The Effect of Hematoporphyrin Dimethyl Ether on the Protein Level of Cyclin D3 and Cyclin-Dependent Kinase Inhibitor p27Kip1 in Ehrlich Ascites Tumor Cells

Zita Krivickienė[1]*,
Vilma Urbonavičiūtė[1],
Vytas Tamošiūnas[1],
Živilė Lukšienė[2]

[1] *Institute of Immunology of Vilnius University, Molėtų pl. 29, LT-2600 Vilnius, Lithuania*
[2] *Institute of Materials Science and Applied Research, Saulėtekio 9, LT-2040 Vilnius, Lithuania*

The phenomenon of radiosensitization described in our previous studies (1–4) is not yet understood. Due to the fact that Hpde per se might induce cell cycle perturbations (3), one of the possible reasons for the radiosensitization by hematoporphyrin dimethyl ether (Hpde) might be its effect on cell cycle regulation. In the present study we examined the effect of HPde on several cell cycle control proteins: cyclin D3 and cyclin-dependent kinase inhibitor (CKI) p27Kip1 in EAT cells. For the first time it has been demonstrated that the protein level of cyclin D3 and Cdk inhibitor p27Kip1 decreases following treatment with HPde in Ehrlich ascite tumor (EAT) cells, suggesting that HPde exerts a significant effect on cell cycle regulation by reducing the protein levels of cyclin D3 and CKI p27Kip1. It may contribute to increased cell radiosensitivity and subsequent inhibition of tumor growth.

Key words: HPde, cyclin D3, p27Kip1, Ehrlich ascites tumor cells

INTRODUCTION

Photodynamic therapy (PDT) has been described as a promising new modality for the treatment of cancer. PDT involves a combination of a photosensitizing agent (photosensitizer), which is preferentially taken up and retained by tumor cells, and visible light of a wavelength matching the absorption spectrum of the drug. Each of these factors is harmless by itself, but when combined they ultimately produce, in presence of oxygen, cytotoxic products that cause irreversible cellular damage and tumor destruction. So far, several lines of evidences indicate that some photosensitizers, hematoporphyrin dimethyl ether (HPde) and aminolevulinic acid (ALA) (1, 4), can act as radiosensitizers. Total Ehrlich ascites tumor (EAT) growth inhibition was observed combining HPde with low doses of gamma-radiation (1). The mechanisms of Hpde radiosensitization are still unclear. It is known that radiosensitization and cell growth inhibition are related with cell cycle regulation (1, 5 and our unpublished data). So, the examination of cell cycle control proteins is well-timed. In this study we examined the effect of HPde on the cell cycle G1 phase control proteins cyclin D3 and cyclin-dependent kinase inhibitor p27Kip1 in EAT cells.

During the early G1 phase, cells integrate external information derived from mitogenic stimuli and nutrient availability to prepare themselves for passing through the various phases of cell cycle (6). The D-type cyclins (D1, D2, and D3) are important integrators of mitogenic signaling (7), as their synthesis is one of the main end points of the RAS/RAF/MAPK pathway (8). When quiescent cells enter the cycle, genes encoding D-type cyclins (D1, D2, and D3) are induced in response to mitogenic signals, and the cyclins assemble with their catalytic partners, Cdk4 and Cdk6, as cells progress through G1 phase (11). D-type cyclins are similar in structure and biochemical functions, but they are expressed in a lineage-specific manner. Cyclin D1 is a rather unstable molecule transported from the nucleus to the cytoplasm, where it is targeted by the SCF ubi-

* Corresponding author.

quitin ligase for proteolysis. Nuclear export is mediated by the glycogen synthase kinase 3β (GSK-3β), a kinase that is inhibited by the RAS/PI3K/AKT pathway (11, 13). So, Cyclin D1 availability is controlled by a balance between the RAS/RAF/MAPK (cyclin D1 synthesis) (8, 13) and RAS/PI3K/AKT (cyclin D1 stability) mitogenic pathways (11, 13), as well as by GSK-3β and SCF activity (cyclin D1 degradation) (9, 10, 13). When D-type cyclins illegitimately activated and/or overexpressed, they can contribute to cellular transformation (19).

The other important regulators of G1 cell cycle phase are cyclin-dependent kinase inhibitors (CKIs) (12). They bind and inactivate various CDKs and cyclin–Cdks complexes. CKIs in all eukaryotes appear to play related roles in the control of progression through the G1 phase (13). The CKIs of mammals are grouped into two major structural families, each with a distinct mechanism of Cdks inhibition (12). Members of the CIP/KIP family, including p21Cip1, p27Kip1, and p57Kip2, control a broad range of cyclin–Cdks complexes by interacting with both subunits in the complex. In contrast, members of the INK4 family, including p15, p16, p18, and p19, display a clear specificity for the monomeric forms of Cdk 4/6 and act in part by reducing cyclin binding. Notably, the assembly of cyclin D and Cdk4/6 complexes requires assistance from CIP/KIP proteins, which enhance binding by interacting with both subunits of the complex without destroying their catalytic activity (13).

P27Kip1 belongs to the class of CKIs (14). The differences in the protein levels have been shown to be caused by the rate of translation and the rate of protein degradation (15, 16). The p27Kip1 protein is degraded by a ubiquitin-dependent pathway (15). The RAS/RAF/ERK pathway is involved in the degradation of p27Kip1 by the ubiquitin–proteasome pathway (17, 18).

In this paper we report the alterations in the protein levels of cyclin D3 and CKI p27Kip1 following treatment of Ehrlich ascites tumor cells with the radiosensitizer Hpde, which might influence EAT cell radiosensitivity.

MATERIALS AND METHODS

Reagents. RPMI1640 Medium was from Biochrom (Germany). Anti-cyclin D3 and anti-p27Kp1 were obtained from Transduction Laboratories (Lexington, KY). FBS was from SumBiotech (USA). BCIP/NBT was purchased from Bio-Rad Laboratories (USA). All other materials were purchased from Sigma (USA).

Cell culture. The photosensitizer was injected i.p. (60 mg/kg body weight) on the 7th day after Ehrlich ascite tumor (EAT) inoculation in mice. After 3 hours of incubation EAT cells were extracted from the intraperitoneum and a cell suspension with the optical density (OD) (λ = 590 nm) = 0.6 (3.7 × $\times 10^6$ cells/ml) was prepared.

Western blot analysis. After treatment the cells ($7.5 \cdot 10^6$) were harvested and washed with ice-cold phosphate buffer containing 100 μM Na_3VO_4, 137 mM NaCl, 2.7 mM, 4.3 mM Na_2HPO_4: $7H_2O$, 1.4 mM KH_2PO_4 (pH 7.3) and then lysed at 4 °C in a lysis buffer containing 1% Nonidet P-40, 50 mM NaCl, 10 mM Tris (pH 7.2), 5 mM EDTA, protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulphonyl fluoride) and phosphatase inhibitors (1 mM Na_3VO_4, 50 mM NaF). After removal of cellular debris by centrifugation at 14000 g for 14 min at 0 °C, supernatants were collected and protein concentrations determined using the Bio-Rad protein assay. Then the lysates were boiled for 5 min in a reducing 2 × SDS sample buffer (62 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 50 mM DTT; 0.1% bromphenol blue). The lysates were subjected to SDS–PAGE (50 μg of protein per lane), transferred to the PVDF membrane and analysed with anti-cyclin D3, anti-p27Kip1 and anti-Erk2.

RESULTS AND DISCUSSION

The protein level of cyclin D3 is downregulated following HPde treatment of Ehrlich ascites tumor cells. Cyclins are well-known effectors of cellular proliferation. To determine possible direct or indirect HPde targets in the cell, the effect of HPde on the protein level of cyclin D3 in EAT cells was examined. Whole cell lysates were prepared from EAT cells before and after treatment with HPde and analysed by Western blotting for cyclin D3. The data presented in Fig. 1. show that the protein level of cyclin D3 is downregulated following HPde treatment of EAT cells for 3 h suggesting that HPde action

Fig. 1. The effect of Hpde on the protein level of cyclin D3 in EAT cells.
EAT cells were left untreated (lane 1) or treated with Hpde for 3 h (lane 2). Whole cell lysates were prepared and assayed by immunoblotting for cyclin D3 protein. This is a representative result of three independent experiments

on the cells influences the availability of cyclin D3 protein by direct or indirect participation in protein level regulation of cyclin D3.

HPde treatment on Ehrlich ascites tumor cells attenuates protein levels of CKI p27Kip1. As the radiosensitivity of tumor cells depends on cell cycle, we analysed the relative protein levels of the other cell cycle control protein, CDK inhibitor p27Kip1, found in untreated and Hpde- treated EAT cells. Immunoblotting for p27Kip1. performed with whole cell lysates prepared from EAT cells before and after treatment with HPde. The results presented in Fig. 2. show that the reduction in the protein level of p27Kip1 was associated with HPde treatment of EAT cells for 3 h, implying direct or indirect HPde action on the protein turnover of p27Kip1. At present we do not know by which mechanism reduction in p27Kip1 protein levels occurs following treatment with HPde.

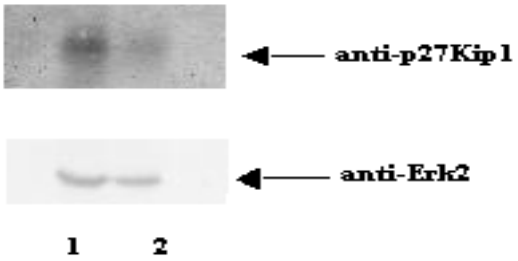

Fig. 2. The effect of Hpde on the protein level of p27Kip1 in EAT cells.
EAT cells were left untreated (lane 1) or treated with Hpde for 3 h (lane 2). Whole cell lysates were prepared and assayed by immunobloting for p27Kip1 protein. This is a representative result of three independent experiments

In conclusion, the results show that the photosensitizer/radiosensitizer hematoporphyrin dimethyl ether (HPde) *per se* exerts some cellular effects. First of all, according to our data, HPde influences the protein levels of G1 regulators cyclin D3 and Cdk inhibitor p27Kip1. These effects on cell cycle control proteins might contribute to the increased EAT cell radiosensitivity and subsequent inhibition of tumor growth.

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Z. Krivickienė., V. Urbonavičiūtė., Ž. Lukšienė, V. Tamošiūnas

HEMATOPORFIRINO DIMETILO ETERIO POVEIKIS LĄSTELIŲ CIKLO KONTROLĖS BALTYMAMS EHRLICHO ASCITINIO NAVIKO LĄSTELĖSE

S a n t r a u k a

Neseniai gauti duomenys išryškino, kad fotosensibilizatorius hematoporfirino dimetilo eteris (HPde) gali veikti ir kaip radiosensibilizatorius. Po HPde ir nedidelės dozės gama spinduliuotės poveikio gauta totalinė Ehrlicho ascitinio naviko (EAT) augimo inhibicija (1–5). Gautos radiosensibilizacijos veikimo mechanizmas nėra aiškus. Šiame darbe mes tyrėme HPde poveikį ląstelės ciklo kontrolės baltymams: ciklinui D3 ir nuo ciklinų priklausomų kinazių (Cdk) inhibitoriui p27Kip1 EAT ląstelėse. Iš gautų duomenų matyti, kad HPde sumažino ciklino D3 ir Cdk inhibitoriaus p27Kip1 baltymų kiekį EAT ląstelėse. Tai leidžia teigti, kad HPde tiesiogiai ar netiesiogiai veikia ląstelių ciklą mažindamas ciklino D3 ir Cdk inhibitoriaus p27Kip1 baltymų kiekį EAT ląstelėse. Dėl šio poveikio gali padidėti ląstelių jautrumas gama spinduliuotei ir kartu naviko augimo inhibicijai, kuri stebėta ankstesniuose darbuose (1, 5).