viability of L1210 leukemic cells, nitric oxide (NO) generation, p38 mitogen-activated protein kinase (MAPK) activity and cell cycle distribution.** ***Methods:*** **Cell viability was assessed by MTT test. Light-emitting diode lamp (λ = 410–700 nm, 2.45 J/cm^2) was used for C_{60} fullerene photoexcitation. Nitrite level and NO-synthase activity were measured by Griess reaction and by conversion of L-arginine to L-citrulline, respectively. p38 MAPK activity was assessed by Western blot analysis. Cell cycle distribution was determined by flow cytometry.** ***Results:*** **It was shown that light irradiation of C_{60} fullerene-treated L1210 cells was accompanied by 55% decrease of their viability at 48 h of culture. Nitrite level measured as an index of reactive NO generation was increased at the early period after C_{60} fullerene photoexcitation due to activation of both constitutive and inducible NO-synthase isoforms. The simultaneous activation of p38 MAPK was detected. Accumulation of L1210 cells in sub-G_1 phase of cell cycle was observed after C_{60} fullerene photoexcitation.** ***Conclusion:*** **Photoexcited C_{60} fullerene exerts cytotoxic effect, at least in part, through triggering production of reactive NO species and activation of p38 kinase apoptotic pathways in L1210 leukemic cells.**

Key Words**: C_{60} fullerene, photoexcitation, L1210 cells, NO radicals, p38 MAPK, cell cycle.**

The direct influence on the signalling pathways involved in coordinated control of cells proliferation and apoptosis is considered to be promising in tumor growth inhibition. It is assumed that intense reactive oxygen species (ROS) production due to imbalance of prooxidant — antioxidant equilibrium, which precedes caspase activation could be the inductor of receptor-independent apoptotic pathway in cancer cells. Carbon nanostructure C_{60} fullerene and its derivatives are shown to be the effective regulators of cell oxidant status and the perspective compounds for photodynamic killing of cancer cells [1–3].

Due to the extended π-conjugated system of molecular orbitals, C_{60} effectively absorbs UV/visible light and shifts to triplet state. In the presence of electron donors in the medium the excited C_{60} is reduced and converted into anion radical $C_{60}^{\cdot-}$ with further transferring of electron to molecular oxygen and producing singlet oxygen, superoxide radical anion, hydroxyl radical, which play an important role as regulators of cell death signalling [1, 4].

The studies of biological activity of C_{60} fullerenes were focused mainly on water-soluble chemically modified derivatives. But substantial modification of fullerene core appears to cause perturbation of its electronic structure and hence to reduce the level of its photoexcitation [1, 5]. Therefore, photodynamic potential of pristine (non-modified) C_{60} fullerene needs detailed investigation.

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Abbreviation used: cNOS – constitutive NOS isoform; iNOS – inducible NOS; MAPK – mitogen-activated protein kinase; NO – nitric oxide; NOS – NO-synthases; ROS – reactive oxygen species.

Using fluorescent-labeled C_{60} fullerene, obtained by covalent conjunction of C_{60} with rhodamine B isothiocyanate we have demonstrated accumulation of carbon nanostructure inside the leukemic cells [6]. We have shown previously apoptosis induction in human leukemic cells treated with pristine C_{60} and irradiated in UV-visible range [7]. But detailed ROS-dependent mechanisms involved in C_{60}-induced photocytotoxic effect are still to be elucidated.

Cytotoxic effect of ROS could be amplified by its interaction with reactive nitric species, nitric oxide (NO) and NO radicals, that is followed by the formation of peroxynitrite ions ($ONOO^-$), irreversible modification of proteins tyrosine and cysteine residues and oxidative/nitrative stress [8, 9]. One of the important redox-sensitive and NO-dependent components of mitogen-activated protein kinases (MAPK) signalling pathways is p38 MAPK, which is involved in the control of cell cycle and apoptosis [10–12].

The aim of the study was to estimate the combined action of C_{60} fullerene and light irradiation on NO generation, p38 MAPK activity and cell cycle distribution in order to elucidate the possible mechanisms involved in photocytotoxic effect of C_{60} fullerene against murine L1210 leukemic cells.

MATERIALS AND METHODS

Water colloid solution of C_{60} fullerene (10^{-4} M, purity > 99.5%, nanoparticle average size 50 nm, stability — 12 months) was synthesized and characterized in Technical University of Ilmenau (Germany) as described in [13].

The murine L1210 leukemic cell line was obtained from the Bank of Cell Lines from Human and Animal

Tissues of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine. Cells were incubated in RPMI 1640 medium with 5% FBS. The cells of the control group were incubated for 2 h without treatment, of second group were irradiated (100 mW, during 2 min), of the third group were incubated with C_{60} (10^{-5} M), of the fourth group were incubated with C_{60} fullerene with further irradiation.

Efficiency of C_{60} fullerene photoactivation strongly depends on optical absorption of the molecule, which is highest in UV region, but the tail stretches into red region. To exclude UV irradiation, which is damaging to cell and to maximize the efficiency of C_{60} fullerene photoexcitation by visible light we use the range of 410–700 nm.

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 ($1 \cdot 10^5$ cells) were placed into the 96-well microplates, 20 µl of MTT solution (4 mg/ml) was added to each well and the plates were incubated for 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 µQuant microplate reader (BioTek, USA).

Nitrite level was measured by Griess reaction [14]. An aliquot (0.5 ml) of the cell suspension (10^6 cells/ml) was mixed with an equal volume of Griess reagent (sulfanilamide 1% w/v, naphthylethylenediamine dihydrochloride 0.1% w/v and orthophosphoric acid 2.5% w/v) and incubated at room temperature for 10 min prior to measurement of absorbance at 546 nm. The amount of nitrite formed was compared to those of known concentrations of sodium nitrite and normalized to the protein content.

NO-synthase (NOS) activity was measured spectrophotometrically by the conversion of L-arginine to L-citrulline [15, 16]. Cells (10^6 cells per probe) were incubated in buffer containing 50 mM KH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM NADPH, 2 mM L-arginine, pH 7.0 for 60 min at 37°C. The reaction was stopped by adding 2N $HClO_4$. The content of L-citrulline was determined in the protein free supernatant. To determine the activity of Ca^{2+}-independent inducible NOS (iNOS) 2 mM EDTA was added instead of $CaCl_2$. Activity of Ca^{2+}-dependent constitutive NOS isoform (cNOS) was calculated as the difference between total NOS activity and iNOS activity. The level of L-citrulline was determined spectrophotometrically [17]. Protein free aliquot was mixed with reagent (59 mM diacetyl monooxime, 32 mM antipyrine and 55 mM iron sulfate (II) in 6N H_2SO_4) and boiled during 15 min. After cooling the extinction at 465 nm was measured. Amount of L-citrulline was determined using a calibration curve for L-citrulline.

After irradiation of cells treated with C_{60} fullerene the aliquots of suspension ($5 \cdot 10^6$ cells) were taken at 30; 60 and 120 min for Western blotting. The cells were centrifuged at 600 *g*, washed with PBS, lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM o-vanadate, 50 mM NaF, 2 mM EDTA, 1 mM PMSF, complete protease inhibitor cocktail) and centrifuged at 12 000 *g* for 20 min at 4 °C. Protein content in supernatants was determined using Pierce™BCA Protein Assay kit (Thermo Scientific, USA). Proteins (30 µg per sample) were separated by electrophoresis on 10% polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated with anti-phospho-p38 kinase antibodies (Cell Signaling, USA) and anti-β-actin antibodies (Sigma, USA) overnight at 4 °C. As secondary antibodies peroxidase-conjugated anti-rabbit or anti-mouse IgG were used, respectively. The immunoreactive bands were visualized using enhanced chemoluminescence detection reagent (Amersham Pharmacia Biotech, USA). Densitometric analysis was performed using the Gel-Pro analyzer software (Media Cybernetics, Silver Spring, USA).

For cell cycle analysis cells ($1 \cdot 10^6$) were resuspended in 0.1 ml PBS (pH 7.4), fixed by adding 0.9 ml of 90% ethanol at –20 °C overnight and centrifuged at 13 000 *g* for 1 min. The fixed cells were rinsed twice with PBS and resuspended in propidium iodide (10 µg/ml) solution containing RNase A (100 µg/ml, Sigma, USA) in PBS without calcium and magnesium. The stained cells were analyzed by a COULTER EPICS XL™ (Beckman Coulter, USA) and FCS Express 3 Flow Cytometry Software (DeNovo Software, USA).

Data processing and plotting were performed by IBM PC using specialized applications Excel 2010. Statistical analysis was performed using *Statistica 6.0* computer program (StatSoft Inc., USA). Paired Student's *t*-tests were performed. Difference values $p < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

To evaluate photocytotoxic effect of C_{60} fullerene, nanostructure-treated L1210 cells were incubated for a long-term period after irradiation (Fig. 1). Cells survival after light irradiation *per se* was not less than 80% when the time of incubation was extended to 48 h. C_{60} fullerene in concentration 10^{-5} M didn't change the viability of L1210 cells. This effect is harmonized with data about lack of C_{60} fullerene cytotoxicity in low concentration range [18–20]. In contrast, cells responded to the combined action of C_{60} fullerene and irradiation by time-dependent decrease of cell viability. After 48 h of culture, viability of leukemic cells declined to 55% as compared to control.

Free radical NO and its various forms are known to be involved in the early phases of cell death. NO can either suppress apoptosis or activate cell death pathways depending on its concentration and cell redox potential [8, 9, 21]. Direct evaluation of the NO level in cell population is complicated by its short lifetime and quick metabolism. At the same time, the level of its stable metabolite nitrite anion is proved to be an adequate index of NO generation [22]. No changes in NO_2^- level were detected in cells treated with C_{60} alone, while after C_{60} fullerene photoexcitation investigated index was increased at 1 and 3 h and was substantially

higher than in control or in irradiated cells (Fig. 2, *a*). These data indicated that photoexcited C_{60} fullerene potentiates NO generation in leukemic cells.

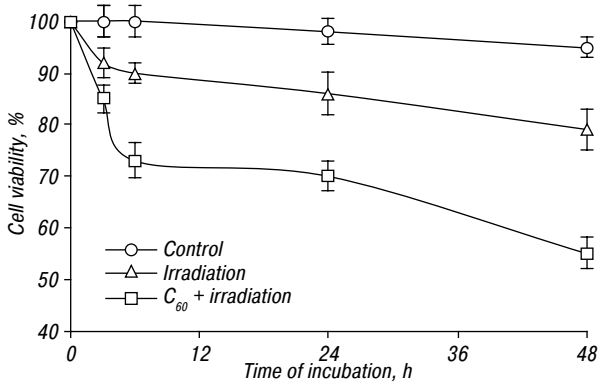

Fig. 1. Viability of L1210 cells after combined action of C_{60} fullerene (10^{-5} M) and light irradiation. M ± m, n = 8

NO is generated due to oxidation of L-arginine to L-citrulline by NADPH-dependent NOS family, consisting of two main isoforms. cNOS could be quickly activated in a Ca^{2+}-dependent manner and is associated with plasmalemma, while iNOS is cytokine-dependent and needs several hours to reach maximum activity and is located predominantly in the inner mitochondrial membrane [23]. The data presented at Fig. 2, *b*, *c* show that C_{60} *per se* had no effect on activation of NOS isoforms, whereas photoexcitation of accumulated C_{60} fullerene was followed by early activation of both cNOS and iNOS. Enzymes' activity was increased already at 1 h and was further enhanced at 3 h after C_{60} fullerene photoexcitation. This observation is in a good agreement with the data presented in Fig. 2, *a* concerning enhanced NO_2^- level and NO generation induced by photoexcited C_{60} fullerene in the leukemic cells.

Taking into account that NO generation coincides with increased ROS production and disturbance of antioxidant system (inhibition of glutathione peroxidase against superoxide dismutase activation, which was detected in L1210 cells at 3 h after combined action of C_{60} fullerene and irradiation) [24] we suggested the possibility of oxidative modification of the components of MAPK signalling pathways. Previous investigations indicate that p38 kinase is stress-activated, redox-sensitive, NO-dependent and could promote cell death [10–12]. We examined p38 MAPK activity by estimation the level of its active phosphorylated form (pp38 MAPK) using Western blot analysis. As shown in Fig. 3, irradiation alone or in combination with C_{60} fullerene activated p38 MAPK at 1 h while C_{60} fullerene treatment was ineffective. Whereas the phosphorylation level of p38 MAPK in cells irradiated without C_{60} fullerene was normalized at 2 h, in cells treated with photoexcited C_{60} fullerene a pronounced 2.5 fold increase in pp38 MAPK level was observed at this time point (Fig. 3, *b*, *c*). There are data that p38 MAPK pathway is engaged in caspase-3, -8 and -9 activation during NO-dependent apoptosis of different cell types [10, 12]. It is also shown that C_{60} fullerene exert photocytotoxicity in the MCF-7 cancer cell line through modulation of ROS and p38 MAPK activation [25]. Our results allow to suggest that activation of p38 MAPK can be involved in redox-dependent cytotoxic effect of photoexcited C_{60} fullerene in L1210 cells.

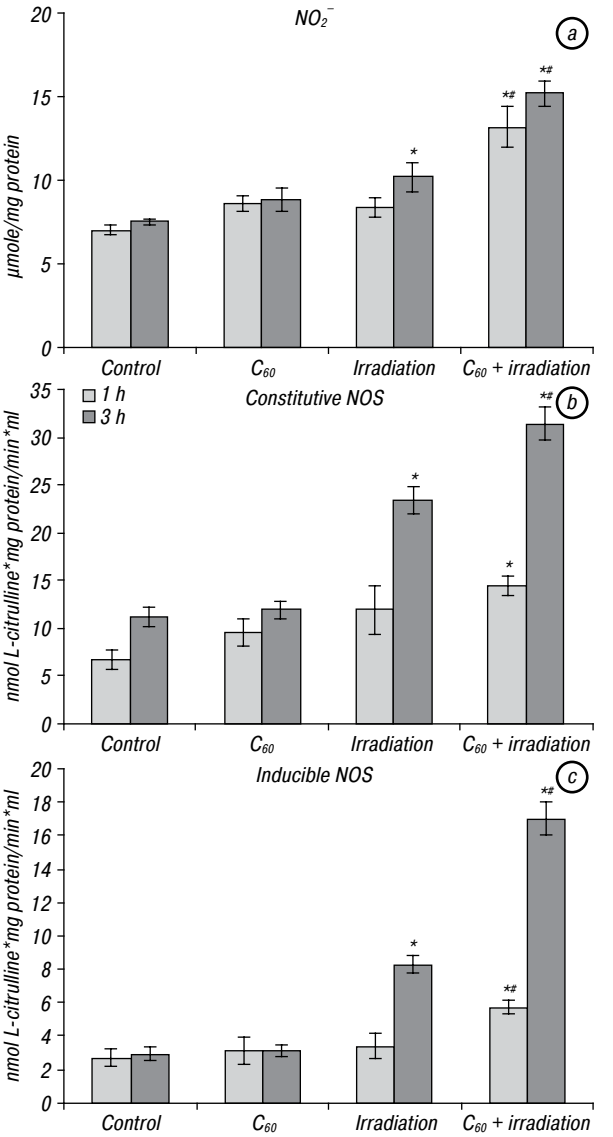

Fig. 2. The level of NO_2^- (*a*) and NOS activity (*b*, *c*) in L1210 cells after combined action of C_{60} fullerene and light irradiation. M ± m, n = 6; $^*p < 0.05$ compared to control cells; $^\#p < 0.05$ compared to irradiated cells

Because transcription factors and cyclin-dependent kinases are among the substrates of activated p38 kinase we examined the cell cycle distribution as the possible long-term effect of C_{60} fullerene in L1210 cells. Flow cytometric analysis showed that treatment with C_{60} fullerene or irradiation alone had little effect on cell cycle profile at 24 h of incubation. Photoexcited C_{60} fullerene was proved to influence accumulation of cells in different cell cycle phases (Fig. 4). A decreased number of cells in G_2/M phase (12.1% *vs* 20.6% in control) was accompanied by an increased number in sub-G_1 phase (7.3% *vs* 3.1% in control). Since increase of sub-G_1 cell fraction is shown to correlate with apoptotic DNA fragmentation [26], the data obtained indicate that photoexcitation of C_{60} fullerene

is followed by cell cycle redistribution of L1210 cells with accumulation of apoptotic cells.

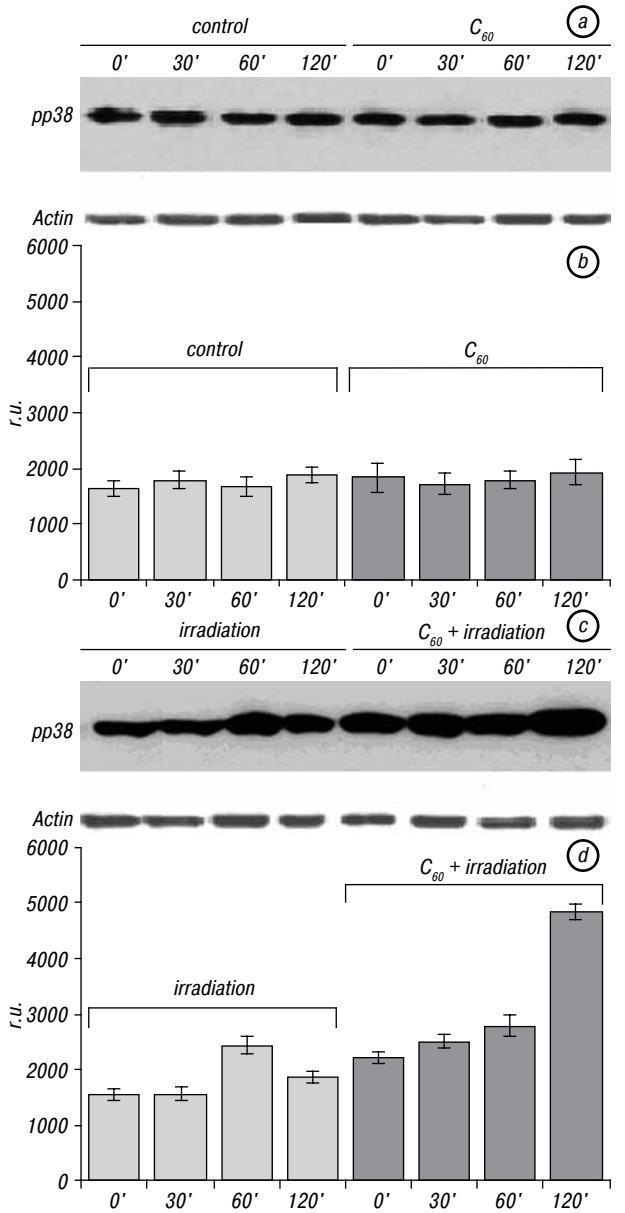

Fig. 3. Activation of p38 MAPK in leukemic cells after C_{60} fullerene photoexcitation: *a*, *c* — Western blot analysis of the pp38 MAPK level (typical blotogram); *b*, *d* — quantitative analysis of the fold increase of pp38 MAPK level. Actin was used as loading control

The results of this study demonstrate that the cytotoxic effect of photoexcited C_{60} fullerene against L1210 leukemic cells is realized through a number of apoptosis-associated pathways. It was shown that visible light irradiation of cells treated with C_{60} fullerene was accompanied by activation of both constitutive and inducible forms of NOS and generation of reactive NO species. The simultaneous activation of p38 MAPK was also detected. The L1210 cell cycle is shown to be disturbed after C_{60} fullerene photoexcitation with cell accumulation in sub-G_1, which is the marker of apoptosis.

CONCLUSION

In summary, our results suggest that photoexcitated C_{60} fullerene induces cytotoxic effect at least in part through triggering production of reactive NO species and activation of p38 MAPK-dependent apoptotic pathways in leukemic cells. The cytotoxic effect of photoexcited C_{60} fullerene could be used for designing and development of complex approached in anticancer therapy.

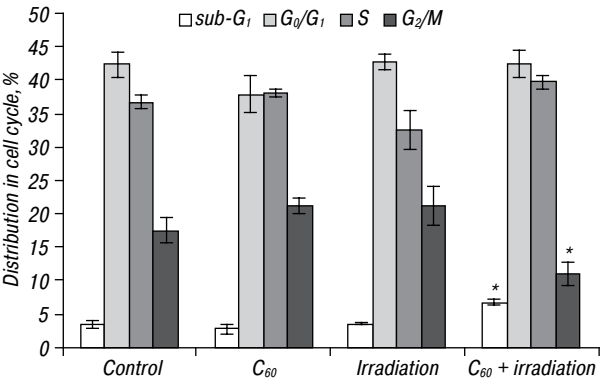

Fig. 4. The effect of C_{60} fullerene photoexcited on cell cycle phase distribution in L1210 cells (M ± m, n = 4; *$p < 0.05$ compared to control cells)

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