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Effects of radiosensitization on proliferation-related events in Ehrlich ascites tumor cells

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Ehrlich ascites tumor cell damage after ionizing radiation (2 Gy) and radiosensitization (HPde 2 Gy) was investigated. According to the data obtained, 2 Gy induced 7% of multinuclear cells, 15% inhibited DNA synthesis, but no significant apoptosis/necrosis was observed after these cell injuries.

After radiosensitization (HPde 2 Gy) the number of multinuclear cells did not change, whereas DNA synthesis was inhibited up to 48% and mostly dead EAT cells were detected.

Key words: hematoporphyrin dimethyl ether, photodynamic therapy, Ehrlich ascites tumor

Abbreviations: Hpde – hematoporphyrin dimethyl ether, PDT – photodynamic therapy, EAT – Ehrlich ascites tumor

INTRODUCTION

Total EAT growth inhibition was observed when hematoporphyrin dimethyl ether (HPde) was combined with low doses of γ-radiation [1]. This combination together with photodynamic therapy (PDT) can be a promising approach in the effort to reduce the dose of photosensitizer, to avoid prolonged skin photosensitivity and eventually to damage more effectively deeper tumors [1, 2]. Since the nucleus is thought to be the main target for ionizing radiation, it was of interest to investigate some proliferation-related events in EAT cells after radiosensitization.

MATERIALS AND METHODS

Materials

The stock solution of hematoporphyrin dimethyl ether (HPde) (a gift from prof. G. V. Ponomarev, Russia) was prepared in physiological saline ($2.5 \times \times 10^{-3}$ M) and stored in the dark below 10 °C, [^{3}H-6] Thy (528 GBq/mmol) was obtained from Chemapol (Czech Republic), other chemicals were of highest quality.

Experimental design

Mice bearing 7th day EAT were injected with 40 mg/kg body weight HPde to reach the clinically accepted concentration. Following 3 hours cell suspension D = 0.6 O.D. (λ = 590 nm) ($3,7 \times 10^6$ cells/ml) was prepared and irradiatedl with 2Gy. γ-Irradiation for EAT cells suspension was performed by 60 CO irradiation at a dose rate from 1.0 to 1.62 Gy/min. The cuvette (λ = 1 mm) with cell suspension was used for irradiation. The dose applied to Ehrlich ascites tumor cell suspension was 2 Gy [3].

DNA synthesis level was evaluated as [^{3}H-6] Thy incorporation [4]. Briefly, 2 μCi/ml of [^{3}H-6] Thy was added to 2 ml suspension of EAT cells ($3.7 \times \times 10^6$ cells/ml) for 30 min at 37 °C. The medium was removed by centrifugation (5 min, 1000 g) and cells were twice washed with ice-cold PBS. The cells suspended in 2 ml ice-cold PBS were transferred to Millipore GS 0.22 μm filters and washed with PBS.

Then filters were exposed to 5 ml 5% trichloroacetic acid (TCA) for 10 min at 4 °C. The TCA solution was filtered through the same filter. The radioactivity on the filters which corresponded to the amount of [^{3}H-6] Thy incorporated into DNA was measured in toluene scintillation liquid (0.4 g PPP, 0.2 g POPOP in 1 l of toluene) with a LS-100C radiospectrometer (Beckman).

Apoptosis detection

Twenty hours after treatment nonpermeabilized cells (10^6 cells) were incubated with 4 μg/ml ethidium bromide in culture medium for 15 min at room temperature, in the dark as described in [5, 6]. The samples were analyzed with a FACSort instrument (Becton Dickinson). Apoptotic cells were detected on FSC/FL2 dot plot as an EB (ethidium bromide)^{dim} area. As a negative control, a sample with viable cells was used. Orange/red fluorescence of EB was collected through 585/42 nm bandpass. Up to 10000 cells were detected on the FCS/FL2 dot plot: R1 area – necrotic, R2 – apoptotic, R3 – viable [1].

Multinuclear cells

Slides were prepared from 7-d Ehrlich ascites tumor cells, fixed in ethanol for 20 min, hydrolyzed in 1 N HCl for 8 min and stained with Methylene Blue. The percentage of multinuclear cells was counted.

RESULTS AND DISCUSSION

It is commonly accepted, that the main target for ionizing radiation is the cell nucleus. For instance, 250 Gy delivered to cytoplasma had no effect upon the proliferation of these cells, whereas the lethal dose to the nucleus is less than 1.5 Gy. Consequently we observed DNA synthesis in the 2 Gy irradiated and HPde-2Gy irradiated cells. The data obtained are presented in Table.

Table. **Incorporation of [^{3}H]Thy in DNA of EAT cells 24 h after treatment**

Treatment type	% of control
Control	100
2Gy γ-irradiation	85 ± 8
60 HPde + 2Gy γ-irradiation	52 ± 13

* The DNA synthesis level was calculated as % of control cell DNA synthesis level in 4 independent experiments.

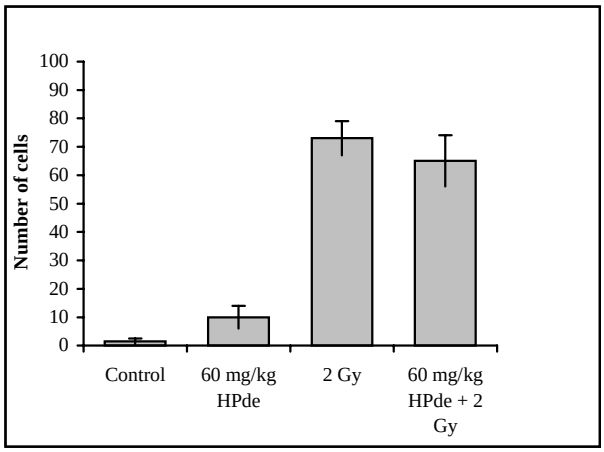

Fig. 1. Number of multinuclear EAT cells after different treatments (from 1000 observed)

It is evident that 2Gy γ-ionization itself has no significant effect on DNA synthesis (inhibition 15%) if compared with control cells (as measured 24 hours after treatment). So far after combined treatment (60 HPde + 2 Gy) cells showed a significantly lower activity of DNA synthesis in comparison with freshly 2 Gy-irradiated cells (48% inhibition).

Multinuclear cells are characteristic of the effects of radiation upon mitotic division of cells, thus, when the process of mitosis is inhibited, all other metabolic functions especially macromolecular synthesis, are continuing. Figure 1 presents the percentage of multinuclear cells after HPde treatment, after γ-irradiation (2 Gy) and after combined treatment (HPde +2 Gy). It is obvious that two different treatments induced the formation of multinuclear cells to a very similar extent.

So far, it must be emphasized that inhibition of tumor growth after radiosensitization is well documented [1]. What pathway of death does occur in

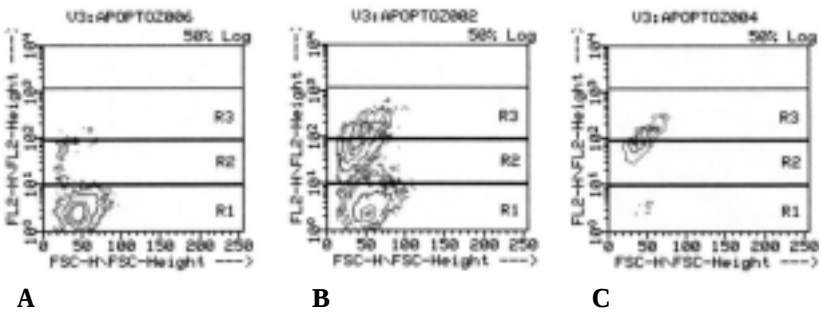

Fig. 2. Development of apoptotic process in EAT cells 20 h after HPde 2Gy treatment (R1 – viable cells, R2 – apoptotic cells, R3 – necrotic cells; A – control, not treated, B – 30HPde + 2Gy, C – 60Hpde + 2Gy)

these cells after different types of treatment? Following 24 h a marked redistribution of cells in the subpopulation of viable, apoptotic and necrotic cells was observed (Fig. 2).

According to our both previous and recent data, control cells preserved their viability, and only small subpopulations of apoptotic cells were detected (2%). 60 HPde did not change the initial distribution of cells in subpopulations, 2 Gy irradiation was not able to damage the cells, either. In the case 60 HPde 2 Gy treatment most of EAT cells were dead: 47% apoptotic, 46% necrotic and 7% of alive cells were detected.

Thus, our, data clearly show that following HPde 2 Gy treatment some radiosensitization does occur in EAT cells. Thus, inevitably the question arises: What is the mechanism of this radiosensitization?

It is commonly known that the best clinical radiosensitizer is oxygen dissolved in the tissue at physiological concentrations [7]. Thus, alternative sensitizers to oxygen must not only act by intracellular binding of naturally occurring radioprotectors (cysteine), but may also act by swinging the radiation chemical competition in favor of the oxidative pathway against the reductive. Only a compound that is electronaffinic will tend to be radiosensitizing [7]. This is the mechanism of action of the nitroimidazole class compounds, which have been used clinically. Bioreductive drugs also belong to this class, *i.e.* they are activated by metabolic reduction in tumor cell to form highly effective cytotoxins.

On the other hand, compounds that interfere with DNA synthesis may also be sensitizers, and so may the other cytotoxic chemotherapeutic agents used in combination with radiotherapy. In principle, the biochemical approach to radiosensitization is to use anticancer compounds (toxic), which mostly effect DNA synthesis or cell proliferation [7]. Thus, another concept might be derived from our observations: sometimes sublethal damage with a low dose of γ-radiation in combination with sublethal damage of HPde might produce a synergistic lethal damage of cells, which results in the total tumor growth inhibition. Thus, attempts to clarify this phenomenon are necessary.

In this study we tried to examine some changes in the cellular level following 2 Gy irradiation and combined HPde + 2 Gy treatment. According to the data obtained, 2 Gy treatment induced some increase in the formation of multinuclear cells (7%) and a certain inhibition of DNA synthesis (15%). So far no serious apoptotic/necrotic process has been detected in EAT cells after 2 Gy irradiation. When 2 Gy was combined with HPde, the formation of multinuclear cells did not increase, whereas inhibition of DNA synthesis reached 48%. Moreover, 20 h after treatment mostly dead EAT cells were found (47% of apoptotic, 46% of necrotic and 7% of alive).

CONCLUSIONS

It has been found before that no fast events at cell membrane level occur after radiosensitization (HPde + 2Gy) [1]. Nevertheless, drastic changes are obviously starting in the cell nucleus. DNA synthesis is disturbed, multinuclear cells appear and following induction apoptosis was detected. It presumably shows that a certain impact of high doses of HPde on cellular events in combination with low doses of γ-radiation might initiate apoptotic cell death. Evidently, further studies are required to elucidate extensively the dark toxicity of the used photosensitizer.

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RADIOSENSIBILIZACIJOS POVEIKIS EAT LÀSTELIØ PROLIFERACIJAI

Santrauka

Darbe tirta Erlicho ascito làsteliø þûtis po jonizuojanèios spinduliuotës (2 Gy) ir radiosensibilizacijos (2 Gy + HPde). Nustatyta, kad 2 Gy spinduliuotë ið dalies inhibuoja DNR sintezæ, indukuoja daugiabranduoliø làsteliø susidarymà, taèiau neaktyvina apoptozës/nekrozës procesø. Minëti procesai stipriai aktyvuojami radiosensibilizavus làsteles (2 Gy + HPde).