Chapter 6
Effects of Photoexcited Fullerene C_{60}-Composites in Normal and Transformed Cells

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Abstract The supramolecular composites containing fullerenes C_{60} immobilized at nanosilica were used for the design of the molecular systems that can be an effective agent in cancer photodynamic therapy (PDT). In particular, it was shown that photoexcited fullerene C_{60}-containing composites decrease viability of transformed cells, intensify the process of lipid peroxidation (LPO) in cell membranes and accumulation of low-molecular weight DNA fragments, and also decrease the activity of electron-transport chain of mitochondria.

Keywords C_{60} fullerene composites; normal and transformed cells; photoexcitation; reactive oxygen species

6.1 Introduction

Presently photodynamic therapy (PDT) is considered as a perspective way for therapy of different diseases, including cancer. PDT is a method based on the local light-induced activation of photosensitizers able to accumulate selectively in energy-deficient cells (malignant or dysplastic ones) not influencing the remaining normal cells of the body (Pass, 1993). Upon the action of irradiation of certain wavelength characteristic for individual photosensitizer, photochemical reaction occurs

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that leads to selective destruction of malignant cells. Mechanism of PDT action consists in the fact that the molecule of photosensitizer after absorption of quantum of light switches to excited triplet state and enters into two types of reactions (Grossweiner et al., 1982). In the first type, interaction directly with the molecules of biological substrate occurs, thus leading to generation of free radicals. In the second type, interaction of the excited photosensitizer takes place with generation of singlet oxygen that is cytotoxic for vital cells due to the properties of potent oxidizer of biomolecules. At the final stage, photochemical reactions of both types lead to destructive changes of important structures of malignant cells and their death.

Fullerenes C_{60} are considered as perspective compounds for PDT. They are inert compounds that do not reveal toxic properties in low concentration range. Due to small size and hydrophobicity, fullerenes C_{60} are able to interact with biologic molecules, be embedded in membranes, and cause biologic effects (Foley et al., 2002; Kamat et al., 2000; Wilson, 2000). They are characterized by significant reducing potential and are able to absorb free radicals (Kamat et al., 2000; Tabata and Ikada, 1999). From the other side, cytotoxic effects of fullerenes C_{60} upon their photoexcitation has been revealed. Upon the action of ultraviolet or visible light irradiation, C_{60} molecule switches to triplet state. As a result of energy transfer (its efficacy reaches nearly 100%) from the excited fullerene C_{60} to molecular oxygen, intense generation of singlet oxygen takes place. Also the excited C_{60} molecule may be an acceptor of electrons. In the presence of donors of electrons in the medium (e.g., NADH $\rightarrow$ NAD^+, H^+) the excited fullerene C_{60} may be reduced due to electron transfer and be converted to anion radical $C_{60}^{\bullet -}$, that in turn transfer electrons to molecular oxygen with generation of superoxide anion $O_2^{\bullet -}$ (Kamat et al., 2000; Guldi and Asmus, 1999; Hamano et al., 1997).

For potentiation of photosensitizing properties of fullerenes C_{60} to the composition of their molecules antenna is being introduced, for example, porphyrine, and anthracenal (Arbogast and Foote, 1991).

Apart from this, among the means to promote accumulation and retention of preparations applied in PDT, in tumor tissue, the increase in their molecular size via conjugation with inert carrier could be used. For example, upon intravenous introduction of fullerene C_{60}–polyethylene glycol (PEG) conjugate to mice it has been shown that this conjugate was accumulated in tumor tissue in large amount and was retained longer than in healthy tissue. This conjugate was not accumulated in other organs. The histology has proved that administration of irradiated C_{60}–PEG conjugate is causing notable necrosis of tumor without affecting healthy tissues (Tabata and Ikada, 1999).

So, fullerenes C_{60} and their derivatives may be potential damaging agents of biologic systems upon PDT, because upon irradiation C_{60} molecule is able to generate singlet oxygen, selectively penetrate tumor cells, and be accumulated there and could be easily removed from biologic medium.

Low affinity to polar solvents and fullerenes aggregation in water limit their use in biologic systems. To increase water solubility of fullerenes, few ways are used: solubilization with the use of some water-soluble polymers like PDT or polyvinilpyrrolidone, generation of complexes with cyclodextrines or calixarenes, and

covalent modification of surface with polar substitutes (Isaacs et al., 1997; Yamakoshi et al., 1994; Yoshida et al., 1994). However, the presence of large functional groups protecting spheroid of fullerene from water as well and numerous substitutes on its surface are influencing the properties of the molecule and may decrease its photosensitizing potential as well as ability to interact with biologic molecules (Da Ros et al., 2001).

For optimization of introduction of fullerenes in aqueous medium, prevention of aggregation, elevation of contact area, and for providing equable placement and specific interaction of fullerenes C_{60} in the zone of contact with biologic material, we have carried out immobilization of C_{60} molecules on the spheric particles of silicon oxide – aerosyl (the mean diameter of about 9 nm), which is a highly dispersed chemically inert material with hydrophilic surface (Chuyiko, 2003). Generation of such composites is a perspective approach, because their content could be enriched by introduction of different components, in particular, the structures able to entrap light (porphyrines and anthracenal) and elevate photosensitizing effect of fullerenes.

The other way to introduce fullerenes C_{60} to biomedium, that we have used, was based on the transfer of these molecules from organic solvent (e.g., toluene) to the water using ultrasound sonication (Scharff et al., 2004).

The aim of the work was evaluation of the ability of photoexcited fullerene C_{60} and synthesized fullerene C_{60}-containing composites to generate reactive oxygen species (ROS) and to perform comparative analysis of the state of the cells of two types (normal ones – thymocytes, and malignant ones – the cells of ascite Erlich carcinoma [EAC] and leucosis L1210) by such indexes as viability, content of LPO products, MTT test, and DNA fragmentation upon incubation in the presence of photoexcited fullerene C_{60}.

6.2 Materials and Methods

Thymocytes were obtained by grinding thymus of rats of Wistar line weighing 120–150 g through nylon lattice in the buffer A (3 mM Na_2HPO_4, 5 mM KCl, 120 mM NaCl, 1 mM $CaCl_2$, 10 mM glucose, 1 mM $MgSO_4$, 4 mM $NaHCO_3$, 10 mM HEPES, pH 7.4). Malignantly transformed cells were obtained at days 8–12 after intraperitoneal transplantation of ascitic Erlich's carcinoma cells to inbred mice (weighing 20 g), and leucosis L1210 cells – to hybrid mice of F_1 line (DBA2 × C57Bl/6). Animals were maintained on the standard chow diet.

Water colloid solutions of fullerenes C_{60} (10^{-4} M) were prepared as described in Scharff et al. (2004). Fullerene-aminopropylaerosyl (fullerene C_{60}-composite-1) was synthesized (Golub et al., 2003) by the introduction of aminopropyl chains oriented ad extra by amine groups (0.9 mM/g), to the surface layer of silicon dioxide nanoparticles that were bound to fullerene C_{60} (0.12 mM/g) (Fig. 6.1). Fullerene-anthracenaliminopropylaerosyl (fullerene C_{60}-composite-2) was composed also from anthracenalimine (0.2 mM/g) that was introduced via azomethine condensation of aldehyde group of anthracenal with surface amino group.

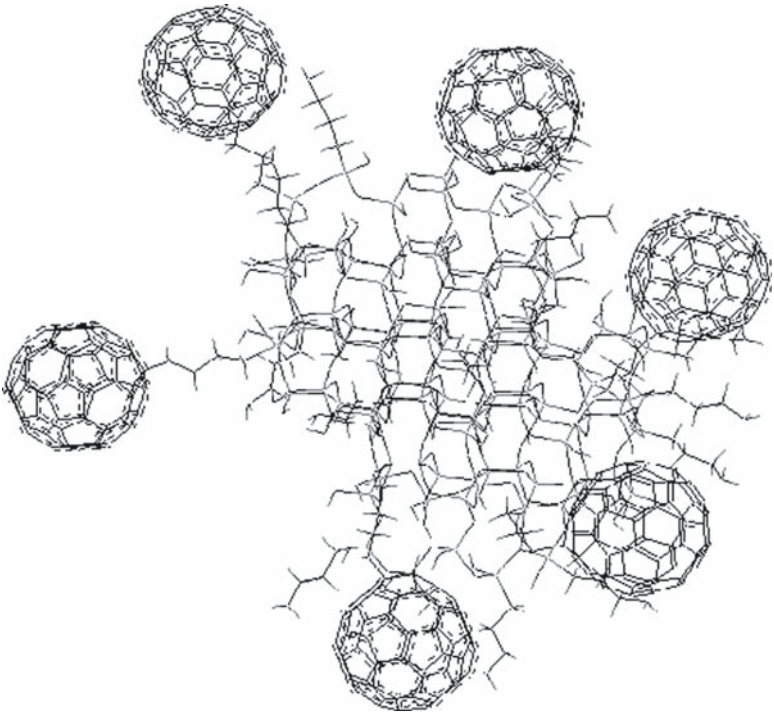

Fig. 6.1 Molecular model of C_{60} fullereneaminopropylaerosyl

Registration of ROS was carried out by electron paramagnetic resonance (EPR) technique using spin trap 1-hydroxy-2,2,6,6-tetramethyl-piperidine-4-OH (2×10^{-3} M). EPR spectra in the samples were registered at room temperature in quartz cuvette with the volume of 200 µl (Burlaka et al., 1994).

The cells ($1–3 \times 10^6$ cells/ml) were incubated for 24 h at 37 °C in RPMI 1640 medium supplemented with 8 mM $NaHCO_3$, 20 mM HEPES, 5% FCS, 10 µg streptomycin, and 10 U/ml penicillin without agents or in the presence of fullerenes C_{60}. The number of viable cells was counted in hemocytometer using 0.4% solution of trypan blue.

After addition of solutions of fullerenes C_{60} or fullerene C_{60}-containing composites to the cell medium, the concentration of silicon dioxide was 0.02%, and that of fullerenes C_{60} – 10^{-5} M. Samples were placed in a glass tube and irradiated for 2 min by mercury-vapor lamp (power 24 W) at the distance of 5 cm.

The content of the products of lipid peroxidation (LPO) was determined after 1 h of incubation of the cells in a buffer A at 37 °C. Aliquot of cell suspension (100 µg protein) was treated with heptane/isopropyl alcohol mixture at the ratio of 1:1. The content of Schiff bases in heptane phase was analyzed on fluorimeter RF-510, Shimadzu (Japan) at λ_{exit} = 360 nm and λ_{emis} = 420 nm (Kolesova et al., 1984). The content of diene conjugates was determined by spectrophotometry (Gavrilov et al., 1988) at the wavelength of λ = 245 nm using spectrophotometer Scinco (Germany).

The content of low-molecular weight DNA fragments – polydesoxyribonucleotides (PDN) – in the cells after 5 and 20 h of incubation in the presence or absence of fullerenes C_{60} was evaluated after treatment with lyzing buffer (10 mM EDTA, 10 mM Tris-HCl (pH 7.4), 0.5% Triton X-100), and centrifugation (15,000 *g*, 20 min). PDN content in supernatant was determined using reaction with diphenylamine (Burton, 1956).

Reaction with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, "Sigma") was carried out in 96-well plate at 37 °C in thermostat (Carmichael et al., 1987). The cells were incubated for 0.5, 2, and 5 h in RPMI 1640 medium in the presence or absence of fullerenes C_{60} samples, then MTT was added, and incubation continued for 2 h. The content of generated formazane was evaluated by spectrophotometry at the wavelength of $\lambda = 570$ nm at the digital spectrophotometer IFCO-2 (ABOTEK, Russia).

Statistical analysis of the results was performed using applied program "Microsoft Excel 98".

6.3 Results and Discussion

Biologic effects of non-excited fullerenes C_{60}, that are revealed at the concentration range lower than 10^{-4} M, are mostly positive, but depend on the type of cells and the way of modification of fullerene C_{60} (Yamakoshi et al., 1994). As we have shown earlier, upon the presence of 10^{-6} M fullerenes C_{60} in incubation medium, resistance of erythrocytes to hemolysis is not altered, whilst at the concentration of 10^{-5} M fullerenes C_{60} the hemolysis rate is accelerated. Hemolytic effect was not revealed if fullerene C_{60} at the concentration of 10^{-5} M was introduced to the content of aminopropylaerosyl (i.e., upon the presence of fullerene C_{60}-composite-1). Cytotoxic influence was not found if thymocytes and EAC cells were incubated with fullerenes C_{60} (10^{-5} M) or fullerene C_{60}-containing composites for 24 h (Prylutska et al., 2006). That is why the study of the influence of irradiation on biologic activity of fullerenes C_{60} was carried out at their concentration of 10^{-5} M.

It is known that fullerene C_{60} molecule maximally absorbs light at the range of $\lambda = 220–345$ nm, and is low at $\lambda = 450$ nm (Scharff et al., 2004). The rate of ROS generation upon irradiation of fullerenes C_{60} with visible light that deeper penetrates tissues and possesses lower damaging effect compared to ultraviolet is studied insufficiently. That is why we have studied the effects of fullerene C_{60} and fullerene C_{60}-containing composites irradiated in the range of $\lambda = 320–580$ nm.

After irradiation of fullerenes C_{60} in the cell medium, ROS production has been detected, and this index increased as follow: fullerene C_{60} < fullerene C_{60}-composite-1 < fullerene C_{60}-composite-2. As one may see from the data presented in Table 6.1, the rate of ROS generation elevated nearly twice after absorption of light in predetermined range by fullerenes C_{60}, if fullerene C_{60} was bound to the surface of aminopropylaerosyl, and threefold, if anthracenal that absorbs at $\lambda = 357$ nm was introduced to the content of composite.

Table 6.1 The rate of generation of reactive oxygen species (ROS) and cell viability after irradiation of samples of fullerenes in incubation medium

Experimental conditions	nmole ROS/ min.10^6 cells	Viability cells in 24 h incubation (%) (M ± m, n = 7)
Samples of fullerenes		
C_{60}	2.9 ± 0.2	
C_{60}-composites-1	6.3 ± 0.2	
C_{60}-composites-2	11.0 ± 0.4	
Suspension of thymocytes	2.3 ± 0.2	97 ± 6
+C_{60}	4.3 ± 0.3	100 ± 3
+C_{60}-composite-1	5.4 ± 0.3	90 ± 5
+C_{60}-composite-2	7.8 ± 0.4	88 ± 3
Suspension of ascitic Erlich's carcinoma cells	1.4 ± 0.1	100 ± 5
+C_{60}	3.0 ± 0.2	80 ± 6
+C_{60}-composite-1	6.1 ± 0.4	71 ± 5*
+C_{60}-composite-2	7.2 ± 0.2	66 ± 7*
Suspension of leucosis L1210 cells	1.2 ± 0.1	98.0 ± 2.0
+10^{-5}M C_{60}	2.6 ± 0.2	88.0 ± 3.0
C_{60}-composite-1	5.5 ± 0.6*	77.0 ± 6.0*
C_{60}-composite-1	6.7 ± 0.7*	71.0 ± 4.0*

*$P \leq 0.05$ compared to the control.

Upon the absence of fullerenes C_{60}, insignificant level of ROS generation in the suspensions of thymocytes, EAC, and L1210 cells has been registered (Table 6.1). After irradiation of fullerenes C_{60} in the cell suspension, elevation of ROS generation rate was observed, but by absolute value the indexes were lower than it could be expected accounting the mentioned data on the rate of ROS generation in water solutions. Certainly, it may be explained by the fact that viscosity of cell suspension is higher than that of water solutions, thus determining the period of existence of fullerenes C_{60} in triplet state (Irie et al., 1996). Upon the presence of photoexcited fullerene C_{60}, the rate of ROS generation in the suspensions of thymocytes, EAC, and L1210 cells increased equally – twice compared to the control. After irradiation of fullerene C_{60}-composite-1 and fullerene C_{60}-composite-2 in the cell medium, the rate of ROS generation increases by 2.3- and threefold in the suspension of thymocytes, and by 4.4- and 5.1-fold in the suspensions of tumor cells, respectively. The higher effect in both cases was observed for fullerene C_{60}-composite-2 that contains anthracenal.

The influence of photoexcited fullerene C_{60} on viability of thymocytes, EAC, and L1210 cells was studied after 24 h of incubation, considering the content of viable cells at incubation as 100% (Table 6.1). After irradiation of fullerenes C_{60} in the cell medium, significant decrease in the content of viable cells in the suspension of thymocytes was not registered, whilst the number of viable malignant cells decreased. Upon the presence of photoexcited fullerene C_{60} in incubation medium, the number of viable EAC cells decreased by 20%, and L1210 by 12%, while in the

presence of photoexcited fullerene C_{60}-composite-1 by 29% and 23%, respectively, and photoexcited fullerene C_{60}-composite-2 by 34% and 29%, respectively compared to the samples without addition of fullerenes C_{60}.

Taking into account that ROS produced by irradiated fullerenes C_{60} may act only in the radius of their short diffusion existence, one may suppose that cytotoxic effect is determined by the interaction of fullerene C_{60} with the surface of cells and initiation of chain reactions of free radical peroxidation in membranes. That is why the influence of photoexcited fullerene C_{60} on the course of LPO process was studied and evaluated by the content of generated primary (diene conjugates) and final (Schiff bases) products. The content of diene conjugates in thymocytes was 17.7 ± 4.2, in EAC cells was 21.1 ± 1.3, and in L1210 was 12.8 ± 3.1 nM/mg protein, and Schiff bases –56 ± 7.9, 46.5 ± 4.5, and 36.6 ± 4.6 rel. units/mg protein, respectively, and did not alter during 1 h incubation of the cells.

In the case of photoexcited fullerenes C_{60} and fullerene C_{60}-containing composites in incubation medium of thymocytes, the indexes of LPO did not alter compared to the control too (Fig. 6.2A). Upon incubation of EAC cells in the presence of photoexcited samples of fullerenes, the decrease in the content of diene conjugates by 35% in the presence of fullerene C_{60} and by 20% in the presence of fullerene C_{60}-composite-1 and fullerene C_{60}-composite-2 was observed (Fig. 6.2B). The presence of photoexcited samples of fullerenes in the suspension of L1210 cells influenced LPO indexes only in the presence of fullerene C_{60}-composite-2, when the content of diene conjugates increased by 35% (Fig 6.2C).

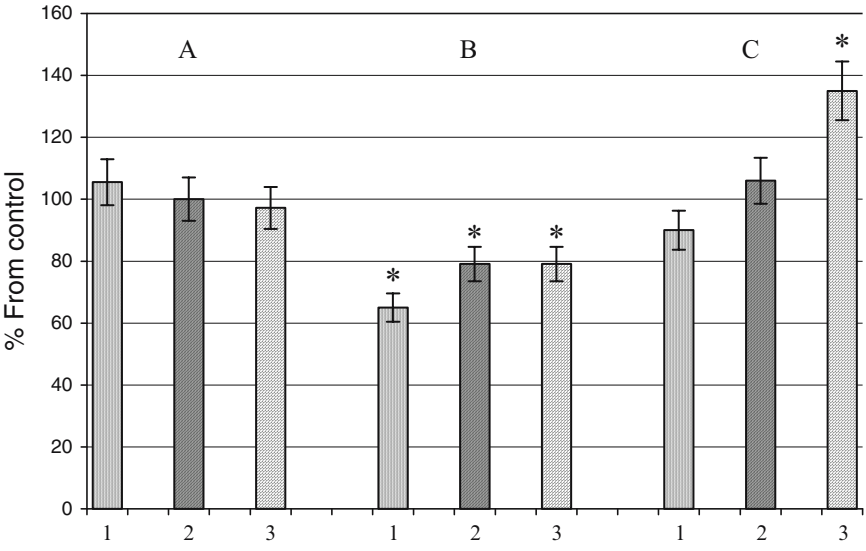

Fig. 6.2 Content of diene conjugates (% from control) in thymocytes (A), EAC (B), and L1210 cells (C) after 1 h of incubation in the presence of photoexcited samples of fullerenes (1 – fullerene C_{60}, 2 – C_{60}-composite-1, 3 – C_{60}-composite-2). *$P \leq 0.05$ *compared to the control*

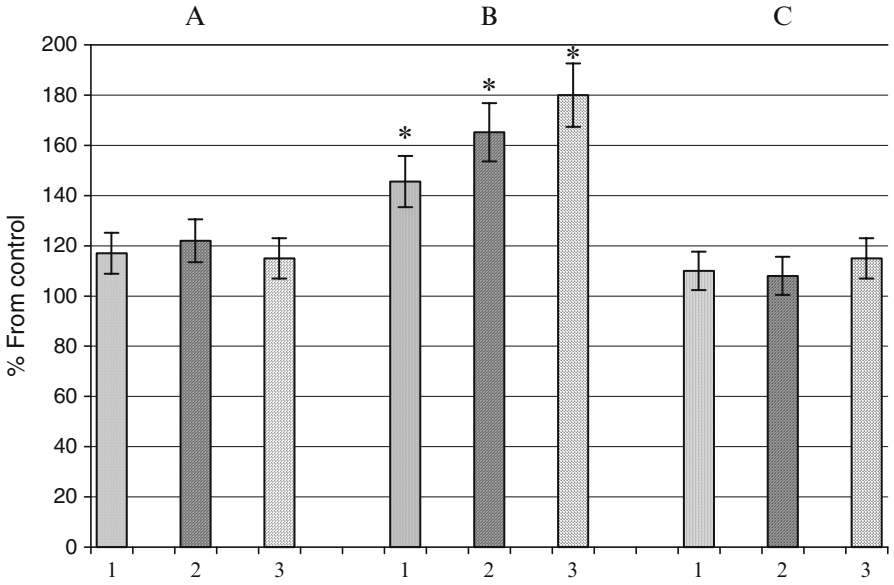

Fig. 6.3 Content of Schiff bases (% from control) in thymocytes (A), EAC (B), and L1210 cells (C) after 1 h of incubation in the presence of photoexcited samples of fullerenes (1 – fullerene C_{60}, 2 – C_{60}-composite-1, 3 – C_{60}-composite-2). *$P \leq 0.05$ *compared to the control*

Upon the presence of the fullerenes C_{60} in incubation medium of thymocytes and L1210 cells, the content of Schiff bases did not alter compared to the control (Fig. 6.3A, C – 1, 2, 3).

However, upon incubation of EAC cells in the presence of photoexcited samples of fullerenes the increase in the content of Schiff bases was observed by 46% in the presence of photoexcited fullerene C_{60}, by 65% – in the presence of photoexcited fullerene C_{60}-composite-1, and by 80% – in the presence of photoexcited fullerene C_{60}-composite-2 (Fig. 6.3B).

Simultaneous decrease in the content of diene conjugates and increase in the content of Schiff bases evidence the quick shift of pro-/antioxidant equilibrium, generation of reactive radicals, and damage of cell membranes in EAC cells, because Schiff bases, generated as a consequence of interaction of malonic dialdehyde with aminogroups of phospholipids and proteins, are highly reactive compounds causing polycondensation of molecules and formation of intermolecular bonds.

It has been shown that the influence of photoexcited fullerene C_{60} on the processes of free-radical oxidation depends on the type of the cell and on the composition of composite. So, in thymocytes in the presence of photoexcited fullerene C_{60} as well as fullerene C_{60}-containing composites, the content of primary and final LPO products did not alter compared to the control. In malignant cells the intensification of LPO processes was registered, and its level depends on the type of cells. So, in thymocytes in the presence of photoexcited fullerene C_{60} in suspension of EAC cells the decrease in the content of diene conjugates simultaneously with the increase in the

final LPO products – Schiff bases – was observed. Pro-oxidant effect was more pronounced upon the use of photoexcited fullerene C_{60}-composite-2. In the presence of photoexcited fullerene C_{60} composite-2 in L1210 cells the increase in the content of primary LPO products was registered, but the increase in the content of final LPO products was not detected.

Literature data on cytotoxic effects of photoexcited fullerene C_{60} are controversial. In the studies on transformed B-lymphocytes of Raji line, phototoxic action of water-soluble carboxy-C_{60} was not revealed even upon its concentration of 5×10^{-5} M (Irie et al., 1996). In the study (Kamat et al., 2000) damaging effect of fullerenes C_{60} in dependence on intensity of irradiation toward CHO cells has been demonstrated. Using microsomal fraction of rat liver that was treated with C_{60}–cyclodextrin complex, it was shown that already in 5–30 min after UV-irradiation the accumulation of LPO products occurs that is suppressed by antioxidants like ascorbic acid and α-tocopherol. Similar effect of fullerenes C_{60} has been revealed in microsomal fraction of the cells of ascitic sarcoma 180 (Kamat et al., 2000).

As a consequence of accelerated generation of oxygen-containing radicals and intensification of LPO reactions the alteration of the structural state of DNA may appear. To perform quantitative biochemical evaluation of the state of DNA of thymocytes, EAC, and L1210 cells upon the presence of photoexcited fullerene C_{60}, the content of the products of DNA degradation – low-molecular weight polydesoxyribonucleotides (PDN) after 5 and 20 h of incubation – has been analyzed. The portion of degraded DNA in the control cells after 5 and 20 h of incubation in the thymocytes is composed of 8 ± 2 and 10 ± 2%, correspondingly, in the EAC cells – 5 ± 1 and 8 ± 1%, correspondingly, in L1210 cells – 5 ± 1 and 7 ± 1%, correspondingly, from the general DNA content. It turned out that the thymocytes are characterized by the high degree of DNA degradation in comparison with the tumor cells. This effect can be explained by the fact that the thymocytes are the incompletely differentiated cells with the unstable genome and possess the low activity of the reparation systems of the single-stranded breaks of DNA. The level of PDN generated upon incubation of cells in the absence of C_{60} fullerenes (control) was considered as 100%.

Upon 20 h of incubation of thymocyte suspension in the presence of photoexcited fullerene C_{60}, the part of the degraded DNA increased insignificantly. As one may see from Fig. 6.4a, upon the presence of fullerenes C_{60}, the content of PDN increased by 10%, fullerene C_{60}-composite-1 by 14%, and fullerene C_{60}-composite-2 by 13% compared to the control.

Irradiation of fullerene C_{60}-containing composites (1 and 2) in the suspension of L1210 cells caused more significant damage of the structural state of DNA compared to that in thymocytes, while fullerene C_{60} did not affect this index in L1210 cells, also. Upon 5 h of incubation of L1210 cells in the presence of photoexcited fullerene C_{60}, the content of PDN increased by 12% in the presence of fullerene C_{60}, by 18% in the presence of fullerene C_{60}-composite-1, and by 24% in the presence of fullerene C_{60}-composite-2 compared to the control. During 20 h of incubation of L1210 cells, insignificant increase in the content of generated PDN was observed: by 14% in the presence of fullerene C_{60}, by 20% in the presence of fullerene

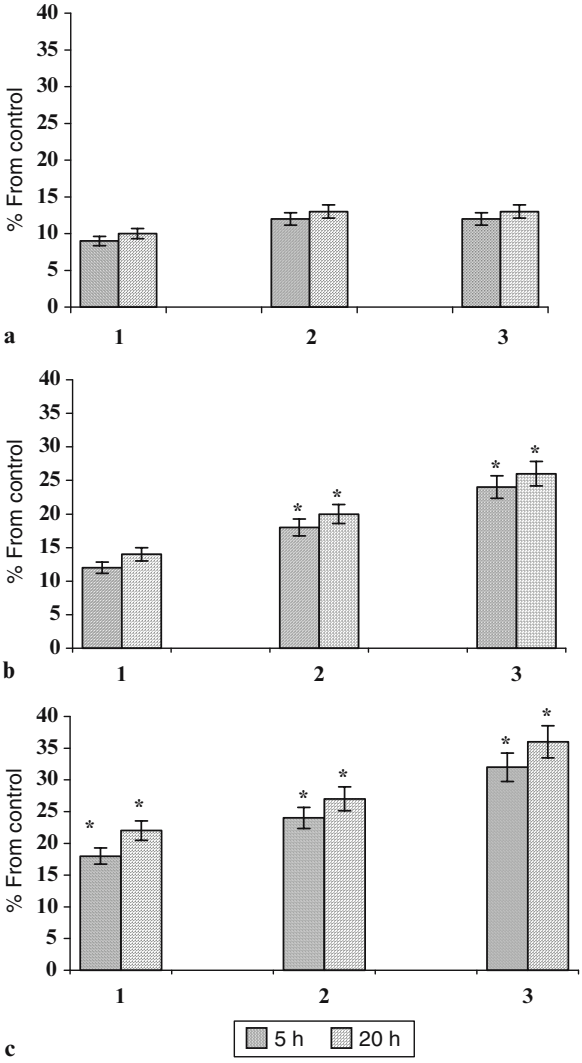

Fig. 6.4 DNA fragmentation (% from control) in thymocytes (a), L1210 (b), and EAC cells (c) incubated for 5 and 20 h in the presence of photoexcited samples of fullerenes (1 – fullerene C_{60}; 2 – C_{60}-composite-1; 3 – C_{60}-composite-2). *$P \leq 0.05$ compared to the control

C_{60}-composite-1, and by 26% in the presence of fullerene C_{60}-composite-2 compared to suspension of L1210 cells incubated without addition of the samples of fullerenes (Fig. 6.4b).

The most intense alteration of the structural state of DNA in the presence of photoexcited fullerene C_{60} was observed in EAC cells. As one may see from Fig. 6.4c, during 5 h the increase in the content of DNA degradation products was observed:

by 18% in the presence of fullerenes C_{60}, by 24% in the presence of fullerene C_{60}-composite-1, and by 32% in the presence of fullerene C_{60}-composite-2 compared to the cells without samples of fullerenes. In the case of 20 h of incubation of EAC cells in the presence of photoexcited fullerene C_{60} the accumulation of DNA degradation products continued to rise: in the presence of fullerenes C_{60} by 22%, fullerene C_{60}-composite-1 by 27%, and fullerene C_{60}-composite-2 by 36% compared to the cells without addition of the samples of fullerenes.

Thus, photoexcited samples of fullerenes are able to promote degradation of DNA and accumulation of low-molecular weight DNA fragments in L1210 and EAC cells already after 5 h of incubation, but more intense DNA damage is observed upon the presence of C_{60}-containing composites, evidencing the promotion of damaging effects of fullerenes C_{60} due to introduction of anthracenal to the content of composite.

From the literature reports it is known that some derivatives of fullerenes C_{60} upon light irradiation are able to alter the structural state of DNA (Samal and Geckeler, 2001). There are the data on specific damage of DNA structure by guanine by irradiated derivatives of fullerenes, and on electron transfer from guanosine to photoexited fullerene C_{60} derivative that occurs with the involvement of singlet oxygen. For example, fullerene C_{60}, solubilized in polyvinylpyrrolidone, upon irradiation caused damage of DNA with the formation of 8-OH-dG (Boutorine et al., 1994). Photoexcited C_{60}–PEG conjugate damaged DNA structure in C_8 position of guanine (Chi et al., 2002).

It is proposed that the cleavage of oligonucleotides occurs as a consequence of photoexcitation of fullerene C_{60} via its transfer in triplet state $^3C_{60}$ and generation of singlet oxygen $^1O_2$, which interacts with oligonucleotide. However, apart from oxygen, acceptors of electrons may also be aromatic rings of tertiary amines, including those of guanine in the content of nucleotide chain (Tokuyama et al., 1993).

For evaluation of influence of the samples of fullerenes C_{60} on total metabolic state of thymocytes, EAC, and L1210 cells we have used MTT test based on the reduction of MTT by reductase system that consists, in particular, of mitochondrial succinatedehydrogenase.

From the data of literature it is known that water-soluble derivatives of fullerenes are able to be localized in mitochondria and influence their state as well as enzyme system (Foley et al., 2002). Such intracellular localization of fullerenes C_{60} could explain biologic effects under irradiation, because generation of free oxygen radicals in the cells occurs during emission of electrons from electron-transport chain of mitochondria.

The rate of MTT reduction was determined adding the agent to cell incubation medium on 0.5, 2, and 5 h after irradiation of samples. In the case of non-irradiation of fullerenes C_{60} in incubation medium, the indexes of total metabolic state of thymocytes, EAC, and L1210 cells did not alter compared to the control.

As one may see from the data presented in Fig. 6.5a, upon the presence of photoexcited samples of fullerenes in incubation medium of thymocytes, no significant changes in the rate of MTT reduction were observed during 5 h. Upon the presence of photoexcited fullerene C_{60} in incubation medium of suspension of L1210 cells,

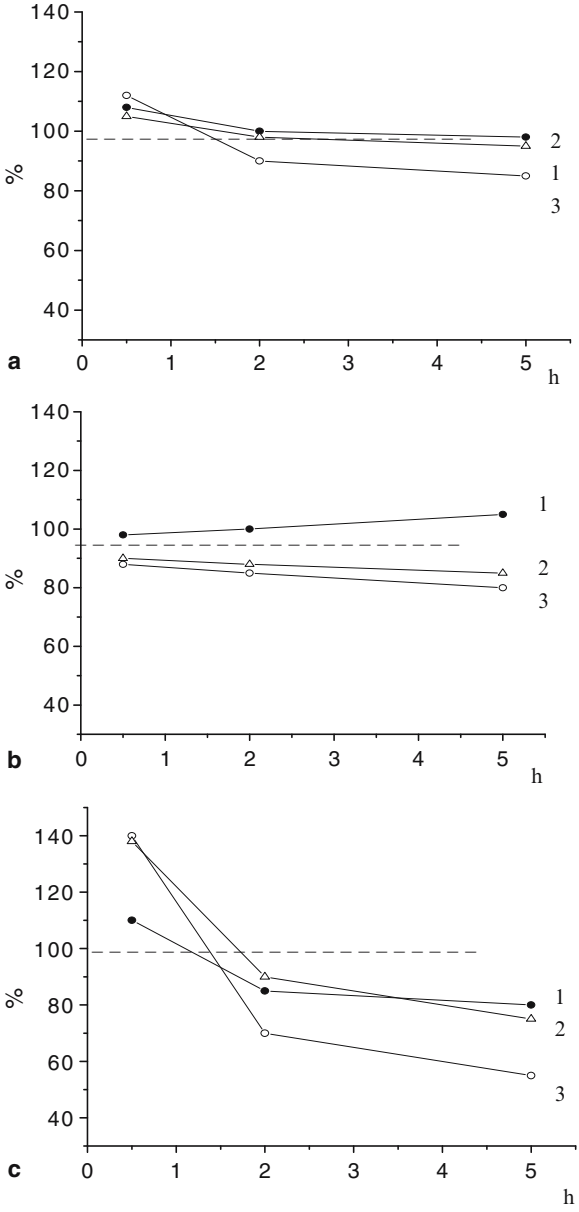

Fig. 6.5 Dependence of the level of MTT reduction (% from control) in thymocytes (a), L1210 (b), and EAC cells (c) incubated in the presence of photoexcited samples of fullerenes (1 – fullerene C_{60}; 2 – C_{60}-composite-1; 3 – C_{60}-composite-2). $P \leq 0.05$ *compared to the control*

no change in the rate of MTT reduction during 5 h of incubation was revealed (Fig. 6.5b, curve 1). But in the case of incubation of L1210 cells in the presence of fullerene-containing composites insignificant decrease in the rate of MTT reduction was observed already during the early stage of incubation (0.5 h) (in the presence of fullerene C_{60}-composite-1 and 2 by 10%) (Fig. 6.5b, curves 2, 3) that further continued to decrease. During 5 h of incubation of L1210 cells in the presence of fullerene-containing composites-1 and 2 the rate of MTT reduction decreased by 18% and 20%, respectively compared to the control. So, during 5 h of incubation irradiated samples of fullerene C_{60}-containing composites decrease the rate of MTT reduction in L1210 cells.

As one may see from the data presented in Fig. 6.5c, in the case of 0.5 h incubation of EAC cells elevation of the rate of MTT reduction was observed upon the presence of fullerenes C_{60} by 10%, fullerene C_{60}-composite-1 by 38%, and fullerene C_{60}-composite-2 by 40% compared to the control. After 2 h of incubation of EAC cells after irradiation of samples, the rate of MTT reduction decreased (in the presence of fullerenes C_{60} by 15%, fullerene C_{60}-composite-1 by 10%, and C_{60}-composite-2 by 25% compared to the control). In the case of 5 h of incubation of EAC cells after irradiation of samples, the rate of MTT reduction decreased at higher degree (in the presence of fullerenes C_{60} by 12%, fullerene C_{60}-composite-1 by 20%, and fullerene C_{60}-composite-2 by 42% compared to the control). One should pay attention to the fact that the rate of MTT reduction markedly elevated at early period after irradiation of fullerene C_{60}-containing composites in the culture medium (by 38% and 40% compared to the control). Such effect could be determined by elevation of electron-transport activity of fullerene C_{60} after irradiation. There are the data showing that fullerene C_{60} accelerates electron transfer from oxidized compounds, in particular, from such physiologic substrate as NADH (Yamakoshi et al., 1994), thus influencing the rate of redox processes. At this moment fullerene C_{60} transforms to anion radical $C^{\bullet-}_{60}$, that in turn transfer electrons to molecular oxygen with generation of superoxide anion $O^{\bullet-}_{2}$. In the case of large production of superoxide its toxic effect may be exerted, what is supported by the data on the fall of the rate of MTT reduction along with the increase in the period of incubation of EAC cells with irradiated composites. It is known that mitochondria are most sensitive to the action of superoxide radicals, where upon the development of oxidative stress the destruction of the barrier function of membranes and the damage of respiratory chain are taking place (Vladimirov, 2002). Lipophylic nature of fullerene C_{60} promotes its penetration in membrane structures. For example, using fluorescent probes (erythrosine and pyrene) incorporated in bilayer of phosphatydilcholine liposomes, it has been shown that upon interaction of membranes of liposome the complex of C_{60}–polyvinilpyrrolidone is broken, and fullerene C_{60} penetrates the membrane. During this process, the polymeric matrix remains outside, whilst fullerene C_{60} diffuses in membrane. The study of subcellular distribution of water-soluble derivative of fullerene $C_{61}(COOH)_2$ by the method of fluorescent microscopy using monoclonal antibodies against fullerene C_{60} and labeled ^{14}C analog has shown that this compound may penetrate plasma membrane and enter the cells where it is bound mainly by mitochondria (Foley et al., 2002).

6.4 Conclusion

So, in this work it has been shown that after irradiation of 10^{-5} M fullerene C_{60} and fullerene C_{60}-containing composites (λ = 320–580 nm) in aqueous solutions and cell suspensions, the generation of reactive oxygen species is observed, and the rate of their generation increases in the case of introduction of fullerenes C_{60} to the content of aminopropylaerosyl and anthracenaliminopropylaerosyl.

It has been shown that the influence of photoexcited fullerene C_{60} on the indexes of metabolic processes depends on the type of the cells and on the content of composite. The specificity of the effects of photoexcited fullerene C_{60}-containing composites is not observed in normal cells (thymocytes), but appear in malignantly transformed cells – Erlich ascite carcinoma (breast cancer) and L1210 (leucosis). It is shown that photoexcited fullerene C_{60}-containing composites decrease viability of transformed cells, intensify the process of lipid peroxidation in cell membranes and accumulation of low-molecular weight DNA fragments, and decrease the activity of electron-transport chain of mitochondria. The presented data point out the possibility of application of fullerene C_{60}-containing composites for photodynamic therapy.

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