Письма в ЭЧАЯ. 2016. Т. 13, № 2(200). С. 427–433

THE EFFECTS OF LIPID A ON GAMMA-IRRADIATED HUMAN PERIPHERAL BLOOD LYMPHOCYTES *IN VITRO*

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The modulatory effects of lipid A (diphosphoryl lipid A (DLA) and monophosphoryl lipid A (MLA)) on apoptosis induction and DNA structure damage (single- and double-strand breaks (SSBs and DSBs, respectively)) in peripheral human blood lymphocytes are studied for ^{60}Co gamma irradiation. It is shown that in the presence of these agents the number of apoptotic cells increases compared with the irradiated control samples. The effect is most strongly pronounced for DLA. In its presence, a significant increase is observed in the number of radiation-induced DNA SSBs and DSBs. Possible mechanisms are discussed of the modifying influence of the used agents on radiation-induced cell reactions.

Изучено модифицирующее влияние липида А (дифосфорил липид А (DLA) и монофосфорила липида А (MLA)) на индукцию апоптоза и нарушений структуры ДНК (одно- и двунитевых разрывов) в лимфоцитах периферической крови человека при γ-облучении ^{60}Co. Показано, что в присутствии использованных агентов увеличивается количество апоптотических клеток по сравнению с облученными контрольными образцами. Наиболее выраженный эффект отмечен для DLA. В присутствии этого агента наблюдается значительное увеличение количества индуцируемых радиацией однонитевых и двунитевых разрывов ДНК. Обсуждаются возможные механизмы модифицирующего влияния использованных агентов на клеточные реакции при облучении.

PACS: 87.14.Cc

INTRODUCTION

One of the main tasks of radiation biology is a search for new ways of modulating the radiosensitivity of the organism's cells and tissues. Such studies are yet more important for the development of approaches to the modulation of immune system cell radiosensitivity. In recent years, specialists' attention has been drawn to endotoxins — thermally stable products that are inherent only in Gram-negative bacteria and have a broad spectrum of their biological activity [1]. From the chemical point of view, endotoxins are lipopolysaccharides (LPS) and are the main component of the outer membrane of Gram-negative bacteria localized on their cell wall. The endotoxic center of LPS is its lipid A. Generally, the natural lipid A of

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Escherichia coli consists of β-1′, 6-linked D-glucosamine (GlcN) disaccharide carrying amide- and/or ester-linked (R)-3-hydroxymyristic acids at 2,3-tetradecanoic acid at positions 2, 2′ and dodecanoic at positions 3, 3′ of the glucosamine group, and phosphate groups at positions 1 and 4′ [1]. Lipid A in low doses shows high biological activity on the host organism and can be the modulator of the immune response, because it binds the CD14/TLR4/MD2 receptor complex in many cell types, but especially the immune cells [2, 3]. Among the immune system cells, the high sensitivity of lymphocytes to gamma radiation is well known [4]. In this connection, it is interesting to study the influence of lipid A on human lymphocyte sensitivity to ionizing radiation.

Binding the complex with lipid A leads to the activation of NF-κB and consequently to an increased production and secretion of pro-inflammatory cytokines (IL-6, TNF, IL-1β) [5].

As is known, apoptosis is an important mechanism of tissue homeostasis support. Apoptosis is the physiological type of cell death; its main function is balancing the cell proliferation effect and elimination of damaged, functionally defective cells. Ionizing and ultraviolet (UV) radiations are efficient inductors of apoptosis. It has been shown that under UV irradiation, lipid A has a protective effect as it influences the development of the apoptotic death of neutrophils. However, for gamma irradiation, both activating and inhibiting effect of lipid A on apoptosis is observed depending on the radiation dose [6]. The controversial effect of lipid A on apoptotic cell death for UV and gamma irradiation can be connected with the different character of the molecular disorders involved in apoptotic process initiation. In the case of UV exposure, as is known, those are pyrimidine photodimers; under exposure to ionizing radiations, DNA single- and double-strand breaks (DSBs) are induced. It is DNA DSBs that are the molecular substrate initiating apoptosis in cells.

Taking it into account, the influence of lipid A on apoptosis induction and formation of DNA strand breaks in human peripheral blood lymphocytes was studied *in vitro* after gamma irradiation. Two lipid A derivatives were used in the experiments: monophosphoryl lipid A (MLA) and diphosphoryl lipid A (DLA). Monophosphoryl lipid A (MLA) differs from native diphosphorylated lipid A by the absence of phosphate ester on position 1 of the reducing-terminal glucosamine. MLA has been proven to be significantly less toxic than diphosphorylated lipid A [1]. This TLR4 ligand is at least 100-fold less pyrogenic than LPS yet and maintains many of the immunomodulatory properties of LPS [7]. In any case, MLA is safe in humans and has been administered as a component of several vaccine formulations [8]. Thus, as a DLA is the minimal toxic unit of endotoxin, the MLA molecule retains the endotoxin's immunomodeling properties, but does not have its toxic properties. The aim was to compare these lipids A together as potential protectors of the cells after irradiation.

1. MATERIALS AND METHODS

1.1. Cell Suspension Preparation. Human peripheral blood lymphocytes were isolated by Boyum's method [9], which is based on sedimentation in single-stage ficoll density gradient ($\rho = 1.077$ g/ml, PanEco). The obtained cell suspension was diluted by the RPMI 1640 nutrient medium (PanEco) containing penicillin/streptomycin (167 units/ml) and HEPES (20 mM) with addition of fetal calf serum (10% FBS, PanEco) up to the concentration of $2 \cdot 10^6$ cells/ml. Cell suspension with the modifying agents monophosphoryl lipid A from *E. coli* F583 (1 μg/ml, Sigma–Aldrich) and diphosphoryl lipid A from *E. coli* F583 (1 μg/ml, Sigma–Aldrich) was incubated for an hour at 37 °C.

1.2. Irradiation. ^{60}Co gamma irradiation was performed at the Rocus-M facility for remote radiation therapy at doses of 3, 5, 7, and 10 Gy with a dose rate of 0.3 Gy/s. The samples were kept in 0.5 ml plastic Eppendorf tubes. For the morphological analysis of the cell suspension, cells were irradiated at room temperature; for a DNA comet assay, the tubes were placed into an ice vessel to inhibit the repair processes. After irradiation, the lymphocytes were incubated at 37 °C for 24 h.

1.3. Morphological Analysis of the Cell Suspension. To study apoptotic cell death induction, necessary volumes were taken from the cell suspension to prepare slides — immediately and after 24 h. The suspension was stained with a mixture of the fluorescent dyes acridine orange and ethidium bromide (1:1; Sigma–Aldrich). The apoptotic, necrotic, and living cells were classified depending on lymphocyte morphology and staining [10]. For each experimental point, about 300 cells were analyzed. The statistical analysis of the obtained results was done using the OriginPro 8.0 program.

1.4. DNA Comet Assay. DNA molecular disorders in the irradiated lymphocytes were studied with DNA comet assay. Experiments were conducted in neutral (pH = 8.3) [11, 13] and alkaline (pH > 13) conditions [12, 13]. To study repair kinetics, samples for preparing slides were taken at certain times during 24 h from tubes held in a thermostat. The obtained slides were stained with propidium iodine (6 μg/ml, Sigma–Aldrich).

To visualize DNA comets, an AxioScope A1 fluorescent microscope (Carl Zeiss) was used with an AxioCam ICc3 CCD camera. Each comet was characterized by the generally accepted parameters: integral fluorescence M_c, which is equivalent to DNA content in the comet, and the comet tail end moment mt, which is the product of the DNA fraction in the comet tail F_t and the tail median X_m. The parameters were calculated as follows:

$$mt = X_m \cdot F_t, \quad X_m = \frac{\sum\limits_t (I_i \cdot X_i)}{\sum\limits_t I_i}, \quad F_t = \frac{\sum\limits_t I_i}{\sum\limits_c I_i}, \tag{1}$$

where I_i is fluorescence intensity at the point i; X_i is the distance from the comet head median to the point i. The summing indices denote the summing domains: t suggests summing only within the comet tail; c, within the whole comet. The images of DNA comets were processed using CASP software [14]. The measurements results were arranged as an Excel table, on the basis of which the mean value $\overline{x}$ and standard deviation STDEV were calculated:

$$\mathrm{STDEV} = \sqrt{\frac{(\overline{x} - x_i)^2}{n-1}}, \tag{2}$$

where n is the number of measurements.

The dose dependences were built with OriginPro 7 software.

2. RESULTS AND DISCUSSION

Figure 1 shows the apoptotic cell frequency dependence on the radiation dose for the cell cultivation under the normal conditions and in the presence of lipid A. As is seen, the number of apoptotic cells in the samples taken immediately after irradiation does not change with

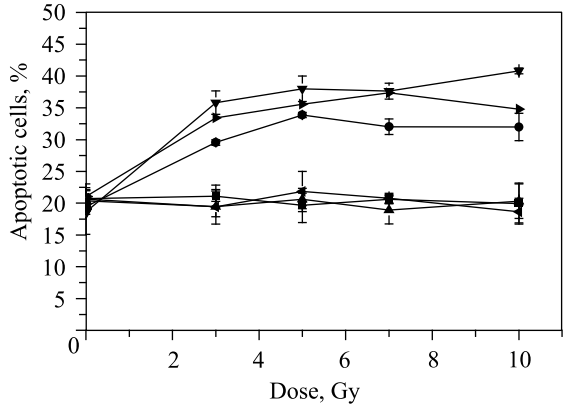

Fig. 1. The influence of the modifying agents MLA and DLA on the apoptotic death of blood lymphocytes for ^{60}Co gamma-ray irradiation: ■ — control 0 h; ● — control 24 h; ▲ — DLA 0 h; ▼ — DLA 24 h; ◀ — MLA 0 h; ▶ — MLA 24 h

increasing the dose, which reaches 10 Gy — both for samples irradiated under the normal conditions and in the presence of lipid A. However, 24 h after irradiation, an increase was observed in the number of apoptotic cells with increasing of the gamma-radiation dose. An increase in the number of apoptotic changed cells is typical of both the control the samples (without lipid A) and the samples with the immunomodulator. It should be noted that both agents used in this study (MLA and DLA) increase the cells' apoptotic response to irradiation compared with the control samples, DLA's effect being pronounced somewhat stronger.

Figure 2 shows the results of a study of the action of the used agents on DNA SSB and DSB yield under radiation exposure. As is seen, with increasing the radiation dose, the number of SSBs and DSBs increases both in the control samples and in the samples with MLA and DLA. Along with this, the effect of the used agents on damage yield is different.

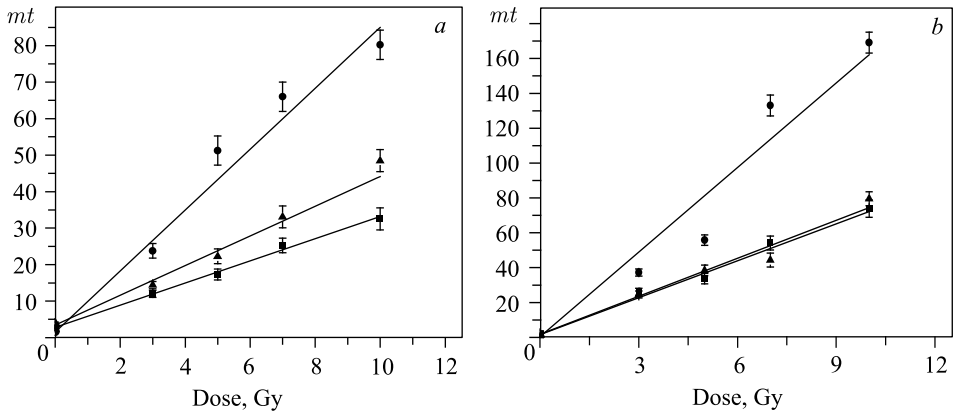

Fig. 2. The action of the modifying agents MLA and DLA on DNA DSB (*a*) and SSB (*b*) yield for irradiation with ^{60}Co γ rays: ■ — control; ▲ — MLA; ● — DLA

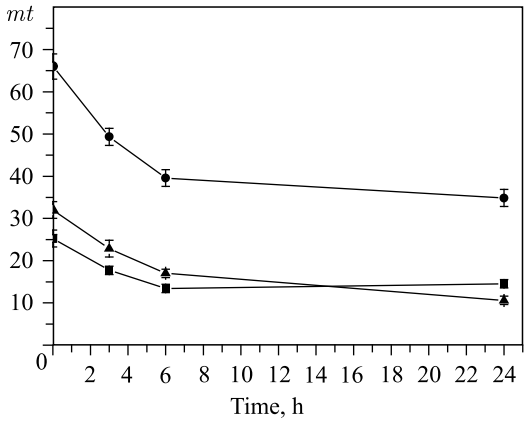

Fig. 3. DNA DSB repair kinetics for irradiation with ^{60}Co γ rays at 7 Gy: ■ — control; ▲ — MLA; ● — DLA

In the presence of DLA, as opposed to MLA, the number of the induced SSBs and DSBs sharply increases — approximately 2.5-fold. The genotoxic action of this agent is realized immediately after irradiation and persists up to 24 h of cell incubation (Fig. 3). Unlike this, DSB repair kinetics in the presence of MLA is similar to that in the control samples.

DLA's genotoxic effect on DNA SSB and DSB yield is observed immediately after cell irradiation, but SSB repair in the presence of this agent, like in the presence of MLA and in irradiated control samples, is completed in the first hours after irradiation (Fig. 4). Thus, the DLA effect on DNA SSB and DSB repair kinetics in irradiated cells is significantly different. Unlike efficient SSB repair in the first hours after irradiation, DSBs remain for a long time in irradiated cells in the presence of DLA.

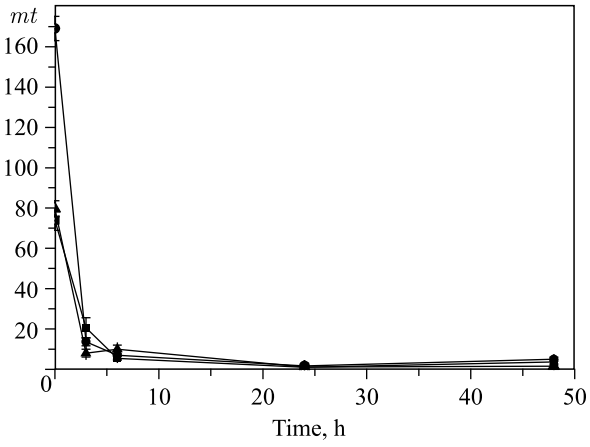

Fig. 4. DNA SSB repair kinetics for irradiation with ^{60}Co γ rays at 7 Gy: ■ — control; ▲ — MLA; ● — DLA

Thus, the used agents (lipid A derivatives: monophosphoryl lipid A (MLA) and diphosphoryl lipid A (DLA)) have a clear effect on cell genetic structures under gamma irradiation. It appears as a sharp growth of the number of DNA SSBs and DSBs compared with damage yield in the irradiated control samples, DLA's effect on DNA DSB yield being stronger than MLA's one. It is probably this fact that causes a higher frequency of apoptotic cell formation in the presence of this agent compared with the irradiated control samples.

As was already said, LPS — a compound of lipid A and polysaccharide — is a unique molecule that is common for all Gram-negative bacteria. Its presence in the host cell is a signal for triggering the organism's response. A prompt and strong antibacterial response started by this signal can be mediated by the immune system (cytokines and other cell mediators). These substances can have an immediate bactericidal effect or involve other cells to eliminate microorganisms. It was established that special CD14 receptor proteins detect and connect LPS with the following signal transduction. LPS induces the synthesis and secretion of pro-inflammatory cytokines (such as interleukin (IL-1, IL-6) and tumor necrosis factor (TNF), etc.) [15]. The action of LPS is greatly enhanced by the toll-like receptor. The recent discovery suggests that CD14 interacts with toll-like receptors to transmit a signal [16, 17]. This recruitment ultimately couples the recognition of LPS with the activation of the transcription factor complex NF-κB [18]. The activated transcription factor complex translocates to the nucleus, where it activates the promoters of many inducible genes, such as pro-inflammatory cytokines, in immune cells [19]. Ionizing radiation exposure is known with the ability to modulate transcript and/or protein levels of several cell cycle regulators (CDKN1A (p21), GADD45a, Cyclin G1 (CCNG1), CHK2-thr68) and apoptosis genes (BAX and BBC3) in diverse cell and blood model systems [20]. LPS treatment induced an inflammatory response in white blood cells by the secretion of IL-6 and TNF into all tested donors' plasma. Significant changes were observed in two biomarkers: an increase in CDKN1A and reduced expression in BBC3. The strongest effect of LPS on the radiation responses was seen for CDKN1A and BBC3.

The activation of the NF-κB complex can promote leukocyte apoptosis in certain contexts and contribute to the resolution of inflammation. The enhancement of the apoptotic action of ionizing radiation on cells in the presence of DLA and MLA agents, as is seen in Fig. 1, might be explained by the influence of the synthesis and secretion of pro-inflammatory cytokines IL-1 and IL-6, the NF-κB complex, etc. On the other hand, as follows from materials in Fig. 2, under the influence of lipid A derivatives, the frequency of the formation of DNA DSBs, which are an apoptosis inductor, increases. These facts seem to underlie the increasing of the apoptotic cell death frequency under exposure to radiation in the presence of the immunomodulator. The obtained results will probably be interesting for planning studies of a combined effect of radiation and immunomodulators on the formation of cells' apoptotic reactions for the whole organism irradiation.

Acknowledgements. The authors thank the Representative of the Government of the Slovak Republic (the Government Office for Cooperation with JINR) for financial support in 2012–2013. This work was also supported by the theme "Study of the Biological Action of High-Energy Heavy Charged Particles of Different Energies" (04-9-1077-2014/2016) of the Joint Institute for Nuclear Research (Dubna).

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Received on September 16, 2015.