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INFLUENCE OF EXTRACELLULAR MAGNESIUM ON THE ANTITUMOUR EFFICIENCY OF PHOTODYNAMIC THERAPY

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Abstract. This study was designed to evaluate the influence of extracellular Mg on the antitumour efficiency of haematoporphyrin derivative (HpD)- or chlorin-e_6 trimethyl ester (E6)-based photodynamic therapy (PDT). In vitro experiments showed that in a calcium/serum-free medium the addition of $MgCl_2$ (from 1 up to 5 mM) to Ehrlich ascites carcinoma (EAC) cells induced (as measured by trypan blue test) a 1.5-fold increase in HpD–PDT efficacy. Moreover, during the HpD–PDT the presence of $MgCl_2$ enhanced the inhibition of both glycolytic and dehydrogenase activity of EAC cells, stimulated the photo-oxidation of cellular proteins and lipids, which was associated with a more considerable decrease in the reduced glutathione content. In the experimental conditions, Mg itself was practically nontoxic towards the cells at all concentrations investigated. Nevertheless, at a physiological concentration (1 mM) of Mg^{2+}, only a weak (about 10%) increase in the rate of HpD-photosensitized inactivation of EAC cells was observed. The influence of extracellular Mg on the efficiency of PDT did not depend on the nature of the sensitizer used; the in vitro effects with E6 were similar to those seen for HpD. However, in contrast to the in vitro experiments it was found that in vivo an elevated level of extracellular Mg increased essentially the viability of EAC cells pretreated (before intraperitoneal inoculation) with HpD–PDT. Although the exact molecular mechanisms are largely unknown, the in vivo results suggest that Mg is, most probably, a serum factor decreasing the antitumour efficiency of HpD–PDT.

Key words: haematoporphyrin derivative, chlorin-e_6, photodynamic therapy, tumour, magnesium.

Abbreviations: EAC = Ehrlich ascites carcinoma; E6 = chlorin-e_6 trimethyl ester; GSH = reduced glutathione; HpD = haematoporphyrin derivative; HBSS = Hanks' balanced salt solution, without phenol red, supplemented with 10 mM Hepes buffer, pH 7.4; MDA = malondialdehyde; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NKH = 154 mM NaCl, 6.2 mM KCl and 10 mM Hepes, pH 7.4; $^1O_2$ = singlet oxygen; $OH^\bullet$ = hydroxyl radical; $O_2^{\bullet-}$ = superoxide anion radical; PBS = phosphate-buffered saline; PDT = photodynamic therapy; PS = photosensitizer; PrSH = protein-bound sulfhydryl groups; TB = trypan blue; Trp = tryptophan.

INTRODUCTION

Photodynamic therapy (PDT) is a new promising method for the treatment of malignant tumours involving the administration of a tumour-localizing photosensitizer (PS) and its subsequent activation by light. Haematoporphyrin derivative (HpD) and its improved version, known as Photofrin-II, are still the most widely used PSs for PDT of tumours. Photoexcitation of tumour-localizing components of HpD leads to the formation of singlet molecular oxygen ($^1O_2$), which is reported to be the main agent responsible for induction of tumour necrosis [1]. The mechanism of PDT is not fully understood, but may involve a direct tumouricidal effect (damage to organelles within the malignant cells [2, 3]) and also microcirculatory changes resulting in reduced blood flow [4].

The tumouricidal effects of HpD–PDT depend on the presence of oxygen [5], the pH value of tumour tissue [6], and other factors. Among them a substantial influence on the efficiency of PDT may be exerted by calcium and magnesium (main divalent cations in body fluids and cells). It has been amply documented that calcium plays a role (both at cellular and tissue levels) in the mechanisms of PDT-mediated tumour ablation [7]. For instance, it was reported that a photodynamically induced increase in cytoplasmic free Ca in HpD-loaded T24 human bladder transitional carcinoma cells (mainly due to influx of extracellular Ca) protect these cells against photodynamic cell killing [8]. However, in the literature we did not find any information about an influence of Mg^{2+} cations on the efficiency of PDT.

Magnesium, a natural Ca-blocker at either cell or organism level [9], is deeply involved in the modulation of a variety of biochemical processes. For example, Mg^{2+} is an absolute requirement for transphosphorylation reactions [10], which are fundamental to cell energy metabolism. A decrease in Mg^{2+} availability to cells inhibits nucleic acid and protein synthesis as well as transport and utilization of glucose [11]. Mg^{2+} is also an essential cofactor in glutathione biosynthesis [12]. A significance of Mg^{2+} in cytotoxicity induced by different xenobiotics [13, 14] and oxidizers has been also proved. For instance, in hepatocyte and endothelial cell cultures, the rate of iron-dependent lipid peroxidation is inversely related to medium Mg^{2+} concentration [15, 16]. On this basis, it could be assumed that Mg^{2+} may play a role in the protection of tumour cells against HpD-mediated PDT. On the other hand, in a previous study [17] we found (in vitro) that the potentiating effect of Lonidamine (an antineoplastic drug acting via inhibition of the energy metabolism) on HpD–PDT of tumour cells is more pronounced in the presence of extracellular Mg^{2+}. It was also shown by others [18] that under various cytotoxic treatments an elevated level of cytosolic free Mg may mediate (by stimulation of Mg^{2+} dependent endonucleases) apoptotic cell death. Such a mode of cell death (via apoptosis) after PDT has been demonstrated both in vitro [19] and in vivo [20]. These data suggest that Mg^{2+} may promote HpD-sensitized photo-inactivation of neoplastic cells.

The aim of the present study was therefore to evaluate the role of extracellular Mg in PDT-induced cytotoxicity using HpD or chlorin-e_6 trimethyl ester (E6) as a PS. Due to their better spectral properties certain chlorin-e_6 derivatives are proposed as alternative PSs for PDT of cancer [21].

MATERIALS AND METHODS

Chemicals

HpD was prepared from haematoporphyrin IX dihydrochloride (Aldrich) by the method of Lipson et al. [22] modified by Kessel et al. [23]. The obtained product was diluted with 0.9% NaCl solution (pH 7.4) to a final porphyrin concentration of 5 mg ml^{-1}, and stored in the dark at −70°C. The working solutions of HpD, at a concentration of 0.1 to 1.0 mg ml^{-1}, were prepared immediately before use by further diluting of the stock solution with a medium (see below). E6 was synthesized from pheophytin-a extracted from nettle leaves according to the procedure of Lötjönen & Hynninen [24]. Working solutions of E6 were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 2 mM and were used on the same day as prepared. $MgCl_2 \cdot 6H_2O$ and all other chemicals (of analytical grade or better) were purchased from Sigma, St Louis, USA. For in vivo experiments, all solutions, including HpD preparations, were sterilized by filtration through 0.22 μm filter units.

Animals and cells

White, mongrel, three month old female mice obtained from the Institute of Experimental and Clinical Medicine (Tallinn, Estonia) were used in the experiments. The animals were fed ad libitum on standard pellets and had permanent access to water. Ehrlich ascites carcinoma (EAC) cells obtained from the Institute of Chemical Physics and Biophysics (Tallinn, Estonia) were weekly transplanted intraperitoneally (i.p.) into mice, 3×10^7 cells per animal.

Light source

In all the experiments a 1 kW xenon arc lamp, which has a continuous character of light emission from 200 nm up to the infrared (IR) domain of the solar spectrum, equipped with glass filters (KS-10 together with SZS-25) that transmit 50% of light at 630 nm (the range between 590–830 nm), and a focusing optical system served as the radiation source. The IR radiation was removed by a 4 cm water filter. The flux of light was focused as a spot (2.54 cm^2) and directed on the front face of a quartz cuvette containing EAC cell suspension or HpD solutions. The intensity of the emitted light at 630 nm was always 220 mW with a power density of 86.6 mW cm^{-2}, as measured by an IMO-2N radiometer (Russian Federation). The EAC cell suspension was also irradiated with 665 nm light (when E6 was used as a PS), isolated by means of the glass filters (KS-14 together with SZS-25; bandpass range 630–830 nm). In this study the intensity of the emitted light at 665 nm was 170 mW with a power density of 67 mW cm^{-2}.

Preparation of cell suspensions and the irradiation conditions

For in vitro experiments, six to eight days old EAC cells were withdrawn with a syringe from the abdominal cavities of the mice and a five-fold amount of cooled NKH medium (154 mM NaCl, 6.2 mM KCl and 10 mM Hepes, pH 7.4) or Ca/Mg-free phosphate-buffered saline (PBS), pH 7.4, was added to the cells. The cell suspension was then stirred and centrifuged for 8 min at 4°C. The packed cells were resuspended in one of the isotonic solutions described above to give a concentration of 1.2×10^8 cells ml^{-1} and kept in an ice bath until use. The viability of the cells was about 95–98%, as found by phase-contrast microscopy in the presence of trypan blue (TB). Finally, the cells were diluted with a medium to a concentration of 0.5 to 4×10^7 cells ml^{-1}. 5 min after the addition of a PS, the cells were irradiated at 665 or 630 nm for E6 and HpD, respectively. Our prior studies [25, 26] showed that after a short incubation time (5 min) approximately 20–25% of the initial content of HpD (1 μg ml^{-1}) or E6 (2 μM) is bound to EAC cells. It was also found that the presence of non-bound PSs in a medium has a negligible effect on the rate of HpD- or E6-photosensitized damage of the cells. The irradiation was carried out in a quartz cuvette (with optical path length of 2 cm; volume 8 ml) by magnetic stirring at 20°C. In all the experiments, the addition of $MgCl_2$ to EAC cells was performed 5 min prior to photoirradiation.

Determination of the amount of injured cells and activity of dehydrogenases

The amount of injured cells in the suspension was determined immediately after irradiation by staining the cells with 0.1% TB in PBS as described earlier in [27]. Additionally, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) was used. This assay is based on the ability of mitochondrial dehydrogenases (at the sites of cytochromes b and c) in viable cells to reduce the yellow form of MTT to a purple formazan [28]. For the determination of the dehydrogenase activity of EAC cells, fractions of 0.02 or 0.1 ml (containing 5×10^5 cells) were withdrawn in duplicate and were placed in test tubes. The cells were then washed once in ice-cold Hanks' balanced salt solution, without phenol red, supplemented with 10 mM Hepes buffer, pH 7.4 (HBSS). Immediately after the addition of 0.5 ml of 2 mg ml^{-1} MTT solution (in HBSS), EAC cells were incubated for 2.5 h in a water bath at 37°C. At the end of the incubation, the tubes with samples were immersed in an ice bath for 5 min, and the cells were harvested by centrifugation at $3000 \times g$ for 10 min at 4°C. The supernatant was removed by aspiration and the pellet was dissolved in 2 ml DMSO. Optical absorbance of the converted dye was measured at 570 nm.

Assay of glycolysis

The rate of glycolysis was estimated by glucose consumption. EAC cells at a concentration of 1×10^7 cells ml^{-1} were incubated for 1 h in a water bath (37°C) in HBSS. At the end of the incubation, 0.5 ml of 9% trichloroacetic acid (TCA) solution was added to 1 ml of cellular suspension. The samples were vortexed

and stored for 1 h at 4 °C. The cells were then sedimented by centrifugation at $3000 \times g$ for 10 min at 4 °C. The glucose concentration in the supernatant was measured colorimetrically by standard *o*-toluidine reagent using a Sigma Kit (procedure No. 635).

Determination of the intracellular content of ATP

Immediately after HpD–PDT, adenosine triphosphate (ATP) was extracted from 1×10^6 cells by the addition of 0.1 ml of an ice-cold solution containing 5% TCA and 4 mM ethylenediaminetetraacetic acid. The cell extracts were then assayed with luciferin/luciferase using Luminometer 1251 (BioOrbit, Turku, Finland), essentially as described by Kahru et al. [29].

Assay for lipid peroxides

The lipid peroxidation in EAC cells was estimated immediately after PDT by measuring the formation of malondialdehyde (MDA) with 2-thiobarbituric acid exactly as described in one of our earlier studies [30].

Reduced glutathione and protein-bound sulfhydryl groups

Reduced glutathione (GSH) and protein-bound sulfhydryl groups in EAC cells were assayed with 5,5'-dithiobis-2-nitrobenzoic acid exactly as described in [26, 31].

Tryptophan content of EAC cells

Tryptophan (Trp) was determined according to the method of Spies & Chambers [32]. For each sample, 2×10^7 cells were collected in 1 ml PBS (154 mM NaCl, 6.2 mM KCl, and 10 mM sodium phosphate buffer, pH 7.4) and 9 ml of 22.3 mM solution of *p*-dimethylaminobenzaldehyde in 21.1 N H_2SO_4 was added (the Ehrlich reagent was prepared immediately before use). After 16 h at room temperature, 0.1 ml of 0.045% $NaNO_2$ solution was added, and 30 min later the absorbance was measured at 590 nm.

In vivo viability of EAC cells subjected (in vitro) to HpD–PDT

For the in vivo experiments, EAC cells (in sterile conditions) were washed once from the ascitic fluid in cold NKH medium with 5.55 mM glucose and were then resuspended in the same medium at a concentration of 4×10^7 cells ml^{-1}. 8 ml of the cell suspension was transferred into a quartz cuvette with 2 cm optical path length (heat-sterilized before use). 5 min after the addition of HpD, up to a final concentration of 5 $\mu g\ ml^{-1}$, the cells were irradiated (without or in the presence of 5 mM $MgCl_2$) with red light (at 630 nm) under magnetic stirring at 20 °C. At irradiation intensity 220 mW, the light exposure time was 6 min (the energetic exposure, 31.2 J cm^{-2}). In the control experiments, EAC cells were incubated for 11 min with the same preparations (HpD only or HpD jointly with $MgCl_2$) but not subjected to photoirradiation. Immediately after these treatments, 0.8 ml of the cell suspension (containing 3.2×10^7 cells) was injected (i.p.) into a

mouse. Female mice with an average weight of 25 g were used. In order to evaluate the impact of PDT on cell survival, we determined the rate of Ehrlich ascites tumour growth by weighing the tumour-bearing animals. In addition, the mice were sacrificed (under anaesthesia) on the eighth day after i.p. inoculation of the cells. The tumour was removed by aspiration; its weight and concentration of carcinoma cells in ascites were measured.

Estimation of the intracellular level of NAD(P)H

Since the reduced forms of nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) are mainly responsible for the fluorescence band at 470 nm in normal as well as tumour cells [33], the level of NAD(P)H in EAC cells was evaluated fluorometrically. Before the measurements, EAC cells were resuspended in 2 ml of NKH medium (without glucose and magnesium) at a concentration of 5×10^6 cells ml^{-1}. The fluorescence of the cell suspension at 470 nm (excitation at 340 nm) was measured in a quartz cuvette with optical path length 1 cm under magnetic stirring at 20°C. The fluorescence intensities were registered by a spectrofluorometer Hitachi 650-60 (Japan) with 5 nm band widths for excitation and detection.

RESULTS

PDT of EAC cells without administration of $MgCl_2$

Figure 1 demonstrates that the time course of EAC cells photo-inactivation by HpD–PDT was S-type. Indeed, after the lag period (12 min) during which the number of stained cells remained at the control level, a strong increase in the rate of the uptake of TB was observed. This indicates an essential damage of plasma membranes in the cells. At the same time, photoirradiation of EAC cells in the absence of HpD, as well as their incubation with the PS in the dark, did not mediate any noticeable increase in the number of injured cells (data not shown). For in vitro experiments, the irradiation time (t_{50}) at which 50% of the cells were stained by TB was utilized as a criterion to evaluate the efficiency of PDT-induced cytotoxicity.

Figure 2 shows that the photoirradiation of EAC cells in the presence of HpD mediated a considerable inhibition of their dehydrogenase activity. The measurements by MTT assay demonstrate that HpD–PDT also caused an essential injury of the mitochondria of the cells. Furthermore, HpD-based PDT led to a substantial inhibition of the glycolytic activity of EAC cells (Fig. 3) and a dramatic (approximately 80%) fall in the intracellular content of ATP (Fig. 4). Thus HpD-induced photosensitization caused a strong injury not only to the membrane structures but also to the energy homeostasis of EAC cells. It is important to note that these disturbances of energy metabolism (the PDT-induced inhibition of glucose consumption and ATP depletion) preceded the disintegration of the outer membrane in EAC cells as estimated by TB test (Figs. 3 and 4).

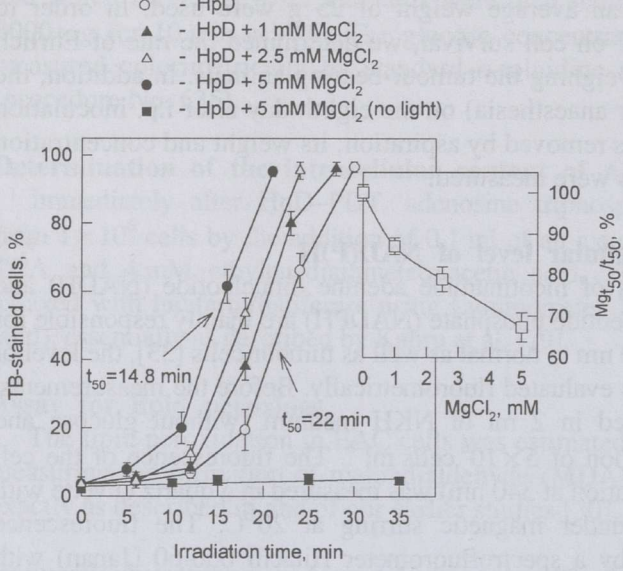

Fig. 1. The rate of trypan blue (TB) staining of EAC cells (5×10^6 cells ml^{-1} in NKH medium with 5.55 mM glucose) after 1 μg ml^{-1} HpD–PDT with or without $MgCl_2$. Inset: Effect of $MgCl_2$ concentration on the efficiency of HpD-sensitized photodamage of EAC cells. t_{50}, irradiation time at which 50% of the cells were stained by TB ($^{Mg}t_{50}$, the same in the presence of $MgCl_2$). Each data point is the mean of 3 separate experiments; bars show the standard error (SE).

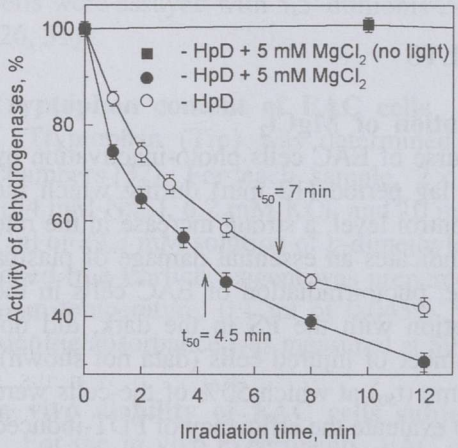

Fig. 2. Effects of HpD-induced photosensitization (HpD, 1 μg ml^{-1}) without or in the presence of $MgCl_2$ on the dehydrogenase activity of EAC cells (5×10^6 cells ml^{-1} in NKH medium with 5.55 mM glucose). The initial activity of dehydrogenases of the non-irradiated cells was determined as 78.6 ± 1.6 nmoles MTT-formazan h^{-1} per 1×10^6 cells; the value was taken as 100%. t_{50}, irradiation time at which 50% decrease of dehydrogenase activity of the cells was observed. Bars, SE from 3 independent experiments.

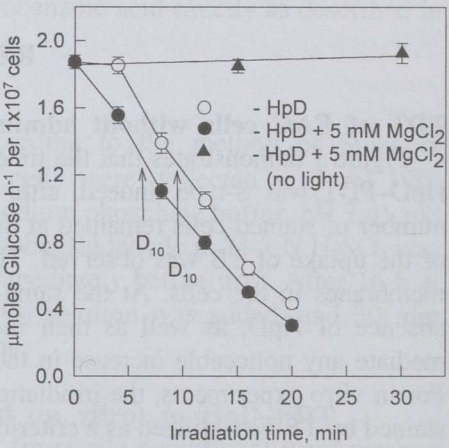

Fig. 3. The rate of glucose consumption of EAC cells (4×10^7 cells ml^{-1} in NKH medium) after 5 μg ml^{-1} HpD–PDT without or in the presence of 5 mM $MgCl_2$. D_{10} is the light dose at which 10% of the cells were stained by TB (before TB-staining EAC cells at a concentration of 1×10^7 cells ml^{-1} were incubated in HBSS for 1 h at 37 °C). Bars, SE from 3 independent experiments.

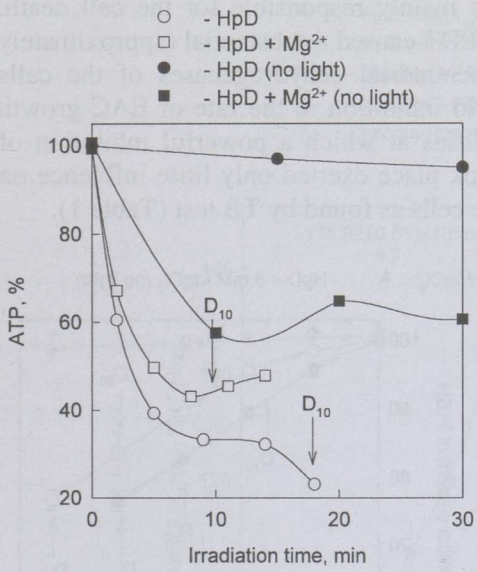

Fig. 4. The ATP content of EAC cells (1×10^7 cells ml^{-1} in PBS with 5.55 mM glucose) after 2 μg ml^{-1} HpD–PDT without or in the presence of 5 mM $MgCl_2$. D_{10} is the light dose at which 10% of the cells were stained by TB. SE<7%.

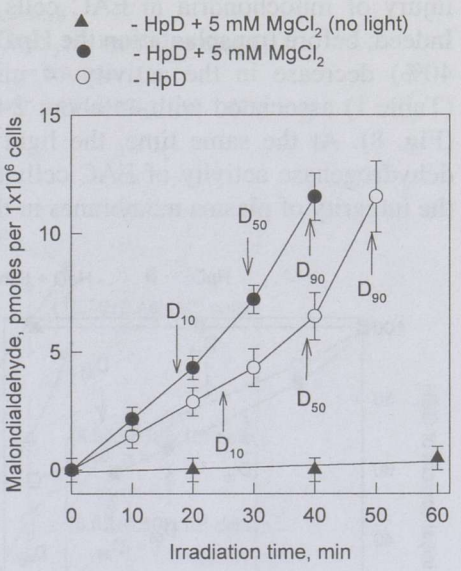

Fig. 5. The kinetics of malondialdehyde formation in EAC cells (4×10^7 cells ml^{-1} in PBS with 5.55 mM glucose) during 5 μg ml^{-1} HpD–PDT. D_{10}, D_{50}, D_{90} are light doses needed for TB-staining of 10, 50, and 90% of the cells, respectively. Bars, SE from 3 independent experiments.

Besides the effects described above, photoirradiation of EAC cells in the presence of HpD caused the stimulation of lipid peroxidation as measured by MDA formation (Fig. 5) and GSH depletion (Fig. 6a). These events were associated with a clearly expressed diminution of the Trp (Fig. 7) and of protein-bound sulfhydryl groups (PrSH) content (Fig. 6b). Moreover, it was established that under HpD–PDT PrSH degrade more easily than Trp residues of proteins. In fact, at a light dose that kills about 90% of the cells (as estimated by TB test) approximately 25% of the PrSH were destroyed and only a small (about 5%) decrease in the Trp content was observed (Fig. 6b and Fig. 7, respectively).

Figure 8 depicts the dynamics of tumour growth after i.p. injection of EAC cells pretreated in vitro with 5 μg ml^{-1} HpD and 6 min of light or by the addition of HpD without photoirradiation. The time after EAC inoculation, when a 10% increase in the weight of tumour-bearing mice was observed (t_{10}), was utilized as a parameter to evaluate the efficiency of HpD-sensitized photo-inactivation of the cells. The measurements of tumour weight and total number of EAC cells in the ascitic fluid indicate that the in vitro HpD–PDT provided an almost 2-fold inhibition in the rate of tumour growth (Fig. 8). Hence, the single HpD–PDT (without addition of $MgCl_2$) mediated an approximately 50% decrease in the number of viable cells. Moreover, our data suggest that under HpD–PDT the

injury of mitochondria in EAC cells is mainly responsible for the cell death. Indeed, before transplantation the HpD–PDT caused a substantial (approximately 40%) decrease in the activity of mitochondrial dehydrogenases of the cells (Table 1) associated with an about 2-fold inhibition in the rate of EAC growth (Fig. 8). At the same time, the light doses at which a powerful inhibition of dehydrogenase activity of EAC cells took place exerted only little influence on the integrity of plasma membranes in the cells as found by TB test (Table 1).

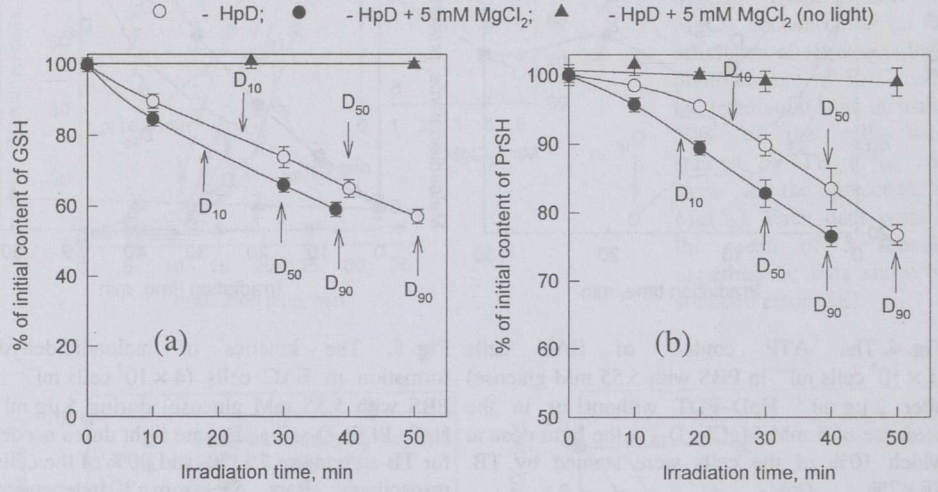

Fig. 6. Influence of $MgCl_2$ on the reduced glutathione (a) and protein-bound sulfhydryl groups (PrSH) content (b) of EAC cells (4×10^7 cells ml^{-1} in PBS with 5.55 mM glucose) under 5 μg ml^{-1} HpD–PDT. The initial levels of GSH and PrSH in the non-irradiated cells were 3.14 ± 0.14 and 17.1 ± 0.49 nmoles per 1×10^6 cells, respectively. D_{10}, D_{50}, D_{90} are light doses needed for TB-staining of 10, 50, and 90% of the cells, respectively. Bars, SE from 3 independent experiments.

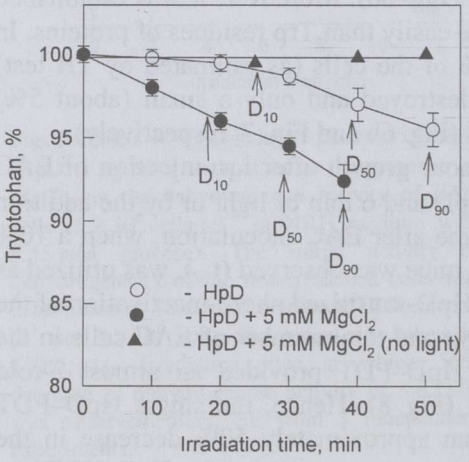

Fig. 7. Effects of HpD-induced photosensitization (HpD, 5 μg ml^{-1}) without or in the presence of 5 mM $MgCl_2$ on the tryptophan content of EAC cells (4×10^7 cells ml^{-1} in PBS with 5.55 mM glucose). The values of the non-irradiated cells were taken as 100%. D_{10}, D_{50}, D_{90} are light doses needed for TB-staining of 10, 50, and 90% of the cells. Bars, SE from 3 independent experiments.

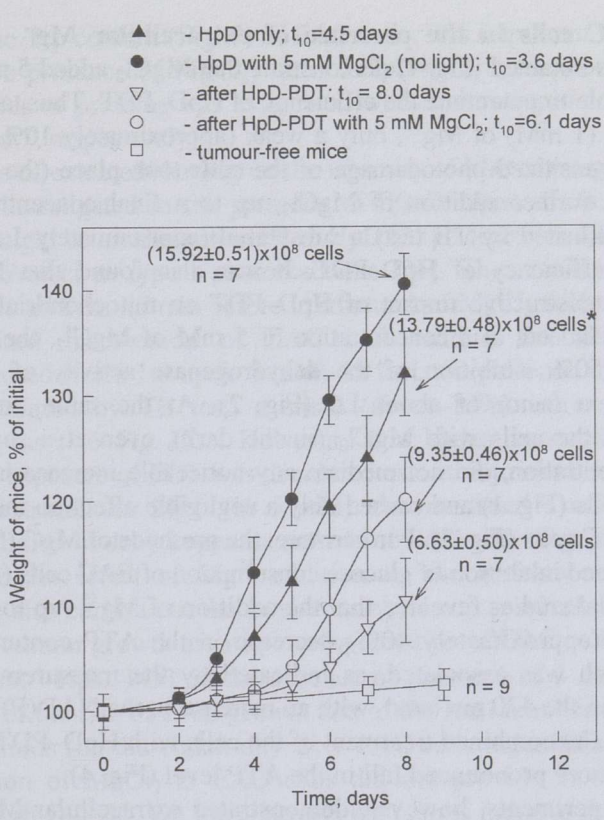

Fig. 8. Change in the weight of tumour-bearing mice after intraperitoneal inoculation of EAC cells subjected (in vitro) to HpD–PDT without or in the presence of $MgCl_2$. t_{10} is the time after EAC transplantation at which 10% increase in the weight of tumour-bearing animals was observed. n, the number of mice in a group. *, the total number of EAC cells in ascites (per mouse) on the eighth day after the tumour transplantation. Bars = SE.

Table 1. The effect of $MgCl_2$ on 5 μg ml^{-1} HpD-photosensitized (irradiation time 6 min with energetic exposure 31.2 J cm^{-2}) injury of EAC cells (4×10^7 cells ml^{-1} in NKH medium with 5.55 mM glucose)

Treatment regime	Activity of dehydrogenases, nmoles MTT-formazan h^{-1} per 1×10^6 cells ±SE	TB-stained cells[a], %±SE
HpD without irradiation (control)	75.8 ± 1.6	2.7 ± 0.9
HpD with 5 mM $MgCl_2$ (no light)	75.0 ± 2.1 (98.9%[b])	2.6 ± 0.8
HpD–PDT only	47.5 ± 1.9 (62.7%)	16.5 ± 2.5
HpD–PDT with 5 mM $MgCl_2$	39.8 ± 2.2 (52.5%)	32.7 ± 3.0

[a] before TB-staining the irradiated and non-irradiated cells (at a concentration of 1.6×10^6 cells ml^{-1} were incubated in HBSS for 2.5 h at 37 °C;

[b] % of control.

PDT of EAC cells in the presence of extracellular Mg

The results obtained (Fig. 1) demonstrate that $MgCl_2$ added 5 min prior to light exposure is able to potentiate the efficiency of HpD–PDT. Thus, at a physiological concentration (1 mM) of Mg^{2+}, only a weak (approximately 10%) increase in the rate of HpD-sensitized photodamage of the cells took place (the inset to Fig. 1). However, the further addition of $MgCl_2$, up to a final concentration of 5 mM, caused (as estimated by TB test) a substantial (approximately 1.5-fold) enhancement in the efficiency of HpD–PDT. It was also found that Mg^{2+} essentially enhanced the destructive impact of HpD–PDT on mitochondrial components of tumour cells. Indeed, at a concentration of 5 mM of $MgCl_2$, the irradiation time required for 50% inhibition of the dehydrogenase activity of EAC cells was decreased by a factor of about 1.5 (Fig. 2). At the same time, a prolonged incubation of the cells with $MgCl_2$ (in the dark), even at a supraphysiological (5 mM) concentration, did not mediate any noticeable increase in the number of TB-stained cells (Fig. 1) and caused only a negligible effect on the dehydrogenase activity of EAC cells (Fig. 2). Furthermore, the presence of Mg^{2+} (5 mM) enhanced the PDT-induced inhibition of glucose consumption of EAC cells (Fig. 3).

Biochemical studies revealed that the addition of Mg^{2+} (up to 5 mM) leads to a substantial (approximately 40%) decrease in the ATP content in EAC cells (Fig. 4), which was associated, as indicated by the measurements of cellular fluorescence in the 470 nm band, with an increase in the NAD(P)H level (Fig. 9). Nevertheless, the combined treatment of the cells with HpD–PDT and $MgCl_2$ did not induce a more pronounced fall in the ATP level (Fig. 4).

Further experiments, however, demonstrated extracellular Mg to be able to enhance the PDT-induced oxidative stress. Indeed (Fig. 6a), during HpD–PDT, $MgCl_2$, at a concentration of 5 mM, mediated a more rapid decrease in the intracellular content of GSH. At the same time, Mg^{2+} itself, without PDT, exerted a minor influence on the GSH level in EAC cells. Moreover, the addition of $MgCl_2$ (up to 5 mM) increased the rate of HpD-photosensitized oxidation of cellular lipids (Fig. 5) and proteins, as indicated by the measurements of PrSH

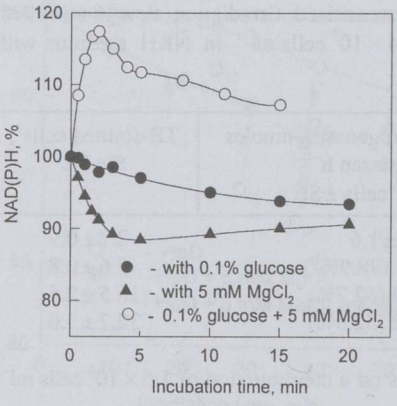

Fig. 9. The effect of $MgCl_2$ on NAD(P)H level in EAC cells. The measurements were performed 10 s after the addition of 10% $MgCl_2$ or 10% glucose solution to the cells. SE<5%.

(Fig. 6b) and the Trp content (Fig. 7) of EAC cells. It is important to note that the elevated (by the presence of $MgCl_2$) rate of HpD-sensitized photo-oxidation of unsaturated lipids or proteins was associated with a more rapid cell inactivation (Figs. 5, 6b, and 7, respectively).

An influence of extracellular Mg on the intensity of photodamage to neoplastic cells was also investigated in the presence of E6. Because of its better spectral properties, E6 might be an alternative PS to HpD [27]. Moreover, E6, in contrast to HpD, is able to react in type I photoreactions [34]. The results obtained show (Fig. 10) that the addition (5 min before irradiation) of $MgCl_2$ enhanced the rate of E6-photosensitized inactivation of EAC cells within the whole range of concentrations used (from 1.0 up to 5 mM). In fact, the presence of 1 mM $MgCl_2$ caused (as measured by TB test) a small (about 10%) increase in E6–PDT efficiency (the inset to Fig. 10a). However, at a higher concentration of Mg^{2+} (5 mM), the t_{50} exposure of PDT was decreased essentially, by about 33%. During E6–PDT, the extracellular Mg^{2+} (in 5 mM) also mediated a more pronounced (1.5 times) inhibition of dehydrogenase activity of EAC cells (Fig. 10b).

To evaluate the effect of Mg^{2+} addition on the antitumour efficiency of HpD–PDT, we also carried out the in vivo assay of cell survival. Figure 8 shows the kinetics of tumour growth after i.p. transplantation of EAC cells subjected (in vitro) to 5 μg ml^{-1} HpD–PDT in the absence or presence of 5 mM $MgCl_2$. The measurements of the rate of EAC growth and of the total number of tumour cells in the ascitic fluids (on the eighth day after EAC cells inoculation) demonstrate that the addition of $MgCl_2$ to EAC cells did not provide an increase in the cytotoxicity of HpD–PDT. On the contrary, the results indicate that the presence of Mg^{2+} (in all the experiments EAC cells were transplanted without washing)

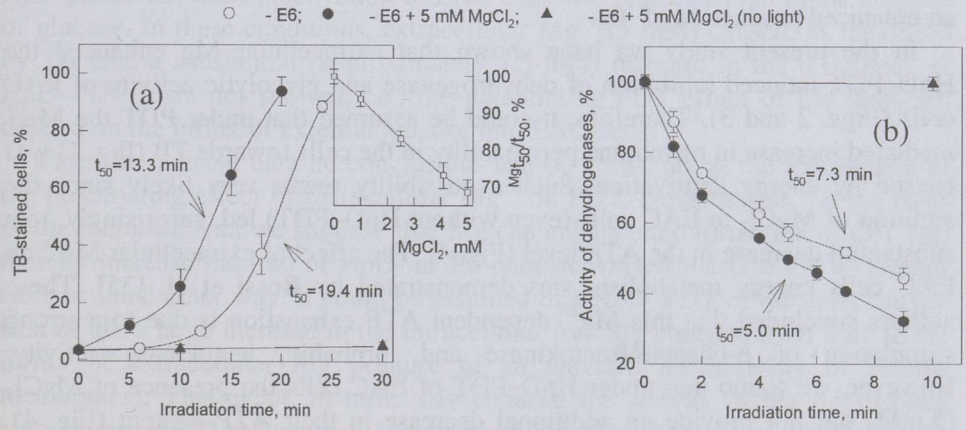

Fig. 10. The rate of trypan blue (TB) staining (a) and dehydrogenase activity (b) of EAC cells (5×10^6 cells ml^{-1} in NKH medium with 5.55 mM glucose) after 2 μM E6–PDT with or without $MgCl_2$. Inset: Effect of $MgCl_2$ concentration on the efficiency of E6-photosensitized damage of EAC cells. t_{50}, irradiation time at which 50% of the cells were stained by TB or 50% decrease of dehydrogenase activity of the cells was observed by MTT assay ($^{Mg}t_{50}$, the same in the presence of $MgCl_2$). Bars, SE from 3 independent experiments.

substantially increased the survival of the cells pretreated by HpD–PDT and to a smaller degree than with HpD alone.

DISCUSSION

In this study it is shown that extracellular Mg may increase the rate of HpD- or E6-photosensitized inactivation of tumour cells. Indeed, as found in vitro by TB test and MTT assay, the HpD– or E6–PDT induced membranous (Figs. 1 and 10a) or mitochondrial damages (Figs. 2 and 10b) acquired a more pronounced character when EAC cells were irradiated in the presence of $MgCl_2$. In the experiments Mg acted synergistically with PDT since extracellular Mg itself, even at supraphysiological concentrations (up to 5 mM), was practically nontoxic towards EAC cells. Several ways can be envisioned in which $MgCl_2$ could enhance cellular photosensitivity in vitro. The addition of $MgCl_2$ to EAC cells could possibly either alter the porphyrins uptake or increase $^1O_2$ production during light exposure. These events would have profound effects on PDT response. However, in a previous work [17] we found that under a long-term (60 min) dark incubation of EAC cells with HpD, the presence of $MgCl_2$ (up to 5 mM) causes a 7% decrease in the cellular HpD content. Thus we cannot explain the potentiating effect of extracellular Mg^{2+} on the efficiency of PDT by its influence on the binding of HpD to the cells. We also found that the presence of Mg^{2+} (from 1 up to 5 mM) had no effect on $^1O_2$ production when the rate of HpD or E6-sensitized photo-oxidation of Trp (a known trap of $^1O_2$) was monitored in simple solutions (data not shown). Hence, it seems unlikely that the potentiating effect of extracellular Mg on the efficiency of PDT was mediated by an enhanced production of $^1O_2$.

In the present study we have shown that extracellular Mg enhanced the HpD–PDT induced inhibition of dehydrogenase and glycolytic activity of EAC cells (Figs. 2 and 3). Therefore, it could be assumed that under PDT the Mg^{2+} mediated increase in membrane permeability in the cells towards TB (Fig. 1) was caused by energy deprivation. Such a possibility seems very likely since the addition of $MgCl_2$ to EAC cells (even without HpD–PDT) led, surprisingly, to a substantial decrease in the ATP level (Fig. 4). The effect of extracellular Mg^{2+} on EAC cells energy metabolism was demonstrated by Bossi et al. [35]. These authors concluded that this Mg^{2+} dependent ATP exhaustion is due to a strong stimulation of 6-phosphofructokinase and, probably, hexokinase activity. However, we found that under HpD–PDT of EAC cells the presence of $MgCl_2$ (5 mM) did not provide an additional decrease in their ATP content (Fig. 4). Hence, there is a very low probability that the potentiating effect of extracellular Mg on HpD–PDT was mediated by energy deprivation.

According to our results Mg^{2+} in vitro acted as a pro-oxidant enhancing the rate of E6- or HpD-sensitized photodamage of tumour cells. In fact, HpD–PDT of EAC cells in the presence of $MgCl_2$ led to a more rapid decrease in the

intracellular content of GSH (Fig. 6a), which was associated with an elevated rate of HpD-sensitized photo-oxidation of cellular lipids (Fig. 5) and proteins (Figs. 6b and 7), while extracellular Mg itself exerted only a minor influence on the cell parameters. Nevertheless, the causes of these Mg^{2+} enhanced photo-oxidative injuries remain unclear. Although the involvement of $^1O_2$ in HpD–PDT is generally accepted, this has not been unambiguously proven. The antitumour effect of HpD–PDT might be due to other reactive oxygen species, such as superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($OH^{\bullet}$) [36, 37]. For instance, $^1O_2$ can be reduced by NAD(P)H and other agents producing $O_2^{\bullet-}$ and hydrogen peroxide (H_2O_2) [38], which may interact in the presence of certain transition metal ions or metal chelates (Fe^{2+}–ADP in cells) through the Fenton–Haber–Weiss reactions to yield $OH^{\bullet}$ [39] as shown below:

$$O_2^{\bullet-} + Fe^{3+}(ADP) \rightarrow O_2 + Fe^{2+}(ADP)$$

$$H_2O_2 + Fe^{2+}(ADP) \rightarrow OH^{\bullet} + OH^{-} + Fe^{3+}(ADP)$$

Hydroxyl radicals are short-lived and extremely reactive. The reaction rate of $OH^{\bullet}$ with most organic compounds approaches their diffusion limit, of the order of 10^7–10^{10} M^{-1} s^{-1}, and exceeds considerably that of $^1O_2$ [40]. Our data indicate that the addition of $MgCl_2$ (up to 5 mM) increased the intracellular level of NAD(P)H (Fig. 9) due probably to the stimulation of glucose consumption by EAC cells [35]. On this basis, we suppose that the potentiating effect of extracellular Mg^{2+} on the efficiency of HpD– or E6–PDT might be explained by an increased (because of NAD(P)H photo-oxidation) production of H_2O_2 and, as a consequence, of very cytotoxic $OH^{\bullet}$. However, further investigations revealed a more complex situation. An enhancing action of $MgCl_2$ on the intensity of HpD-photosensitized inactivation of EAC cells was also observed in the absence of glucose. In these conditions, extracellular Mg^{2+} (5 mM) caused, as measured by TB test, a substantial (approximately 1.5-fold) increase in the efficiency of HpD–PDT (data not shown), i.e., the potentiating PDT effect of Mg does not depend on the influx of external glucose into EAC cells.

From the present data no conclusions can be drawn about the mechanism of the potentiating effect of extracellular Mg^{2+} on PDT. Nevertheless, on the basis of the literature data we suppose that Mg can enhance the formation of $OH^{\bullet}$ and thereby increase the rate of HpD- or E6-photosensitized inactivation of tumour cells in some other way. It could be assumed that HpD–PDT of the cells causes, first of all, a large increase in the intracellular free Mg concentration (due to the influx of extracellular Mg because of an elevated permeability of plasma membranes). This event, in turn, may enhance the activity of the microsomal cytochrome P-450 reductase [41] which, as found by Morehouse & Aust [42], can couple the oxidation of NADPH with reduction of ADP-chelated iron, both aerobically and anaerobically. At the same time, the presence of iron in its ferrous form is needed for the metal-catalyzed reductive cleavage of photodynamically generated H_2O_2 leading to the formation of $OH^{\bullet}$. Moreover, recent studies revealed that Mg itself may promote lipid peroxidation by an

unknown mechanism. In fact, Regan et al. [43] showed that the iron-dependent oxidation of phosphatidylethanolamine liposomes may be weakly enhanced by 0.5–1.0 mM Mg^{2+}. Such a pro-oxidative effect of extracellular Mg might occur during HpD–PDT. However, the hypotheses will be a subject of future investigations.

In contrast to the in vitro experiments, we found that in vivo an elevated level of extracellular Mg surprisingly increased the viability of EAC cells pretreated with HpD–PDT (Fig. 8). Perhaps this effect of Mg^{2+} on cell survival is mediated by stimulation of the cellular processes responsible for repair of HpD–PDT induced injuries. For example, it was established that PDT may cause an essential depletion of cellular glutathione [3] which, as shown by Thomas & Girotti [44], is needed for detoxification of lipid hydroperoxides generated by HpD-sensitized photo-oxidation. At the same time, Mg^{2+} is an obligatory cofactor for γ-glutamylcysteine synthetase and glutathione synthetase [12], which are involved in the biosynthesis of glutathione. The ability of transformed cells to repair sublethal damages following PDT has been demonstrated by others [3].

CONCLUSIONS

The data presented here demonstrate that extracellular Mg has a dual effect on the PDT-induced cytotoxicity. Indeed, in a calcium/serum-free medium the addition of $MgCl_2$ enhanced the rate of HpD- or E6-photosensitized damage of EAC cells. However, at a physiological concentration (1 mM) of Mg^{2+}, only a weak (approximately 10%) increase in the efficiency of PDT (independently of the nature of the PSs used) took place. By contrast, in our in vivo experiments an elevated level of extracellular Mg increased essentially the viability of the cells pretreated (before intraperitoneal inoculation) with HpD–PDT. The net effect of extracellular Mg on HpD–PDT, therefore, appears to be the sum of both stimulatory and protecting activities. However, a comparison of the in vitro results (Table 1) with those obtained in vivo (when EAC cells were treated with HpD–PDT and transplanted at a higher concentration of $MgCl_2$ (Fig. 8)) suggests that the protective activity of Mg^{2+} is predominating. While the exact molecular mechanisms remain to be determined, the in vivo results suggest that Mg is, most probably, a serum factor decreasing the antitumour efficiency of HpD–PDT.

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EKSTRATSELLULAARSE MAGNEESIUMI MÕJU FOTODÜNAAMILISE TERAAPIA KASVAJAVASTASELE EFEKTIIVSUSELE

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Hinnati ekstratsellulaarse Mg toimet fotodünaamilises teraapias (FDT) kasutatavale hematoporfüriini derivaadile (HpD) ja kloriin-e_6 trimetüülestrile. *In vitro* katsetes suurendas $MgCl_2$ (1–5 mM) lisamine Ehrlichi astsiitse kartsinoomi (EAK) rakkude kaltsiumist ja seerumist vabasse meediumisse 1,5 korda HpD fotodünaamilist efekti (hinnatud trüpansinise testi abil). HpD–FDT käigus inhibeeris $MgCl_2$ EAK rakkudes täiendavalt nii glükolüüsi kui ka dehüdrogenaaside aktiivsust ning stimuleeris raku valkude ja lipiidide fotooksüdatsiooni, mis oli seotud redutseeritud glutatiooni taseme olulise vähenemisega. Kogu uuritud kontsentratsioonivahemikus oli Mg enda tsütotoksiline toime EAK rakkudele ebaoluline. Samas suurendas Mg^{2+} HpD fototsütotoksilist toimet füsioloogilisel 1 mM kontsentratsioonil nõrgalt (10%). Mg mõju fotodünaamilisele toimele ei sõltunud kasutatud fotosensibilisaatori tüübist: efektid olid *in vitro* katsetes sarnased nii HpD kui ka kloriin-e_6 trimetüülestri puhul. Vastupidiselt *in vitro* katsetele leiti *in vivo* eksperimentides, et Mg taseme suurendamine (enne intraperitoneaalset manustamist) tõstis oluliselt EAK rakkude vitaalsust HpD–FDT suhtes. Kuigi täpne molekulaarne mehhanism on suures osas teadmata, viitavad *in vivo* katsed sellele, et Mg seerumi faktor vähendab HpD–FDT kasvajavastast efektiivsust.