DNA damaging capability of hematoporphyrin towards DNAs of various accessibilities

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Abstract

In this work we wanted to verify that photoactivation of DNA-non-binding porphyrin derivative hematoporphyrin IX (Hp) is able to induce damages in DNAs of various accessibilities such as B-conformation and superhelical isolated DNA, nucleoprotein complex and intracellular DNAs. It was found that photodynamic reaction of Hp results significant changes in thermal stability of isolated T7 DNA and induces single strand breaks in supercoiled Bluescript plasmid isolated from Escherichia coli cells. As optical melting measurements revealed, the irradiation of photosensitized T7 nucleoprotein complex leads to a destabilization of the protein capsid. The photodynamic reaction affected both the protein structure and DNA-protein interaction, however, the parameters corresponding to the DNA denaturation are not influenced. The accumulation of Hp in HeLa cells was followed by laser scanning confocal microscopy. The picture received is typical for lipophilic dyes. When Hp loaded cells were irradiated, a reduction of viability could be observed in a concentration and a light dose dependent manner; 12 μM porphyrin induced almost complete cell killing after 30 min irradiation. After similar treatment, alkaline agarose gel electrophoresis of isolated nuclear DNA did not show the presence of single strand breaks. The alkaline comet assay also failed to demonstrate any DNA damage in HeLa cells. We also considered the possibility of the generation of damages in intracellular SV40 DNA. According to the electropherograms there was no difference between the patterns of DNAs from treated and control samples.

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1. Introduction

Photodynamic therapy “PDT” is the oxygen-mediated, tumouricidal combination of non-thermal power densities of visible light and non-toxic, light-absorbing chemicals [1,2]. The photodynamic effect is believed to be mediated largely, but almost certainly not exclusively, by the generation of singlet excited oxygen. This species has a short half-life and short diffusion path [3,4], so that primary damage is mainly expressed in the immediate vicinity of the photosensitizer. Therefore the efficiency of the photodynamic process depends strictly on the pattern of dye localization in cells and subcellular compartments [5–7].

The site of localization of photosensitizers and target of photodynamic action within the cell can be various, such as plasma membrane, mitochondria, lysosomes, nucleus, depending on the structure of photosensitizer [8–10]. Hydrophobic photosensitizers such as hematoporphyrin are known to be localised in apolar membrane structures, typically in cellular and mitochondrial membranes. Some results obtained by fluorescence microscopy showed staining of the nuclear envelope or perinuclear region also after incubation with hematoporphyrin derivatives or α-amino-levulinic acid [11,12]. It was also proposed by several
authors that in spite of their membrane localization hydrophobic photosensitizers are able to generate damages also in nuclear DNA [13,14].

The goal of this paper is to characterize the DNA-damaging capability of some photosensitizers towards DNA in different environments. As a starting point of such studies, we wanted to verify that a DNA-non-binding porphyrin derivative hematoporphyrin IX (Hp) is able to induce damages in DNAs of various higher ordered structures such as B-conformation and superhelical isolated DNA, nucleoprotein complex and intracellular DNAs.

The Bluescript plasmid was selected as superhelical DNA, T7 phage was used as nucleoprotein complex (NP) and its isolated nucleic acid as relaxed B-conformation DNA. The advantage of T7 DNA and T7 nucleoprotein complex is that due to the high purity of our preparations their structural changes can be investigated by spectroscopic methods. DNA damages in two types of intracellular DNA were investigated: nuclear DNA of HeLa cells and viral DNA of simian virus (SV40). The damages in plasmid DNA and SV40 DNA were analysed by neutral and alkaline gel electrophoresis; the same methods and Comet assay was used for HeLa cell DNA.

As a later goal, the photodynamic reaction may be applied to the investigation of nuclear DNA structure. Most scientists agree that gene regulation cannot be fully explained by linear, two-dimensional models in which sequence-specific factors bind promoter elements either to induce or to block transcription [15]. A number of studies show that repressed domains have unique positions within the nucleus [16,17]. It was suggested that subnuclear positioning represents a novel means of regulating transcription and recombination. Nuclear periphery is one of the major classes of transcriptionally repressive nuclear subcompartments [18]. This has been well established in Saccharomyces cerevisiae [19,20]. In human nuclei, analysis of whole chromosomes has revealed that a gene-poor chromosome is preferentially localized to the nuclear periphery, whereas a gene-rich chromosome is more centrally disposed [21]. However, it remains to be determined whether gene activity in higher eukaryotes is also regulated by perinuclear localization.

Damages induced by membrane-localizing porphyrin derivative might be a potential tool in the identification of genes localized on the nuclear surface in close vicinity of the nuclear membrane. According to the general view, such photosensitizer localization may facilitate the induction of photodynamic damages in the perinuclear region but not in the central DNA sequences.

2. Materials and methods

2.1. Photosensitizer

The photosensitizer hematoporphyrin IX dihydrochloride (Hp) (molecular weight 671.62) was purchased from Porphyrin Products (Logan, USA). It was stored in powder form at 4 °C or as 3 mM stock solution in methanol. Further concentrations were obtained by dilution of the stock sensitizer solution in RPMI-1640 medium (Invitrogen, Karlsruhe, Germany) containing 2% foetal calf serum or TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5).

2.2. Light source

In all experiments the light source was a 100 W quartz light bulb (Tungsram®). For exposure of samples the light was filtered to exclude wavelengths shorter than 400 nm. The emission maximum of the light source was at 650 nm [22]. The total fluence rate (800 W m⁻²) was determined by a 10A-P Nova laser power/energy monitor (Ophir, Optronix, Jerusalem, Israel) using the pyroelectric detector.

2.3. Plasmid DNA

Bluescript plasmid DNA (about 3000 bp) was prepared using Escherichia coli culture and purified as described by Birnboim and Doly [23]. The plasmid DNA (1.65 μg) was suspended in TE buffer and irradiated in the presence of Hp (6 μM) or in its absence. The products (superoiled, linear and open circle DNA) were analysed using 1% agarose gel in Tris-acetate-EDTA (0.004 M Tris-acetate, 0.001 M EDTA) buffer pH 8. The gels were stained with ethidium bromide and photographed with a CCD camera (Biometra, Göttingen, Germany). Gel photos were quantified using the IQ program from BioImage (Buffalo, USA).

2.4. T7 bacteriophage

T7 (ATCC 11303-B7) was grown on Escherichia coli (ATCC 11303) host cells. The cultivation and purification were carried out according to the method of Strauss and Sinheimer [24]. The phage suspension was concentrated on a CsCl gradient and dialyzed against buffer solution composed of 20 mM Tris–HCl and 50 mM NaCl, pH 7.4. The concentration of T7 bacteriophage was determined from its optical density using a molar absorptivity of ε₂₆₀ = 7.3 × 10³ (mol nucleotide bases⁻¹ cm⁻¹) in phosphate buffer [25].

2.5. Preparation of DNA from T7 nucleoprotein complex

DNA was prepared from phages by incubating with 0.5% SDS (Sigma, München, Germany) for 30 min at 65 °C; then the protein–SDS complex was precipitated with 1 M KCl (Sigma) on ice for 10 min [26]. The solution was centrifuged twice for 10 min in an Eppendorf microcentrifuge at 13000 rpm, and then the DNA was precipitated with ethanol from the supernatant. The pellet was washed with 70% ethanol then suspended in buffer solution 20 mM Tris–HCl, 50 mM NaCl, pH 7.4, and the amount of DNA was determined spectrophotometrically. The quality of DNA was checked by electrophoresis and by its absorption spectrum.
2.6. Cell lines and culture conditions

HeLa cells and African green monkey kidney (RITA) cells were maintained as monolayer culture in RPMI-1640 medium without phenol red and supplemented with 10% foetal calf serum (FCS). The cells were grown in a humidified CO₂ atmosphere at 37 °C in tissue culture flasks and passaged two or three times a week after trypsinisation (0.05% trypsin, 0.02% EDTA) to keep them in the exponential phase. RITA cells were infected at subconfluence with Simian Virus 40 (SV40) at a multiplicity of 1 plaque forming unit (PFU) per cell. DNA was extracted 3 days after infection.

2.7. Optical melting measurements

Prior to the light exposure, the isolated T7 viral DNA or virus suspension was mixed with dye and kept in the dark for 10 min. Base pair concentration of DNA was 2 × 10⁻⁵ M, which corresponds to 5 × 10⁻¹⁰ M phage particle in the T7 solution. Samples were continuously stirred during photo-treatment.

Thermal denaturation curves of T7 DNA or bacteriophage solutions were recorded by absorbance at 260 nm on a Cary 4 E spectrophotometer (Varian, Mulgrave, Australia), equipped with a Peltier thermoregulator. The heating rate was 0.5 °C/min in the temperature range 25–97 °C. Five samples were measured in parallel using an automatic cell changer; the sixth sample holder was used to measure the temperature in an identical quartz cell filled with buffer. The cell holder was insulated to ensure that the temperature did not vary more than 0.1 °C between cells, even above 90 °C. The initial absorbance of the samples was adjusted to approximately 0.2 at 260 nm in quartz cells of 1 cm path length. The absorbance data were collected at every 0.5 °C. Data were treated using the program KaleidaGraph on a Macintosh computer. The curves were normalized to the absorbance at room temperature, smoothed in five point intervals; derivative melting curves were calculated from the differences between adjacent points as implemented in the program and once more smoothed for five points. The peak of the derivative melting curve was accepted as the corresponding melting temperature (T_m).

Another parameter to characterize the transition is its half width defined in the usual way, as the difference between the temperatures, where the derivative reaches half of its extreme value.

2.8. Laser scanning microscopy

Monolayer HeLa cells growing in wells (Nunc, Wiesbaden-Schierstein, Germany) were incubated in RPMI with 2–10 μM Hp for a given time period varying between 0 and 6 h at 37 °C. After incubation with Hp, the cells were washed three times with Hank’s solution. Laser scanning confocal microscopy was used for detection of intracellular porphyrin. A beam scanning unit is attached to the video port of an inverted microscope (IX-70, Olympus Optical, Hamburg, Germany). The 568 nm line of a low noise Ar-Kr laser is coupled into the module through a monomode fiber and illuminates the back aperture of the objective lens. Fluorescence was detected in the range of 608–662 nm with an avalanche photodiode in photon-counting mode behind a pinhole with a diameter of 50 μm. The beam is scanned and positioned using rotating mirrors driven by closed-loop galvanometer scanners M2 (GSI Lumonics, Unterschleißheim, Germany) and a scan lens FVX-1R-PL (Olympus), while a stepping motor moves the objective along the z axis. The control software [27] allows us to acquire transmission images, and to position the laser with 25 nm precision.

2.9. Cell viability following phototreatment

The viability of HeLa cells was measured by trypsin blue exclusion assay. After incubation in RPMI medium with various concentrations of porphyrin (3–20 μM) for 16 h, cells were washed twice with Hank’s solution (Invitrogen) to remove unbound drug, trypsinized and resuspended in fresh medium. After centrifugation the cell pellets were suspended again in RPMI-1640 medium and illuminated in a cuvette with continuous stirring. Trypan blue was added just before counting them under the light microscope. The number of blue stained (dead) and unstained (viable) cells were counted in three fields in two independent experiments. At least 100 cells per square were scored. The cell viability was calculated as the ratio of the number of unstained cells and the total number of cells.

2.10. Gel electrophoretic analysis of cellular DNA

Monolayer HeLa cells growing in Petri dishes of 3 cm diameter were incubated in RPMI-1640 containing various concentrations of the sensitizer (3 μM–20 μM) in the dark. At the end of the 16 h incubation at 37 °C, RPMI-1640 was removed and cells were washed with Hank’s solution to remove unbound dye and illuminated with white light on ice. The treated cells were suspended in lysis buffer consisting of 1% sodium dodecyl sulphate (SDS), 100 μg/ml proteinase K and 10 mM Tris–HCl buffer, pH 7.4. After 24 h digestion in these solutions at 37 °C, the DNA was unwound at pH ≈ 13 by adding NaOH. In each case the whole sample was electrophoresed for 3 h at 8 V/cm in Tris-acetate-EDTA (TAE) buffer using 0.5% agarose gel. The separated bands were stained with ethidium bromide and photographed.

2.11. Gel electrophoretic analysis of SV40 DNA

Monolayers of RITA cells were grown and treated as it was described for HeLa cells. Following irradiation infected RITA cells were lysed in 500 μl of detergent (1% SDS, 10 mM EDTA pH 7.5 and 100 μM/ml proteinase...
at room temperature for 30 min, thereafter at 37 °C for 10 min. Then 125 μl of 5 M NaCl were added to the lysate and the mixture was placed on ice. The lysate was centrifuged, and the supernatant containing viral DNA extracted with phenol and chloroform. The aqueous phase was mixed with NaCl (final concentration 0.1 M), then cold 95% ethanol (final concentration 60%), and kept overnight at −20 °C. After ethanol precipitation the mixture was centrifuged, and then the DNA was dried and dissolved in TE buffer. Samples containing virus DNA were electrophoresed for 3 h at 8 V/cm in Tris-acetate-EDTA (TAE) buffer using 1% agarose gel, and stained with ethidium bromide and photographed.

2.12. Alkaline comet assay of HeLa cells

The alkaline version of comet assay was carried out according to the procedure of Mayer et al. [28] with some modifications. After 16 h incubation with Hp, monolayer HeLa cells were trypsinized, suspended in RPMI-1640 medium, centrifuged, resuspended again in RPMI and 50 μl of the cell suspension was mixed with 350 μl 0.7% low melting agarose. Fifty microlitre aliquots of this cell suspension were spread on 4 areas of CometSlides™ (Trevigen, Gaithersburg, USA) and placed on ice for solidification. Parallel to these procedures untreated, in fresh medium suspended HeLa cells were irradiated with 5 Gy using a 137Cs source (Gammacell 1000, Atomic Energy of Canada, Ltd, Edmonton, Canada) at a dose rate of 12 Gy min⁻¹. Fifty microlitre aliquots were mixed with low melting agarose and transferred on Comet Slides. After illumination of Hp treated HeLa cells all slides were submerged into pre-chilled lysis solution (100 mM Na₂EDTA, 10 mM Tris, 2.5 mM NaCl, 1% sodium sarcosinate, 1% Triton X-100, 10% dimethyl-sulphoxide, pH 10) overnight, then transferred into electrophoresis buffer (300 mM NaOH, 1 mM Na₂EDTA, pH 13) for 20 min to allow denaturation of DNA. For electrophoresis of the DNA, 25 V and 300 mA was applied for 30 min. To prevent additional DNA damage, all steps were conducted under dimmed light. Following electrophoresis, the slides were fixed for 5 min in ice-cold absolute ethanol and dried at room temperature. The DNA was stained with 50 μl SYBR® Green solution (Molecular Probes, Leiden, Netherlands) diluted by four orders of magnitude in TE buffer and 51 comets per slide area were evaluated by fluorescence microscopy and image analysis software (Kinetic Imaging Ltd., Liverpool, UK).

3. Results and discussion

3.1. Photoinduced damages of isolated DNA

Photoinduced structural changes in two types of isolated DNAs of different higher order structures were investigated by gel electrophoresis and optical melting method. Blue-script plasmid has a superhelical DNA while isolated T7 DNA is present in the regular B-conformation under our experimental conditions [29,30].

Prior to photosensitized reaction, plasmid DNA was irradiated without photosensitizer and treated with Hp in the dark. Neither of them caused any strand scission in plasmid DNA (data not shown). Fig. 1 shows the relative number of single strand breaks (ssbs) in photosensitized samples as a function of illumination time. With increasing incident light dose, the amount of open circular form (Form II) increases which is indicative for the induction of single strand breaks. After 2 h of exposure, there is a 13% elevation in ssbs as compared to the control samples.

Recently a comparison of the photoinduced strand breaks generated in plasmid DNA by DNA-binding meso-tetakis[4-(carboxymethyleneoxy)phenyl]porphyrin [H₂T₄CPP] and DNA-non-binding meso-tetakis (4-carboxy-phenyl)porphyrin [H₂CPP] was published by Chatterjee et al. [31]. H₂T₄CPP proved to be more effective than H₂CPP in this process which could be interpreted by the closer proximity of sensitizer to DNA. The ssbs producing efficiencies of non-binding porphyrins H₂CPP and Hp investigated here are very similar to each other. It has been also shown [31] that the profile of plasmid DNA photodamage with special respect to the production of open circular form corresponds to damage caused by singlet oxygen.

To investigate further the possible structural changes induced by Hp photoreaction, the thermal denaturation of isolated T7 DNA was monitored via the changes in its light absorbance at 260 nm, at the maximum of DNA absorption spectrum. The melting derivative curves of native and photochemically treated DNAs are presented in Fig. 2A. They all show the typical hyperchromic transition around 84 °C. This transition is attributed to the denaturation of the DNA double helix: the opening of the H bonds, weakening of the stacking interaction and the separation of the two single strands. (The peak around 95 °C

![Fig. 1. Relative increase of single strand breaks (ssbs) in photosensitized plasmid DNA as the function of illumination period. Hp concentration was 6 μM. The curve was fitted by sigmoidal curve using Microcal Origin fitting routine. Standard deviation of experimental points was smaller than 5%.](image-url)
can be explained by the presence of protein residues from nucleoprotein complex.)

As it is seen in Fig. 2A the phase transition temperature of photosensitized DNA is shifted to lower values with increasing incident light dose. Parallel to the change of phase transition temperature the half-width and the amplitude of melting derivative curves are altered. All of these changes correspond to a destabilization of DNA structure and fragmentation of the DNA chain [32].

3.2. Photoinduced structural changes in NP complex

The melting derivative curve of photosensitized T7 nucleoprotein and its changes during irradiation are presented in Fig. 2B. The dark reaction with Hp alone and irradiation of the phages without sensitizer did not cause significant changes in the thermal denaturation pattern (data not shown).

In the melting derivative curve of T7 three structural transitions can be observed: a hypochromic change between 50 and 60 °C and two hyperchromic transitions around 84 °C and 95 °C. The low temperature transition is due to the disruption of the phage particle [32]. During this transition, the DNA is released from the capsid, it adopts a B secondary structure and loses its higher order arrangement [33]. The transition around 84 °C reflects the denaturation of the DNA double helix as it was described also for isolated DNA. The last melting step is related to the final separation of DNA and capsid proteins.

As Fig. 2B shows, the temperature of the phage disruption shifted from 59.5 °C to 54.5 °C and the last phase-transition temperature is shifted from 94.5 °C to 95 °C after 60 min of irradiation in the presence of 15 μM Hp. Under similar conditions the change of the phase-transition step related to the DNA strand separation is in the range of the experimental error.

Our results indicate that the photoreaction of Hp with the T7 phage results in the destabilization of the protein capsid. The changes observed in the first and last melting temperature suggest that the photodynamic reaction influences both the protein structure and DNA–protein interactions in the NP complex. However, no change was observed in the temperature, width or shape of the transition corresponding to the DNA denaturation.

Very similar results were obtained, when T7 phage was sensitized by another DNA-non-binding porphyrin derivative 5,10,15-(4-β-D-galactosylphenyl),20-(2’,3’,4’,5’-penta-fluorophenyl)porphyrin (TPFP) [34]. TPFP induced photoreaction destabilized the protein capsid and DNA–protein binding, but did not influence the stability of phase DNA.

3.3. Cellular uptake and localization of Hp

The incorporation and subcellular localization of photosensitizers have crucial importance for their efficiency and mechanism of action. Before investigation of the possible damages in nuclear DNA, it was necessary to prove and characterize the intracellular accumulation of Hp under our experimental conditions.
The fluorescence of Hp upon excitation allows the determination of subcellular localization of the dye by fluorescence microscopy. Here we followed the accumulation of Hp in HeLa cells by laser scanning confocal microscopy. Cell monolayers were incubated with photosensitizer for various time periods. After removing the unbound dye, the red fluorescence emitted upon excitation at 568 nm was detected. A continuous increase of intracellular fluorescence was detected up to 6 h incubation period. Fig. 3 shows a typical picture received after 4 h incubation with 10 μM Hp. The fluorescence of the porphyrin is found to be relatively diffuse, suggesting membrane and cytosolic incorporation. A network structure suggests that mitochondria might be more intensely labelled. No fluorescence was found in the nucleus.

The cellular distribution is certainly influenced by the uptake mechanism, which is determined by the lipophilicity of the molecule, the composition of the medium and the carrier system [35,36].

The picture received here is typical for lipophilic dyes [37,38]. Porphyrins, which accumulate mainly via passive diffusion in cells, are known to localize in cytomembranous structures such as endoplasmatic reticulum, lysosomes or mitochondria.

### 3.4. Cellular phototoxicity of Hp

After cellular uptake, we verified the cell killing activity of accumulated porphyrin derivative. It was necessary for two reasons: (1) to confirm the photobiological activity of intracellular Hp, and (2) to select the highest non-phototoxic concentration for the following Comet assay experiments.

In the absence of light Hp cytotoxicity was not significant up to 20 μM concentration. The phototoxic effects of Hp were measured by trypan blue exclusion assay in a concentration range of 3–20 μM. The cell viability was determined after 16 h incubation with the dye in two individual experiments. The effect of combination of porphyrin and visible light is presented in Fig. 4. When cells were irradiated, Hp caused a reduction in the viability of the HeLa cells in a concentration and a light dose dependent manner. After incubation with 6 μM Hp, 10 min irradiation did not reduce cell viability, but the same irradiation period reduced the cell survival if they were incubated with 9 μM porphyrin. Presence of 12 μM porphyrin induced almost complete cell killing after 30 min irradiation.

### 3.5. Photoinduced damage of intracellular nuclear DNA

Photoinduced damages were tested in two types of intracellular DNAs: nuclear DNA of HeLa cells and nuclear virus SV40.

Monolayer of HeLa cells were incubated with various concentrations (3–20 μM) of Hp for 16 h. After removing the unbound sensitizer, cells were exposed to white light for 30 min. During irradiation the cells were kept on ice to avoid DNA repair. After phototreatment cells were lysed and DNA was denatured in alkali. The analysis of the DNA lesions in HeLa cells was carried out by electrophoresis on 1% agarose gel. In Fig. 5 the electropherogram of extracted DNA from HeLa cells incubated with 6 and 15 μM Hp and exposed to 30 min irradiation is presented. DNA from irradiated but not photosensitized cells was used as control. As Fig. 5 shows there is no difference between the DNA patterns of control and irradiated samples.

![Fig. 4. Hp toxicity and photoxicity in HeLa cells measured by trypan blue exclusion assay. HeLa cells were incubated with various concentrations of Hp (3–12 μM) and then irradiated with white light.](image)

![Fig. 5. Agarose gel electrophoresis of intracellular chromosomal DNA isolated from HeLa cells. Lanes from left to right: (1) 10 kb control; (2) none Hp, 30 min irradiation; (3) 6 μM Hp, in the dark; (4) 6 μM Hp, 30 min irradiation.](image)
To investigate the induction of ssDNA in photosensitized cells alkaline agarose gel electrophoresis was also performed. We failed to show any indication of the presence of ssbs even at longer irradiation periods (data not shown).

Comet assay is a sensitive method allowing the characterization of DNA damages in individual cells. The alkaline version of comet assay is a useful technique for the detection of single- and double-stranded breaks [28]. In this work, we studied the possible induction of DNA strand breaks at low light and sensitizer dose combination, i.e. less than any light dose-porphyrin concentration combination leading to the 25% inactivation of cells (LD25).

Our results show (see Fig. 6) that DNA strand break, as judged from the absence of the tail formation, cannot be detected in the light-only or porphyrin-only groups. Neither was significant DNA strand breaks observed for the photosensitized and irradiated groups.

This failure of demonstration of DNA damages is in contrast to some recent observations [11,17,39,40] where it was suggested that the sensitizer located in the nuclear membrane but not in the nucleus, could also photodamage the DNA. On the other hand McNair et al. reported undetectable DNA damages when cells loaded with DNA-non-binding sensitizers were irradiated [40].

The reason for these differences in results is not clear; however the diversity of experimental conditions and cell lines can give some explanation. It is known that the incubation and irradiation protocols are of crucial importance for determining the site of intracellular damages and consequent mechanism of cell death [41].

Dellinger et al. [42] analyzed the possible inactivation pathways at two different incubation protocols. He showed that a long-lasting low dose treatment resulted in apoptosis with significant DNA damages, whereas a short incubation time with high porphyrin concentration induced cell lysis after plasma membrane alteration and necrosis of the cell. It was also demonstrated in other publications that the relative yield of oxidative base modifications decreased at higher light doses [43,44].

The results suggest that a low-dose photodynamic treatment with a membrane localizing agent initiates an apoptotic pathway and DNA damages; meanwhile a high-dose treatment induces membrane destruction and necrotic cell death. Ouedraogo et al. investigating also this possibility, excluded the direct attack by O2 (1' Δg) on nuclear DNA, but they did not prove the apoptotic pathway, either [14].

3.6. Photoinduced damage of intracellular viral DNA

After we failed to demonstrate any DNA damages in HeLa nuclear DNA, we considered the possibility of the generation of damages in another type of intracellular DNA.

SV40 DNA is 5243 nucleotides long and is arranged in a closed circular superhelical conformation. Virus proteins contain "nuclear localization signals". The newly replicated SV40 minichromosomes accumulate at the nuclear matrix and are directly encapsidated into DNA resistant SV40 virions inside the nucleur structure [45]. Assemblies occur mainly in the nucleus; a small fraction of newly replicated minichromosomes [46] leave the nuclear matrix to associate with the cellular chromatin. Before lysis most of the virus particles are enriched in the vicinity of nuclear membrane and they are released when the cell lyses. Because of this special intracellular localization of its DNA, SV40 can be an interesting subject for the investigation on intracellular DNA damages. Viral DNA can be an easier target for the reactive species generated by the extra-nuclear photosensitizer. Moreover, detection of relaxed circular DNA induced by O2 might be more convenient than analysis of whole cell chromatin fragments.

Monolayer of SV40 infected RITA cells was treated as described for HeLa cells above. Fig. 7 shows the electrophoretic separation of isolated SV40 DNA. This method facilitates the detection already one ssb of the SV40 DNA as transition of superhelical form I to the relaxed form II. According to the electropherogram there is no difference between the patterns of DNAs from treated and control samples. In Fig. 7, a line corresponding to relaxed dimer form or replicative intermediates of SV40 DNA are manifested over the marked open circle and supercoiled DNA.

Fig. 6. Comet assay of HeLa cells exposed to (A) positive control (5 Gy γ-radiation), (B) 30 min light exposure, and (C) 30 min light exposure after incubation with 4.5 μM Hp.

Fig. 7. Agarose gel electrophoresis of viral DNA isolated from RITA cells. Lanes from left to right: (1) no Hp, in the dark; (2) no Hp, 30 min irradiation; (3) 6 μM Hp, in the dark, (4) 6 μM Hp, 30 min irradiation; (5) 15 μM Hp, in the dark; (6) 15 μM Hp, 30 min irradiation.
form [47]. However, we did not find indication of the presence of ssDNA even in the sensitized and irradiated samples.

4. Summary

It has been suggested that the photodynamic reaction induced by membrane localizing porphyrin derivative such as hematoporphyrin IX might be a potential tool in the identification of genes localized on the nuclear surface in close interaction with the nuclear membrane. For the possible application of this method, here we wanted to verify that hematoporphyrin IX is able to induce damages in DNAs of various higher ordered structures.

When isolated nucleic acid was dissolved in Hp containing buffer solution, porphyrin mediated photoreaction caused damages both in superhelical and B-conformational DNA. During photodynamic treatment of nucleoprotein complex, porphyrin concentration and light dose inducing destruction of protein capsid caused neither stabilization nor destabilization in intraphage DNA, i.e., the presence of protein capsid protected the phage DNA.

As it was expected, Hp was accumulated by the HeLa cells and induced their photoactivation. However, electrophoretic methods and comet assay were not able to show any indication of photoinduced damages in nuclear DNA even if the applied porphyrin concentration and incident light dose was already phototoxic for HeLa cells. Similar results were received when intracellular SV40 DNA was tested.

From these results we can conclude that reactive species generated by the photoactivated Hp were not able to reach DNA molecules surrounded by protein capsid or localized in the cell nucleus. We lean to an opinion that DNA damages recognized before in cells sensitized by membrane-binding porphyrins are generated by other cellular mechanisms. To clear this point we are planning to perform experiments with other porphyrin derivatives with various hydrophilic–hydrophobic characters.

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References


