Correlation of death modes of photosensitized cells with intracellular ATP concentration

Vida Kirveliene*, Ausra Sadauskaite, Jurgis Kadziauskas, Sofija Sasnauskiene, Benediktas Juodka

Department of Biochemistry and Biophysics, Vilnius University, Ciurlionio 21, LT-2009 Vilnius, Lithuania

Received 26 June 2003; revised 20 August 2003; accepted 5 September 2003

First published online 18 September 2003

Edited by Vladimir Skulachev

Abstract The impact of intensity of glycolysis and oxidative phosphorylation on death of photosensitized murine hepatoma MH22 cells in vitro has been investigated. Cells photosensitized with *meso*-tetra(4-sulfonatophenyl)-porphine localized to lysosomes died mostly by necrosis, and the mode of cell death did not depend on the energy metabolism. Photosensitization with 5-aminolevulinic acid-stimulated endogenous porphyrins localized mainly in mitochondria or 5,10,15,20-tetrakis(*m*-hydroxyphenyl)-chlorine localized to cell membranes, including mitochondria, led to cell death mostly by apoptosis. In this case, the mode of cell death depended on the medium: under conditions unfavorable to glycolysis the ratio apoptosis/necrosis decreased significantly.

Key words: Apoptosis; Photosensitization; ATP; Mitochondrion; Glycolysis

1. Introduction

Apoptosis, programmed cell death, is an essential mechanism in tissue homeostasis. It is well known that apoptosis is an active process that requires ATP for its execution [1] and it has been suggested that the ATP level is a determinant of cell death by apoptosis or necrosis [2]. The cellular ATP pool is maintained by oxidative phosphorylation and glycolysis; the latter is especially active in tumor cells [3]. Early alterations of mitochondrial functions, such as loss of mitochondrial ATP production and membrane potential, have been recognized as hallmarks of apoptosis [4,5]. This might imply the increased importance of glycolysis in the apoptotic cell.

The data regarding the role of glycolysis in apoptosis are rather controversial. It has been shown that only glucose uptake and glycolysis protected cultured neonatal rat cardiac myocytes from hypoxia-induced apoptosis [6]. On the other hand, depletion of ATP prevented neuronal apoptosis triggered by microtubule breakdown, and repletion of ATP by enhanced glycolysis restored all apoptotic features [7]. Recent studies have demonstrated that glycolytic ATP production was required for tributyltin-induced apoptosis [8] and that oxidative stress-induced apoptosis depended on an active glycolytic flux, and it was suggested that the endogenous block of glycolysis that follows oxidative stress might be an active self-protective reaction of the cell [9].

This prompted us to follow the stress-induced changes of ATP under conditions favorable for either mitochondrial or glycolytic ATP production and relate bioenergetic conditions in the damaged cell to the mode of cell death. The stress was induced by light exposure of photosensitized cells. Photosensitization is a phenomenon underlying photodynamic therapy (PDT), the new modality of great promise for therapy of some tumors and other diseases [10]. Photosensitizers exposed to light produce reactive cytotoxic species, mainly reactive oxygen species (ROS), inducing further cellular damage that might lead to cell death [11], and apoptosis and necrosis have been recorded in cells subjected to PDT [12]. Since singlet oxygen, which has been accepted to be the main ROS produced in photodynamically treated cells, has a short lifetime in biological systems and a radius of action shorter than 20 nm [13], the initial cell damage sites are very close to those of singlet oxygen formation and strictly related to the distribution of the photosensitizer in the cell. Therefore, we used photosensitizers, which localize to the different compartments of the cell.

Here, we demonstrate that intracellular ATP concentration correlates with the mode of cell death initiated in murine hepatoma MH22 cells in vitro by photosensitizers of mitochondrial but not lysosomal localization: (1) cells photosensitized with *meso*-tetra(4-sulfonatophenyl)-porphine ($TPPS_4$) localized to lysosomes [14] died mostly by necrosis, and the mode of cell death did not depend on the energy metabolism; (2) cells photosensitized with 5,10,15,20-tetrakis(*m*-hydroxyphenyl)-chlorine (m-THPC) localized to cell membranes including mitochondria [14], or 5-aminolevulinic acid (ALA)-induced endogenous porphyrins predominantly localized in mitochondria under conditions used in the present study [15], led to cell death mostly by apoptosis, however under conditions unfavorable to glycolysis the ratio apoptosis/necrosis decreased considerably.

*Corresponding author. Fax: (370)-5-2398231; www.gf.vu.lt/bbk.
E-mail address: vida.kirveliene@gf.vu.lt (V. Kirveliene).

Abbreviations: ALA, 5-aminolevulinic acid; Ant, antimycin A; DG, 2-deoxyglucose; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; EP, endogenous porphyrins; FCS, fetal calf serum; m-THPC, 5,10,15,20-tetrakis(*m*-hydroxyphenyl)-chlorine; ROS, reactive oxygen species; SRB, sulforhodamine B; $TPPS_4$, *meso*-tetra(4-sulfonatophenyl)-porphine

0014-5793/03/$22.00 © 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)01014-7

2. Materials and methods

2.1. Materials

Fetal calf serum (FCS) and dialyzed FCS were from Gibco. Culture flasks and plates were from BD Falcon. m-THPC was kindly provided by R. Bonnett (Queen Mary's College, University of London, UK). The stock solution was prepared in ethanol (1 mg/ml) and stored at −20°C in the dark. $TPPS_4$ was purchased from Porphyrin Products (USA). The stock solution was prepared in Dulbecco's phosphate-buffered saline (DPBS) (5 mg/ml) and stored at −20°C in the dark. Proteinase K, ethidium bromide and acridine orange were from Merck, Germany. $DiOC_6$ was from Molecular Probes. 4 GeneRuler 1-kb DNA Ladder as a molecular weight standard for DNA fragments was from MBI Fermentas, Lithuania. Other chemicals were from Sigma.

2.2. Cell culture

MH22 cells from murine hepatoma were maintained in monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM glutamine, at 37°C in a humidified atmosphere containing 5% CO_2. Cells were subcultured by dispersal with 0.025% trypsin and replated at 1:4 dilutions twice a week.

2.3. Photodynamic treatment of cells

The culture medium was removed from nearly confluent cell layers and one of the three following steps was carried out: (1) 0.5 μg/ml of m-THPC solution in serum-free medium was added for 18 h; (2) 50 μg/ml of $TPPS_4$ solution in serum-free medium was added for 4 h; (3) 1 mM ALA, a precursor of endogenous porphyrins (EP), in serum-free medium was added for 4 h. Precautions were taken to avoid irradiation of the samples with room light. After following incubation at 37°C in the dark extracellular photosensitizers were removed by rinsing the cell monolayer four times with DPBS. Cells containing the accumulated photosensitizers were exposed to light either in (1) DMEM without glucose, glutamine, and sodium pyruvate, supplemented with 10% dialyzed FCS and substrates of either glycolysis and/or Krebs cycle, as follows: 5.5 mM glucose, 4 mM glutamine, 5 mM pyruvate, or (2) full DMEM supplemented with 10% FCS and inhibitors of either mitochondrial respiratory chain (10 μg/ml of antimycin A, Ant) or glycolysis (5.5 mM 2-deoxyglucose, DG). Cells were exposed to white light from a 500 W halogen lamp. The fluence rate at the level of the cells was 28 W/m^2 as measured with IMO (Russia). The cells incubated with m-THPC were exposed to light for 5 min, the cells with ALA for 10 min, and cells with $TPPS_4$ for 40 min. Heating of the cells during light exposure was prevented by a water filter. After the light exposure cells were incubated in the same medium in the dark before performing further experiments. Cell viability was evaluated after 24 h following light exposure by the sulforhodamine B (SRB) assay [16]. The amount of bound SRB was determined by fluorescence at $\lambda_{ex} = 485$ nm and $\lambda_{em} = 590$ nm using a microplate reader Ascent FL (Labsystems).

2.4. ATP assay

The ATP content of the cells was measured using the ATP Bioluminescence Assay kit (Sigma) based on the luciferin–luciferase reaction [17]. Luminescence measurements were made using a microplate reader Ascent FL (Labsystems). ATP concentration was calculated by using the values measured immediately upon addition of the internal standard. As the yield of ATP differed in the experiments, results were expressed as percentage of control.

2.5. Measurement of $\Delta\psi_m$

Mitochondrial membrane potential $\Delta\psi_m$ was measured with the cationic fluorescent probe $DiOC_6$ [18]. Cells were incubated with 100 nM $DiOC_6$ in DPBS for 15 min at 37°C in the dark. After washing twice with DPBS, the fluorescence was measured using a microplate reader Ascent FL (Labsystems) at $\lambda_{ex} = 485$ nm and $\lambda_{em} = 538$ nm. The change in fluorescence intensity is then interpreted as an indication of $\Delta\psi_m$ dissipation. As the yield of staining with $DiOC_6$ differed in the experiments, results were expressed as percentage of control. The reliability of the results was checked by adding the uncoupler carbonylcyanide *m*-chlorophenylhydrazone to a 100 μM concentration. The fluorescence decrease/increase in treated cells compared to untreated control cells was calculated as follows:

$$\Delta F = (F_{treated} - F_{control})/F_{control}$$

2.6. Morphological changes of nuclei

A monolayer of the photodynamically treated cells was stained with a mixture of acridine orange (1 μg/ml) and ethidium bromide (1 μg/ml) in DPBS [19]. The cells were visualized with a fluorescence microscope Olympus AX70 equipped with an MF filter cube (480–500/550–580 nm for excitation and 515–535/590–625 nm for emission). Green cells with a round nucleus were considered alive, orange cells with a round nucleus were considered necrotic, and cells of either color with a bright fragmented nucleus were considered apoptotic. At least 300 cells from five different areas were counted in each experiment.

2.7. Visualization of photosensitizer localization

Cells incubated with a photosensitizer were visualized with an Olympus AX70 fluorescence microscope equipped with a 60×, NA 1.25 oil immersion lens, and a specially produced filter cube (400–410 nm for excitation and beyond 590 nm for emission). The localization of photosensitizers was compared with specific fluorescent probes, rhodamine 123 (2 μg/ml, 15 min at 37°C) for mitochondria, and acridine orange (5 mg/ml, 2 min at room temperature) for lysosomes (and DNA) using a WIBA filter cube (460–490 nm for excitation and 515–550 nm for emission). The images were recorded with a CCD camera Orca (Hamamatsu) and analyzed with MicroImage version 4.0 (Media Cybernetics) software.

2.8. Gel electrophoresis of DNA

A modified gel electrophoresis procedure was employed to determine fragmentation of DNA as described in [20]. 15×10^6 cells were washed with DPBS, dispersed with 0.025% trypsin, pelleted, and gently opened in 100 μl of lysis buffer containing 1% NP-40 in 20 mM EDTA and 20 mM Tris–HCl, pH 7.5. After centrifugation at $1600 \times g$ for 5 min at 4°C, the extraction was repeated and the supernatants were collected. 10% sodium dodecyl sulfate was added to a concentration of 1% and the probes were treated with 1 μg/μl of RNase A for 1.5 h at 56°C, followed by incubation with 2.5 μg/μl of proteinase K for at least 2 h at 37°C. After a half volume of ice-cold 10 M ammonium acetate was added, the DNA was precipitated by incubation with 2.5 volumes of 96% ethanol for 15 min at −80°C, and the DNA was collected by centrifugation at $7200 \times g$ for 40 min at 4°C. The DNA was suspended in gel loading buffer (0.2 mM EDTA, 1 mM Tris–HCl, pH 8.0) and run at 9–10 V/cm on 1.5% agarose gel containing 0.1 μg/μl of ethidium bromide. The bands were visualized under UV light with an electronic UV transilluminator Ultra Lûm (Paramount, CA, USA).

2.9. Data analysis

The data are presented as means ± S.D. from two to five independent experiments. SigmaPlot 2001 for Windows version 7.101 software was used for the statistical analysis.

3. Results and discussion

We have attempted to reveal whether there is any correlation between cell death mode and the mode of cellular energy metabolism. Since cell bioenergetics is largely determined by the integrity of mitochondria, which may be affected by cell death induction, we employed several sensitizers of different cellular localization, but the same mechanism, namely, light-induced killing of photosensitized cells, for cell damage. The dependence of cell death on the photosensitization protocol has been widely recorded, and the slow proceeding of apoptosis induced by the lysosomally localized $TPPS_4$ [21] as well as the determining role of the mitochondrial localization of zinc-(II) phthalocyanin in promotion of apoptosis [22] was demonstrated.

The localization of the photosensitizers in murine hepatoma MH22 cells was defined by fluorescence microscopy: $TPPS_4$ co-localized with the lysosomal marker acridine orange, and more diffuse fluorescence of m-THPC or EP partially coin-

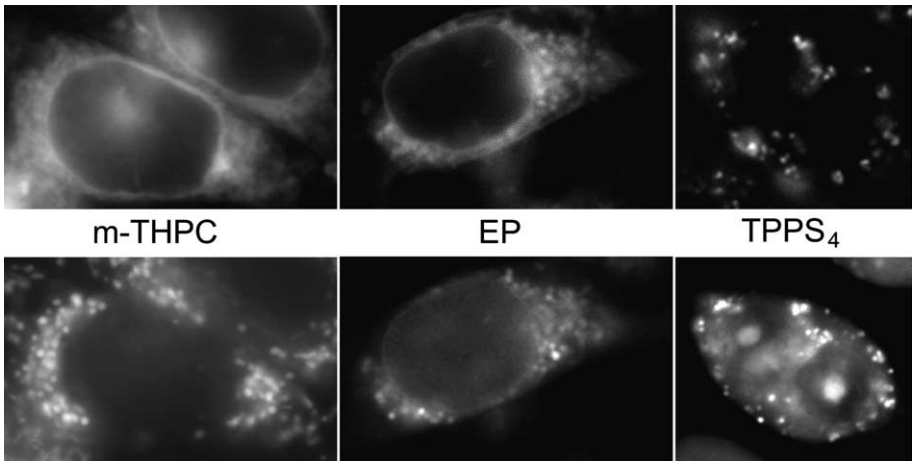

Fig. 1. Cellular localization of photosensitizer fluorescence. MH22 cells were incubated with the photosensitizers as described in Section 2.3, and examined with fluorescence microscopy, as described in Section 2.7. Upper row, fluorescence of the photosensitizers; lower row, fluorescence of the mitochondrial marker rhodamine 123 (in the cases of m-THPC and EP) and the lysosomal marker acridine orange (in the case of $TPPS_4$).

cided with fluorescence of the mitochondrial marker rhodamine 123 (Fig. 1). Accumulation of photosensitizing concentrations of EP was stimulated by ALA, a precursor in the heme biosynthetic pathway [23].

Our investigation was built on two source findings: (1) the high activity of both mitochondrial and glycolytic ATP production in MH22 cells, since we had recorded [24] that the high concentration of 20 μg/ml of oligomycin, a blocker of mitochondrial ATP synthesis, reduced the intracellular ATP of MH22 cells by 40% indicating that in this transformed cell line, a large fraction of ATP was derived from glycolysis, and (2) the occurrence of apoptosis in photodynamically treated MH22 cells, which was demonstrated by 'ladders' on DNA gel electrophoresis, nuclear chromatin condensation, and activation of caspase 3, an executor of apoptotic cell dismantling.

The protocol of photodynamic treatment was adjusted to the equal final loss of viability of the cells incubated with any of the photosensitizers. Cell exposure to light for 5, 10 or 40 min after incubation with m-THPC for 18 h, ALA for 4 h, and $TPPS_4$ for 4 h, respectively, induced the decrease of cell viability to approx. 40–50% as estimated after 24 h following light exposure by the SRB assay (Fig. 2).

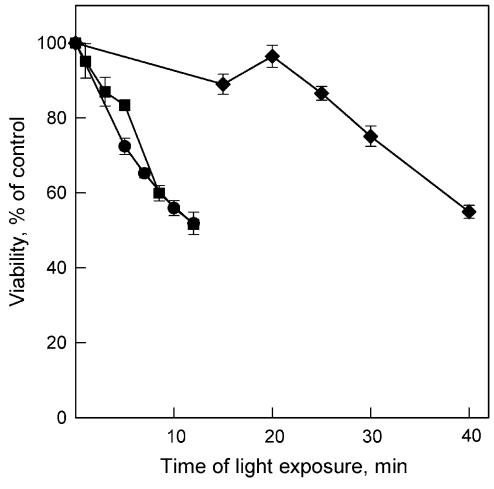

Fig. 2. Viability of photosensitized MH22 cells exposed to light. The cells were incubated with the photosensitizers and exposed to light as described in Section 2.3. Cell viability was assayed by the SRB assay after 24 h incubation in DMEM supplemented with 10% FCS in the dark following light exposure. EP, squares; m-THPC, circles; $TPPS_4$, diamonds; bars, ±S.D.

The bioenergetic parameters were assessed at 30 min following light exposure in order to register the primary metabolic changes preceding morphological changes. We registered two parameters of cellular energy metabolism: intracellular ATP concentration and mitochondrial membrane potential ($\Delta\psi_m$). During cell respiration, an electrochemical gradient of H^+ is generated. Most of the gradient is manifested in the form of $\Delta\psi_m$. Thus, $\Delta\psi_m$ is a good indicator of the energy status of the mitochondrion in particular and of cellular homeostasis in general. Numerous studies have demonstrated recently that cells undergoing apoptosis exhibit a decrease in $\Delta\psi_m$, preceding nuclear signs of apoptosis, suggesting that early alterations of mitochondrial function may be important for the apoptotic process [4].

For assessment of $\Delta\psi_m$ cells were incubated with the lipophilic cationic fluorochrome $DiOC_6$, which accumulates in the

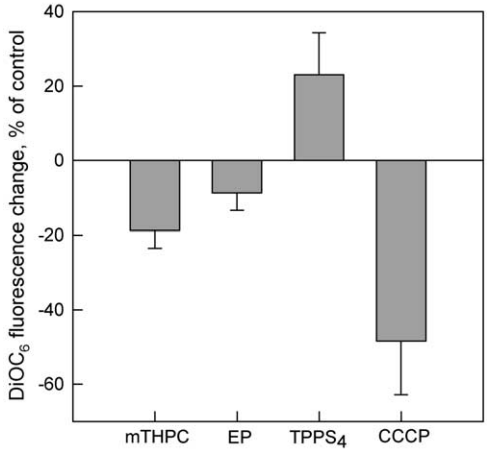

Fig. 3. Changes of mitochondrial membrane potential in photosensitized MH22 cells exposed to light. $\Delta\psi_m$ was measured by fluorescence of $DiOC_6$ accumulated in mitochondria after 30 min following light exposure as described in Section 2.5; bars, ±S.D.

mitochondrial matrix, driven by the $\Delta\psi_m$ (Fig. 3). After 30 min following light exposure, $\Delta\psi_m$ decreased in cells, photosensitized with m-THPC and EP. However, in cells photosensitized with $TPPS_4$ $\Delta\psi_m$ was increased by 20%.

The ATP measurement in photosensitized cells at 30 min following light exposure revealed that the ATP pool was not depleted under any conditions of photodynamic treatment (Fig. 4A, None). On the contrary, intracellular ATP concentration was slightly increased, especially when the stress was mediated by $TPPS_4$. In $TPPS_4$-loaded cells the increase in $\Delta\psi_m$ after light exposure correlated with the increase of the intracellular ATP pool. It could be attributed to the rise of mitochondrial activity following the photodynamic induction of cellular stress, which originated at the lysosomes and had not triggered damage to mitochondria, at the moment of measurement. The slight initial rise of the ATP pool was also registered in photodynamically treated A431 cells loaded with $AlPcS_4$ [25]. However, in m-THPC- and ALA-loaded cells, $\Delta\psi_m$ decreased, but ATP did not. The decrease of $\Delta\psi_m$ should be attributed to the deterioration of mitochondria, induced by the photosensitizers targeted on cellular membranes, including mitochondria, and it should result in reduction of mitochondrial ATP synthesis.

The role of the ATP production pathway in the photodynamically stressed cells was examined by inhibition of either glycolysis with DG or mitochondrial respiration with Ant. ATP levels were lowered by inhibition of either process of cellular energy metabolism, but the inhibition of glycolysis had a stronger effect: the inhibition of mitochondrial electron transport with Ant decreased the intracellular ATP pools by approx. 30% (Fig. 4A, Ant), meanwhile the inhibition of glycolysis with the glucose analogue DG lowered ATP levels by approx. 70% (Fig. 4A, DG).

However, inhibition of glycolysis results in a reduced production of pyruvate, a substrate for mitochondrial respiration, thus, DG inhibits mitochondrial ATP production, as well. On the other hand, DG is a glycosylation antagonist, and thus an inducer of endoplasmic reticulum stress. Therefore, in addition another approach was applied, and photosensitized cells were energized with the glycolytic substrate glucose and/or the mitochondrial substrates pyruvate and glutamine. The latter is especially important for the deviant energetic metabolism of malignant cells [26].

When the photosensitized cells were energized with the glycolytic substrate glucose and/or the mitochondrial substrates pyruvate and glutamine, the ATP concentration in the untreated cells was fully maintained by either glycolysis or oxidative phosphorylation alone (Fig. 4B, Control). Photodynamic treatment with $TPPS_4$, disturbing lysosomes, resulted in a 20% increase of the ATP level in the cells incubated with the full set of energy substrates implying the decrease of ATP consumption or elevation of ATP synthesis for some ATP-demanding repair processes; incubation with mitochondrial substrates did not induce a detectable change in ATP concentration in comparison with untreated cells implying the undisturbed integrity of mitochondria (Fig. 4B, $TPPS_4$). On the contrary, the lack of the glycolytic substrate in the incubation medium of the cells photodynamically treated with m-THPC or EP, disturbing mitochondria, caused a decrease of the ATP levels by $\geq 50\%$ reflecting severe damage to mitochondria (Fig. 4B, m-THPC, EP).

The latter approach is not unambiguous, either. The presence of glucose leads to production of pyruvate and stimulation of mitochondrial respiration. On the other hand, the absence of glucose in the substrate set for mitochondrial respiration could unbalance cellular processes other than glycolysis, since glucose not only serves as a nutrient but also has significant effects on the expression level of some genes and exerts many hormone-like regulatory effects in a wide variety of eukaryotic cell types [27]. Therefore, we compared and analyzed the results of both approaches.

We aimed to detect signs of apoptotic and necrotic cell death following the stress induced by photodynamic treatment. The occurrence of apoptosis in the population of dying cells was confirmed by demonstration of DNA laddering typ-

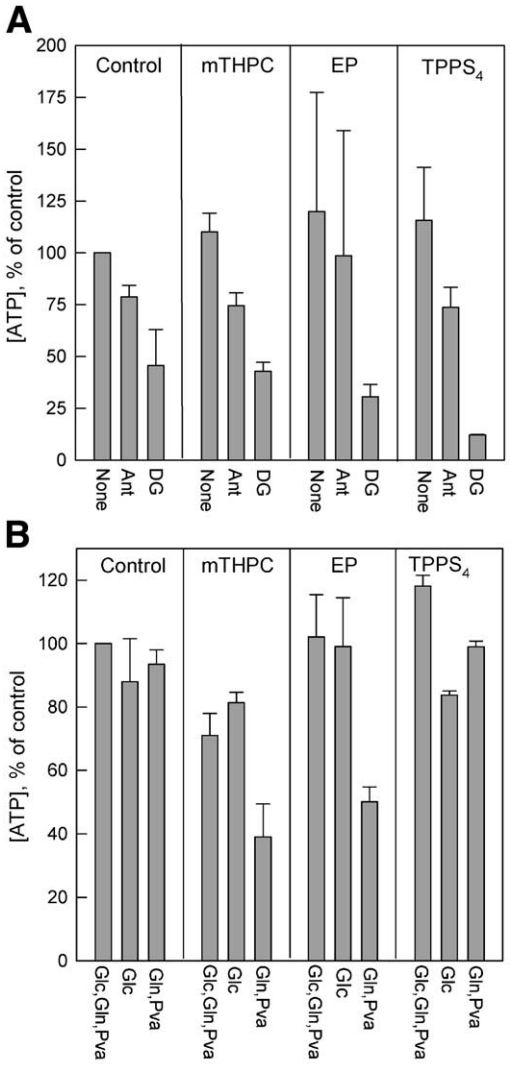

Fig. 4. Changes of intracellular ATP concentration in photosensitized MH22 cells exposed to light and incubated with inhibitors (A) or different substrates (B) of energy metabolism. ATP concentration was measured after 30 min following light exposure. Cells were exposed to light as described in Section 2.3. A: During light exposure and afterwards, cells were incubated in full DMEM supplemented with 10% FCS and inhibitors of either the mitochondrial respiratory chain (10 μg μl^{-1} Ant) or glycolysis (5.5 mM DG) as indicated in the axis title. None, medium without inhibitors. B: During light exposure and afterwards, cells were incubated in DMEM supplemented with 10% dialyzed FCS and substrates of either glycolysis and/or Krebs cycle: 5.5 mM glucose, 4 mM glutamine, 5 mM pyruvate, as indicated in the axis title; bars, ± S.D.

ical of apoptosis (Fig. 5) and the increased activity of caspase 3, which plays a crucial role in the execution phase of apoptosis (unpublished results). Measurement of caspase activity reveals the level of apoptosis reliably, however for evaluation of ratio apoptosis/necrosis, counting of selectively stained cells was preferred.

Apoptotic and necrotic cells were counted by analyzing the images of fluorescence microscopy, when the cells started to detach from the substratum, i.e. at the very beginning of the morphological rearrangement of the dying cells. This time point was chosen for scoring in order to avoid underestimation of apoptosis due to the fast cell fragmentation to apoptotic bodies during the running cell death. Therefore, the results should be considered a momentary section of the process lasting for hours, and the percentage of the cells that displayed the signs of apoptosis or necrosis at the moment of scoring represents a kind of dying rate, but not the final fraction of cells which retained viability. The exact time point was different for the photosensitizers, and the most delayed one (5 h) was for $TPPS_4$ implying the biggest potential of $TPPS_4$-photosensitized cells to counter the damage. It should be noted that no death was detected of unirradiated control cells incubated for 5 h with either the specific media or the inhibitors.

In $TPPS_4$-treated cells with lysosomes as the primary targets of cellular stress, necrosis was the dominant mode of cell death with the ratio apoptosis/necrosis being below 1.0 under all examined conditions (Fig. 6, $TPPS_4$). Meanwhile, the cells photodynamically treated with m-THPC or ALA-derived EP with mitochondria among the primary targets of damage died predominantly by apoptosis in the full medium containing both glycolytic and mitochondrial substrates with the ratio apoptosis/necrosis exceeding 20 (Fig. 6, m-THPC, EP). In this case, the lack of mitochondrial substrates had no significant effect on the mode of cell death. On the contrary, the lack of glucose increased the extent of necrosis severely reducing the ratio apoptosis/necrosis to below 1.0. Recently, it has been shown that glucose as a source of ATP is obligatory for the execution of apoptosis in photosensitized cells [28].

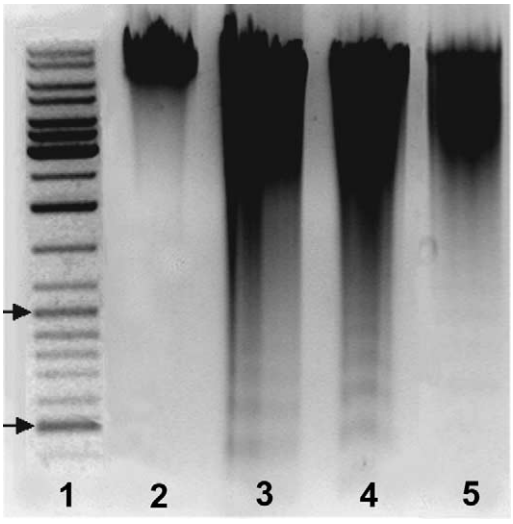

Fig. 5. Agarose gel electrophoresis of DNA extracted from photosensitized cells exposed to light. MH22 cells were incubated with m-THPC for 18 h (lane 3), ALA (lane 4) or $TPPS_4$ (lane 5) and exposed to light for 5, 10 and 40 min, respectively. DNA was extracted after 2, 1.5 and 5 h, respectively, following light exposure, and analyzed in an agarose gel as described in Section 2.8. A molecular weight standard (arrows indicate fragments of 500 and 1000 bp) was loaded on lane 1, and DNA extracted from untreated cells was loaded on lane 2.

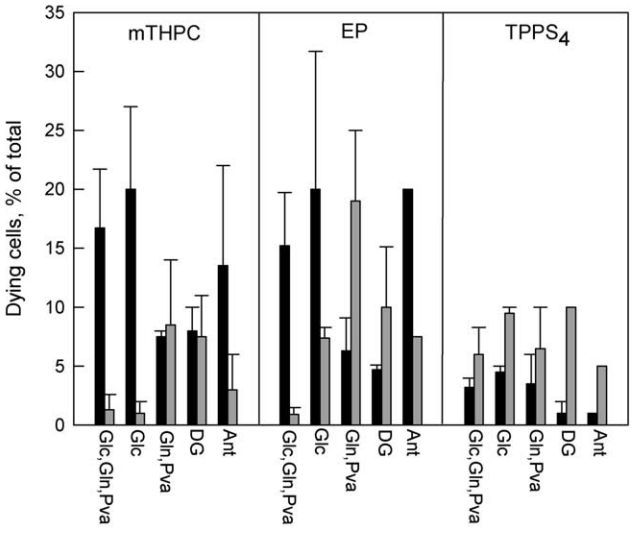

Fig. 6. Apoptosis and necrosis of photosensitized cells. During light exposure and afterwards, MH22 cells were incubated in DMEM supplemented with substrates or inhibitors indicated in the axis title. Morphological changes of nuclei were followed by fluorescence microscopy as described in Section 2.6, at the beginning of cells detachment: m-THPC, 1.5 h; EP, 2.5 h; $TPPS_4$, 5 h after light exposure. Black bars, apoptotic cells; gray bars, necrotic cells; bars, ± S.D.

It was shown that ATP was necessary for apoptosis to proceed [1]. However, in $TPPS_4$-loaded cells the high intracellular ATP concentration did not ensure cell death by apoptosis, since necrosis was the dominant mode of cell death in all media. The prevalence of necrosis in the cells photosensitized with $TPPS_4$, which accumulates in lysosomes, vs. apoptosis in the cells photosensitized with m-THPC and EP, which accumulate in cellular membranes including mitochondria, points out the significance of the target of the damage.

In the cells photosensitized by m-THPC or ALA-derived EP, the lack of mitochondrial substrates had no significant effect on the mode of cell death. However, the lack of glucose increased the extent of necrosis severely. In murine hepatoma MH22 cells the control function of glucose per se does not seem significant, since the presence of glucose did not increase apoptosis of $TPPS_4$-treated cells with, supposedly, sufficient supply of mitochondrial ATP. There could be two alternatives: either the glycolytic production of ATP was essential for apoptosis or there was a limit in the intracellular ATP concentration, beyond which apoptosis could not proceed, and the ATP production by the damaged mitochondria without the input of glycolysis was not enough to support the necessary ATP level.

Regardless of which alternative is true, the results of the present study point to the possibility of modulating the pathway of photosensitized, at least, cell death by adjusting the composition of the incubation medium. This possibility could have great potential from the clinical point of view.

Acknowledgements: We are grateful to R. Bonnett for the generous gift of m-THPC and V. Piskarskiene for the excellent care of the cells.

This work was partly supported by the European Commission, Programme of Centres of Excellence, project CEBIOLA, ICA1-CT-2000-70027, and the Lithuanian State Foundation for Science and Studies, Programme Light in Biomedicine, P-14/01.

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