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Single Cell Responses to Spatially-Controlled Photosensitized Production of Extracellular Singlet Oxygen

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Abstract

The response of individual HeLa cells to extracellularly produced singlet oxygen was examined. The spatial domain of singlet oxygen production was controlled using the combination of a membrane-impermeable Pd porphyrin-dendrimer, which served as a photosensitizer, and a focused laser, which served to localize the sensitized production of singlet oxygen. Cells in close proximity to the domain of singlet oxygen production showed morphological changes commonly associated with necrotic cell death. The elapsed post-irradiation “waiting period” before necrosis became apparent depended on (a) the distance between the cell membrane and the domain irradiated, (b) the incident laser fluence and, as such, the initial concentration of singlet oxygen produced, and (c) the lifetime of singlet oxygen. The data imply that singlet oxygen plays a key role in this process of light-induced cell death. The approach of using extracellularly-generated singlet oxygen to induce cell death can provide a solution to a problem that often limits mechanistic studies of intracellularly photosensitized cell death: it can be difficult to quantify the effective light dose, and hence singlet oxygen concentration, when using an intracellular photosensitizer.

INTRODUCTION

Photodynamic therapy (PDT) is a clinically approved procedure that exploits a photosensitizer, light and oxygen to destroy unwanted cells and tissues (1, 2). We have been particularly interested in the photosensitized production of singlet oxygen, $O_2(a^1\Delta_g)$, the lowest excited electronic state of molecular oxygen, which is produced upon energy transfer from the excited state sensitizer to ground state oxygen, $O_2(X^3\Sigma_g^-)$ (3). Singlet oxygen has unique chemistry that is different from that of ground state oxygen (4, 5). This chemistry can result in oxidative modifications of a variety of biomolecules (*e.g.*, proteins, nucleic acids, and unsaturated lipids) (6–8) which, in turn, can influence mechanisms of both intra- and intercellular signaling (9, 10), all of which can play a role in cell death. Indeed, it is well-established that singlet oxygen is the primary mediator of both necrotic as well as apoptotic photodynamic cell death (11, 12).

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SUPPORTING INFORMATION

Additional images and a histogram showing cell response to irradiation of PD are available through the Photochem. Photobiol. website.

Over the years, data have been compiled to indicate that the site of singlet oxygen production in a cell not only influences the susceptibility of that cell to death, but also influences the mechanism of cell death (11–17). These conclusions have derived from experiments in which the sensitizer has been somewhat preferentially localized in a specific intracellular domain or organelle. For example, it is known that the production of singlet oxygen in the mitochondria can be particularly toxic (11–17). The dependence of toxicity on the location of singlet oxygen generation partly reflects the fact that, with an intracellular lifetime of $\sim 1\text{--}3\mu\text{s}$ in an H_2O -incubated cell, the sphere of singlet oxygen activity has a maximum radius of $\sim 150\text{ nm}$ from its point of production (3, 18). In a D_2O -incubated cell, where the singlet oxygen lifetime is much longer, the radius of singlet oxygen's sphere of activity can be as large as $\sim 500\text{--}600\text{ nm}$ (3). The corollary of this latter statement is that, in a D_2O -incubated cell where solvent-mediated singlet oxygen deactivation is less effective in the kinetic competition with substrate-mediated singlet oxygen deactivation (*e.g.*, reaction with a protein), singlet oxygen will be more cytotoxic (3).

The majority of studies published thus far have focused on the intracellular production of singlet oxygen and its subsequent effects. Only a few studies have addressed the consequences and relevance of extracellular singlet oxygen production (19–26) and, of these, two focus on mechanisms of stress response in the absence of light (24, 25). To our knowledge, only two groups have specifically addressed the issue of cell response to the extracellular photosensitized production of singlet oxygen. In one study (19–21), irradiation of a solid-phase immobilized photosensitizer was used to generate singlet oxygen in the air space immediately above a sample of bacteria cells deposited on a membrane filter. A key feature of this work involved the diffusion of singlet oxygen from its point of production, across the air gap, to the bacteria. This approach was later extended to study the response of mammalian cells that were likewise deposited on membrane filters and, in the absence of a culturing medium, exposed to the gas phase singlet oxygen (26). In a second approach using suspensions of eukaryotic cells (22, 23), singlet oxygen was generated upon thermal decomposition of naphthalene endoperoxides that, depending on their substituents, preferentially localized either inside or outside the cell. To complement these thermal sources of singlet oxygen, an agarose-bound photosensitizer was used to produce extracellular singlet oxygen upon irradiation. It was elegantly shown that in human skin fibroblasts (a) only intracellular singlet oxygen could activate p38 and c-Jun-N-terminal kinases, both of which are mitogen-activated protein kinases that mediate AP-1 gene expression, whereas (b) external singlet oxygen could activate the transcription factor AP-2 but not the p38 and c-Jun-N-terminal kinases.

Further elucidating aspects of the extracellular photosensitized production of singlet oxygen can be important for a number of reasons: (a) Many sensitizers used in photodynamic studies do not selectively localize in a given intracellular spatial domain, but are distributed in appreciable concentrations both inside and outside the cell (27). As such, upon global irradiation of a cell-containing sample, one could generate a non-negligible extracellular concentration of singlet oxygen. (b) Intracellular singlet oxygen produced near or in the cell membrane can diffuse through the membrane to yield an appreciable extracellular concentration (28–30). (c) Proteins exposed on the outer surface of the cell membrane can be significantly different from those on the inner surface (31) and, as such, the products and effects of the reaction of singlet oxygen at these respective protein sites can likewise be different. The fibroblast data discussed above (22, 23) are clearly a corollary of this point.

At present, one of the more important ramifications of the extracellular photosensitized production of singlet oxygen, certainly for experiments involving single cells surrounded by an appropriate culturing medium, is that one can more accurately quantify the amount of singlet oxygen produced for a given dose of incident light. Limiting features of the

production of singlet oxygen using an intracellular sensitizer include (a) light scattering by cell components that precludes an accurate correlation between the number of photons delivered and the number of photons absorbed, and (b) binding of the sensitizer to proteins, for example, or self-aggregation of the sensitizer, both of which can cause a change in the singlet oxygen quantum yield that is difficult to quantify (32–34). In contrast, quantifying the yield of photosensitized singlet oxygen production in the extracellular medium, at least in a microscope-based single cell experiment, is a tenable exercise. In turn, this can be a boon in mechanistic studies of singlet-oxygen-mediated cell death.

We report here the effects of photosensitized singlet oxygen production using, as a sensitizer, a membrane-impermeable palladium-porphyrin dendrimer which was developed as a phosphorescent probe for optical sensing of ground state oxygen (35). When using this molecule in combination with a focused laser for irradiation, extracellular populations of singlet oxygen can be created in a highly controlled manner at a selected distance from the cell membrane. The data presented here show that singlet oxygen generated in this way can indeed cause cell death, and that a key step in this procedure is the diffusion of singlet oxygen through the surrounding medium to a reactive site in or on the cell. This report thus sets the stage for some uniquely methodical and systematic studies of photoinitiated cell death.

MATERIALS AND METHODS

Materials

We have previously described the preparation of a physiologically compatible medium, our so-called Artificial Bath Medium, ABM, also called the Standard Maintenance Medium, SMM (34, 36). The solution was prepared using either H₂O or D₂O, depending on the experiments to be performed. All chemicals were obtained from Sigma-Aldrich except the following: TmPyP (5,10,15,20-tetrakis(*N*-methyl-4-pyridyl)-21*H*,23*H*-porphyrin, Porphyrin systems), D₂O (Euriso-Top, > 99.9% D), MEM solution of 100 mM sodium pyruvate (Invitrogen) diluted by a factor of 10 for use, and catalase (from *Aspergillus niger*, Fluka, with an activity of 50 U/mL used in a given sample in a given experiment).

PdP-AG¹-PEG750 (Fig. 1) consists of a palladium porphyrin (PdP), encapsulated by a poly(arylglycine) generation 1 dendrimer (AG¹), that is modified peripherally with monomethoxypolyethylene glycol groups (PEG750, average molecular weight of 750 Da). PdP-AG¹-PEG750 was synthesized and purified according to a previously published method (35). Throughout the remainder of the text, we abbreviate PdP-AG¹-PEG750 as PD.

Cells

To maintain continuity with our previous work, HeLa cells were used in this study. The preparation of samples has been described previously (34, 36).

The laser-based optical system and methods used

The details of the femtosecond (fs) laser system used for sample irradiation have been previously described (34, 37, 38). The instrumentation and methods used to acquire images and to record time-resolved luminescence data have likewise been described (34, 39). For the microscope-based studies in the present work, cells were housed in an incubator (DH35i, Harvard Apparatus) mounted onto the stage of our inverted microscope, and were maintained at 37 °C throughout the course of an experiment. For video microscopy, data were recorded using a sequence of 30 frames with a rate of 1 frame per 30 s.

The effects of irradiation using a “focused” source were examined using this microscope system. Before photodynamic treatment, the cultivation medium was removed from the cells and replaced with a solution of 5.7 μM PD in ABM (or PD-free ABM for the corresponding control experiments). Bright field images of the cells were recorded using a 640 nm band pass filter (Oriel model 53940, 10 nm FWHM) in the light path to avoid unnecessary excitation of PD (*i.e.*, PD does not absorb light at 640 nm, see Fig. 2). The outer membranes of the cells were identified by visual inspection of the bright field image, and the dish containing the cells was translated such that the laser spot intersected the sample at a specific distance, d , from the selected cell’s membrane. The values of d reported herein were measured from the center of the beam waist. The stage position was then noted using the Scope-Pro application within the Image-Pro software so that the cell could be revisited and observed at later times.

Photodynamic treatment was performed using 420 nm light (*i.e.*, one-photon excitation of PD) with different irradiation times and powers. The diameter of the laser beam in the image plane was $\sim 1.4 \mu\text{m}$ (28). For high light doses (*i.e.*, 215 kJ/cm^2), laser irradiation with the average power of 11 μW (measured at the stage) was applied for 5 min. For low light doses (*i.e.*, 40 kJ/cm^2), the laser power was 5.1 μW and the irradiation time was 2 min. After irradiation near one cell, other cells in different locations (at least 1 mm away) could be similarly treated.

Studies of PD localization

Experiments were performed on the stage of a microscope (Nikon Diaphot 200) to which a fiber-optic-based spectrometer (USB 2000, Ocean Optics) was attached. Bright field and Differential Interference Contrast (DIC) images of a given cell were obtained with the microscope lamp (LQ 2600 Fiberoptic Hein, Switzerland, equipped with a Xenophot HLX 64657 bulb, Osram, Germany) and a digital camera (MV blueFox 124G, 1200×1600 , Matrix Vision GmbH, Germany). To image cells without suffering the adverse effects of PD excitation, a 580 nm bandpass filter (Thorlabs, Sweden, FWHM of 20 nm) was used in the light path (*i.e.*, PD only weakly absorbs light at 580 nm, see Fig. 2).

Emission spectroscopy was used to investigate whether PD was incorporated into and/or associated with HeLa cells. For this study, a coverslip with cells was placed on the temperature-controlled microscope stage (TC-III, Luigs & Neumann, Germany) and rinsed with ABM. The cell was then perfused for 1 min with a solution of 5.7 μM PD in ABM ($\sim 20\text{--}30 \mu\text{L}/\text{min}$). A drug-application device attached to a 13 valve micro-manifold with a 100 μm i.d. output tube (ALA-sciences, New York) was used to apply the PD and ABM solutions to the cells. After the 1 min perfusion with the PD solution, the emission spectrum of the sample was obtained using an integration period of ~ 1 s. A spectrum was recorded again after reperfusion for 1 min with an ABM solution lacking PD. Corresponding emission spectra were recorded in an independent experiment in which the cells exposed to PD were also irradiated for 1.5 min at 425 nm (irradiance of 0.73 W/cm^2 to yield a fluence of 0.07 kJ/cm^2). The spectra were obtained upon excitation with a Nikon fluorescence lamp (HB 10101 AF, equipped with an Osram HBO 100 W/2 mercury short arc lamp) connected to a Lambda 10–2 filter wheel–shutter (Sutter Instruments, California), using a 425 nm excitation filter (Thorlabs, Sweden, FWHM = 20 nm). Emission was recorded using the abovementioned fiber optic spectrometer in combination with a 515 nm long pass filter (Nikon).

The effects of irradiation using a “non-focused” light source were also examined using this microscope system. Cells were first rinsed for 1 min with ABM, then a solution of 5.7 μM PD in ABM was applied (flow rate of $\sim 20\text{--}30 \mu\text{L}/\text{min}$). After 1 min, and maintaining constant perfusion of the PD solution, the system was irradiated at 425 nm using a 40 $\times$

objective (Nikon 121735, 40/DIC/0.55 LWD) for 1.5 min at 0.73 W/cm^2 (i.e., fluence of 0.07 kJ/cm^2). Cells were then examined after different post-irradiation waiting times.

Monitoring cell response

Two approaches were used to assess cell response to irradiation:

- a. Bright field or DIC images were used to record morphology changes (e.g., membrane located vacuole formation, shrinkage, loss of adhesion to the plate, condensation of nuclear chromatin). It is clear that such general morphological features are likely the result of changes in multiple cellular processes. Moreover, after a given cell has been perturbed, the “waiting period” before morphology changes become apparent will depend on which specific cellular processes are involved. Despite the difficulty of determining the onset of these morphological changes, and establishing the pertinent statistics from single cell experiments (*vide infra*), this is nevertheless an established method that combines reasonable accuracy with ease of implementation in assigning both apoptotic and necrotic death (39–41).
- b. In some cases, propidium iodide, PI, was used to assess the integrity of the cell membrane. In a dead cell, where the membrane has degraded sufficiently and lost function, PI can enter the cell and stain the nucleus (42). In the procedure used, PI was added to the cell solution to a final concentration of $2.25 \mu\text{M}$ immediately before the 420 nm irradiation used to perturb the cell. Excitation of PI was performed using a 535 nm bandpass filter (FWHM = 40 nm), and PI emission was monitored using a 600 nm band pass filter (FWHM = 40 nm).

Photophysical characterization

Absorption and emission spectra—UV/VIS absorption spectra were recorded using a Shimadzu UV3000 UV-VIS NIR spectrophotometer. The emission spectrum of PD, recorded from a $5.7 \mu\text{M}$ ABM solution purged with nitrogen for ~ 10 min, was obtained using a Horiba Jobin Yvon spectrometer (FluoroMax P). Spectra obtained matched those previously recorded in water over a wide range of concentrations (35), indicating that neither ABM nor PD itself perturbed the PD emission. The excitation wavelength was 420 nm with an integration time of 0.1 s. The spectra were corrected for detector efficiency.

Singlet oxygen quantum yield, ϕ_{Δ} —The PD-sensitized singlet oxygen quantum yield was determined in bulk solution-phase experiments with air-equilibrated D_2O as the solvent. Experiments were performed using the 1275 nm singlet oxygen phosphorescence as a probe, as previously described (43). Data obtained using TMPyP as the sensitizer served as the standard [$\phi_{\Delta}(\text{TMPyP}) = 0.77 \pm 0.04$ (44)]. Both PD and TMPyP were excited at 535 nm, and the data were recorded as a function of the incident laser power; the latter was controlled by placing neutral density filters in the laser beam. In the 1 cm cuvette used, the absorbance at the excitation wavelength was ≤ 0.15 . The time-resolved 1275 nm phosphorescence signal was integrated, and the integral was normalized by the value of the overlap of the laser spectrum with the absorption spectrum of each sample, thus accounting for the broad spectral output of the fs laser. A typical error for such quantum yield measurements is 10%, and the standard deviation of our data were within that range.

RESULTS AND DISCUSSION

Dendritically-protected Pd porphyrin-based phosphorescent probes for use as oxygen sensors have been described previously (35, 45–48). In these systems, the salient feature exploited for use as a sensor is the fact that the phosphorescent porphyrin triplet state is

quenched by oxygen (49). In the present study, we set out to use one such porphyrin as a specific triplet state sensitizer for the production of singlet oxygen, not as an oxygen sensor. In particular, we wanted to capitalize on the fact that the porphyrin dendrimer chosen for this study (PD, Fig. 1) does not permeate cell membranes (35) and, thus, can act as an extracellular singlet oxygen sensitizer.

It is important to note, at the outset, that our experiments were performed on individual cells. Thus, it is difficult to report observations with the quantitative accuracy and statistics normally seen in measurements on ensembles of cells (with the inference that identical “numerical” results could be similarly obtained in an independent experiment performed in another laboratory) (50). Nevertheless, the general phenomena that we report were repeatable with different experiments performed over an extended period of time.

Properties of PD

The dendritically-protected porphyrin PD has several features that make it useful as a sensitizer in a singlet oxygen study. The absorption and emission spectra of PD are shown in Fig. 2A. The absorption spectrum shows a characteristic intense band centered at ~400 nm corresponding to the S_0 - S_2 transition (Soret band) and the weaker S_0 - S_1 transition at ~530 nm (Q-band), both of which are conveniently accessible wavelengths for our fs laser system. The Soret band has an extinction coefficient of $3.8 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ at peak maximum. The emission spectrum exhibits a band with a maximum at ~707 nm and a shoulder at ~785 nm. Although Pd porphyrins, in general, have small quantum yields of fluorescence ($\sim 10^{-6}$), PD is sufficiently phosphorescent, even in air-saturated solutions (35), which is useful for experiments to visualize its localization in or around a cell. Most importantly, we ascertained that the quantum yield of PD-sensitized singlet oxygen production is 0.85 ± 0.09 (Fig. 2B). Thus, although the dendritic shell modulates oxygen diffusion to the porphyrin chromophore (35, 51), our data clearly indicate that an appreciable amount of singlet oxygen is still produced by PD.

Based on the structure of the dendrimer components of PD (Fig. 1) and on what is known about the reactions of singlet oxygen (5), one would not expect photoinduced singlet-oxygen-dependent changes in the dendrimer shell surrounding the Pd porphyrin. However, if such changes did occur, some of the more likely products that would accumulate (*e.g.*, hydroperoxides, alcohols) would be good quenchers of singlet oxygen. Thus, if photoinitiated degradation of the dendrimer surrounding the chromophore occurred, one would expect to see a decrease in the intensity and lifetime of the PD-sensitized singlet oxygen phosphorescence signal as a function of the elapsed irradiation time. The pertinent experiment was performed in a 1 cm path length cuvette with $5.7 \mu\text{M}$ PD dissolved in D_2O -based ABM. Under relevant conditions (420 nm irradiation at 1 mW with constant stirring of the 2 mL volume), no significant changes in the time-resolved singlet oxygen phosphorescence signal were observed over an irradiation period of 20 min. This is an observation which is consistent with the expectation that, for this work, PD remains intact and should not contribute products of oxidation reactions that, in turn, could influence our cell kill studies.

Verifying the extracellular localization of PD

In general, PEG- and dendrimer-coated porphyrins must be delivered into cells with special techniques; they are not routinely incorporated into a cell upon simple exposure to the cell (52). Nevertheless, we must specifically verify that, under the conditions of our experiments, PD does not enter the cell or associate with the cell membrane.

For this test, we relied on the fact that the detection of PD phosphorescence is a sensitive measure of whether or not there is an appreciable amount of this chromophore present in the system. Fig. 3A shows a representative DIC image of HeLa cells used for this test. The circle represents the area from which the emitted light was collected.

In one approach, we perfused the cell with a 5.7 μM solution of PD in ABM. We then irradiated the sample for 1.5 min at 425 nm (0.730 W/cm^2) and, thereafter, the light emitted from the sample was recorded. The spectrum thus obtained (Fig. 3B, solid trace) matches the phosphorescence spectrum obtained from a bulk solution of PD (Fig. 2A). The cell was then rinsed with ABM for 1 min, and the spectrum of emitted light was re-recorded. The data obtained clearly show the absence of PD phosphorescence (Fig. 3B, dashed trace). These data indicate that, even under conditions where the irradiation of PD could (a) adversely affect cell viability and membrane integrity (*vide infra*) and/or (b) result in a modification of the dendrimer shell of the porphyrin, PD does not associate with, nor does it enter, the cell.

In a different approach, after perfusing the cell with a PD solution for 1 min in the absence of light that could excite PD, we then rinsed the cell with a PD-free ABM solution. The emission spectrum was then recorded. The data obtained indicate that, for a cell that is presumably not perturbed and that functions normally, PD is likewise not incorporated into, or adsorbed onto, the cell.

Thus, upon PD irradiation in our experiments, singlet oxygen will be produced exclusively in the extracellular medium.

Effect of extracellular singlet oxygen production using “non-focused” irradiation

For these experiments with “non-focused” light, the area irradiated at the sample had a diameter of $\sim 270 \mu\text{m}$, achieved using a lamp as the excitation source (Fig. 4). These experiments thus differ from those discussed in the next section where the excitation light is a tightly focused laser beam that irradiates a more confined area of about $1.4 \mu\text{m}$ in diameter.

The cells were perfused with a 5.7 μM solution of PD prepared in H_2O -based ABM. The presence of PD in the samples was verified by recording its phosphorescence, as discussed above. A spatial domain containing a cell was then irradiated at 425 nm with a fluence dose of 0.07 kJ/cm^2 at the image plane of the microscope (*i.e.*, for 1.5 min at an irradiance of 0.730 W/cm^2). To assess cell response, the sample was imaged at a lower magnification so both irradiated and non-irradiated (but PD perfused) cells could be inspected.

Prior to irradiation, the cells appeared to be well-adhered to the coverslip and had a smooth topography (Figs. 4A and 4C). These features point to a viable cell and indicate that perfusion does not disturb the cells. After irradiation and a 10 min “waiting period” in the dark, large vacuoles were clearly visible at the membrane edges and the nucleus appeared to have a distinct border in the PD-containing sample (Fig. 4B). These morphological features are generally characteristic of a dying or dead cell (39, 40). Cells outside the irradiation zone did not show morphological changes over the same 10 min waiting period. Likewise, morphological changes were not observed in the cells upon irradiation of the sample lacking PD (Figs. 4C and 4D). We independently ascertained that, over a longer period of time, PD is not cytotoxic in the dark.

For the PD-free study, two different experiments were done: (a) irradiation of cells exposed only to an ABM solution lacking PD, and (b) initial perfusion of the cells for 1 min with a PD solution, followed by 1 min re-perfusion/wash with ABM lacking PD, and then irradiation. In both cases, no irradiation-dependent morphological changes to the cells were observed up to a period of 10 min after the irradiation ceased. Data recorded from the

second control experiment (*i.e.*, cells that had previously been exposed to PD) are shown in Figs. 4C and 4D. The results of this second control experiment are consistent with the data shown in Fig. 3, and indicate that PD does not associate with the cell membrane, nor does it enter and remain in the cell.

Effect of extracellular singlet oxygen production using “focused” laser irradiation

In an independent series of experiments, we investigated the toxicity of extracellular singlet oxygen generated upon irradiation of PD using a laser focused to a spot whose diameter is $\sim 1.4\mu\text{m}$ at the beam waist in the image plane. In this way, we can produce a localized population of singlet oxygen at any arbitrary distance from the cell membrane simply by laterally moving the sample in the image plane. Moreover, with the comparatively short lifetime of singlet oxygen in the H_2O -based medium ($\sim 3\mu\text{s}$), diffusion of singlet oxygen will not appreciably change its domain of activity relative to the spatial domain irradiated by the laser (18).

Point Spread Function, PSF—In performing these spatially-resolved experiments, it is important to quantify the degree of beam localization (*i.e.*, the point spread function, PSF) under our conditions and, hence, establish the location of singlet oxygen production.

First, we note that, for these experiments, the back-aperture of the microscope objective was underfilled with light. This leads to an expansion of the PSF relative to the theoretical limit defined by the diffraction of light (53). Nevertheless, the focal volume of light delivered to the sample was still sufficiently small compared to the sizes of the cells in our samples (37). This is consistent with an image of scattered light recorded upon the irradiation of a quartz plate with the focused laser (Fig. 5).

Second, upon irradiation of a sample characterized by domains with a different density and refractive index (*e.g.*, a polylysine-coated coverslip covered with ABM), light scattering by the sample can increase the size of the PSF (34, 54). Although we do not directly irradiate the highly-scattering cell in these experiments, and our focal volume is not limited by the diffraction of light (*vide supra*), the spatial domain in the sample actually irradiated could nevertheless be larger than that defined solely by the laser beam profile in a homogeneous medium (*i.e.*, air).

To assess the PSF pertinent for our experiments, we recorded the light emitted from a polylysine-coated plate covered with a PD-containing solution of ABM (*i.e.*, PD phosphorescence recorded upon irradiation in the extracellular domain of our samples). The data recorded show an apparent PSF that is larger than that seen upon irradiation of a bare quartz plate (Fig. 5). However, this broader profile can be largely attributed to the fact that our excitation laser propagated through a PD-containing liquid that was $\sim 1.5\text{ mm}$ thick (z -axis) and light was collected from multiple xy -planes. In the comparatively thin z -layer, whose dimension corresponds to the thickness of a cell ($\sim 10\mu\text{m}$), focused laser irradiation will still define a spatially-resolved profile of photon flux from which useful conclusions can be drawn.

Cell response as a function of the light dose—In these experiments, the laser was focused to a spot a given distance away from the cell in the extracellular medium. In Fig. 6, data recorded upon delivery of a comparatively high light dose (*i.e.*, 215 kJ/cm^2) $\sim 2.4\mu\text{m}$ away from the cell membrane are shown. It is clear that the cell shows an immediate adverse response under these conditions (*i.e.*, vacuoles marked by the arrow in Fig. 6B). In Fig. 7, corresponding data recorded upon delivery of a much lower light dose (40 kJ/cm^2) at a similar distance of $\sim 2.9\mu\text{m}$ away from the cell membrane are shown. In this case, an adverse morphological response first appeared only after, at minimum, a 41 min waiting

period (*i.e.*, apparent condensation of the nuclear chromatin along the nuclear membrane (41)) with vacuole formation clearly apparent in an image recorded after 109 min.

To test whether the morphological changes seen in Figs. 6 and 7 are, in fact, associated with cell death, we performed a PI assay. For this experiment, we used the comparatively low light dose of 40 kJ/cm² delivered ~ 1.9 μm away from the cell membrane. As such, this experiment is similar to those shown in Figs. 6 and 7 where the light was delivered 2.4 and 2.9 μm away from the cell membrane, respectively. The data obtained are shown in Fig. 8. First, we note that, as in the low dose experiment of Fig. 7, the obvious adverse morphological change of vacuole formation only appears after prolonged waiting (see differences between Figs. 8B and 8C). In Fig. 8D, we show an image of this system based on the fluorescence of PI. It is clear that the cell in close proximity to the focused laser spot, and the one that shows clear vacuole formation in the bright field image, yields pronounced PI fluorescence, indicating that the membrane in this cell has indeed been compromised.

To complement the experiments shown in Figs. 6–8, we performed control experiments in which the cells were kept in ABM that lacked PD but were exposed to the same light doses. For experiments performed at both the high and low light doses, no morphology changes characteristic of cell death were seen over the corresponding waiting periods. An example of the data recorded for an experiment with the high fluence dose of 215 kJ/cm² delivered at a spot ~ 1.9 μm from the cell membrane is given in the Supporting Information.

Precluding optoporation and/or heat-mediated cell death—On the basis of the data presented thus far, it is clear that the presence of both light and the photosensitizer are needed to kill cells under our experimental conditions. This is particularly important in that it has been independently shown that, by itself, light from a focused laser pulse can kill cells, even when positioned only into the adjacent extracellular medium (55–57). This phenomenon is a consequence of local plasma formation that, in turn, produces an omnidirectional shock wave that propagates through the medium and that can disrupt the cell membrane. The latter forms the basis for a technique called optoporation whereby a transient pore can be opened in the cell membrane to allow the facile incorporation of extracellular material into the cytoplasm (55–57). With this in mind, our focused laser pulse could also facilitate the incorporation of PD into the cell. However, the data recorded indicate that none of these possibilities were realized. The absence of divots or craters at the point of irradiation on the coverslip is also consistent with the conclusion that a membrane-perturbing plasma is not formed under our conditions.

One must also consider the related possibility that, in the focused laser experiments, a pronounced temperature increase in a localized extracellular domain could likewise perturb a cell. To address this, we performed experiments in which sodium azide, NaN₃, was added to the surrounding medium (final concentration of 10 mM). NaN₃ is an efficient hydrophilic quencher of singlet oxygen ($k_q \sim 5 \times 10^8 \text{ s}^{-1}\text{M}^{-1}$) (58, 59). As such, upon irradiation of PD in an aerated solution containing NaN₃, most of the incident photon energy will rapidly be converted to heat (*i.e.*, internal conversion in PD plus non-radiative quenching of the singlet oxygen produced by PD). We have independently established that, by itself, NaN₃ is not cytotoxic under the conditions used in the present experiments (59).

In D₂O-based systems containing NaN₃, we did not observe signs of cell death upon irradiation of PD, even when the laser focus was positioned right next to the cell membrane (Fig. 9). Although these NaN₃-based experiments allow us to make a substantive conclusion about heat-mediated cell death (*i.e.*, it does not play a role in our present study), the data obtained also constitute a key control experiment to indicate that our cells are responding to the effects of singlet oxygen. We return to this point in subsequent sections.

Finally, it is important to recall that the lamp-based irradiation experiments shown in Fig. 4, where shock wave and localized heat formation are precluded, still result in cell death. Thus, it indeed appears that, in our experiments, the cells are only perturbed by the photodynamic action of extracellular PD.

Cell response as a function of the distance between the cell membrane and the site of singlet oxygen generation—We next set out to monitor cell response as a function of the distance between the cell membrane and the site of singlet oxygen production. Experiments were performed using a fluence of 40 kJ/cm², which corresponds to the fluences used in the experiments shown in Figs 7 and 8.

In Fig. 10, the response of two closely aligned cells to focused irradiation at a distance, d , of $\sim 8 \mu\text{m}$ from the membrane of one of the cells is shown. Under these conditions, we first saw vacuoles characteristic of cell death 90 min after the light dose was delivered. Most interestingly, however, these vacuoles only appeared in the cell closest to the laser focus; the cell that was further away exhibited only slight morphological changes over the 269 min period of post-irradiation observation. (Images recorded at 232 and 269 min after delivery of the light dose, which are not shown in Fig 10, simply reinforce the 195 min image in Fig 10H.) Similar results were obtained with a second pair of cells (Fig. 11). Again, the cell closest to the laser focus ($d \sim 1.7 \mu\text{m}$) showed vacuole formation characteristic of death within 19 min of light delivery (Fig. 11C), whereas the cell further away from the laser focus ($d \sim 20 \mu\text{m}$) exhibited no morphological changes over the 248 min period of post-irradiation observation.

Note that we are making these distance-dependent observations using pairs of cells that have been cultured at the same time in the same medium. In this way, we hope to provide more meaningful results by minimizing the effects of variables that characterize single cell experiments and, in turn, contribute to the scatter of data. In short, it is generally accepted that data recorded in a given experiment from one cell will not necessarily correspond to data recorded from another cell in a second experiment, even if the experiments are performed under presumably identical conditions (34, 50). Indeed, for experiments performed using different batches of cells, the correlation between d and a given measure of cell death (*e.g.*, the time for light-induced vacuole formation) was not as good as one would like, presumably due to the increased susceptibility to statistical variations and the limited number of samples that could realistically be examined (*e.g.*, compare data in Figs. 7–8 to those in Figs. 10–11).

It is valid to ask why a cell can show morphological signs of death when the focused laser is positioned $\sim 1\text{--}10 \mu\text{m}$ away from the cell membrane, given that, in an H₂O-based medium, the sphere of singlet oxygen activity has a radius of $\sim 150 \text{ nm}$ from its point of production. There are, at least, three reasonable possibilities. First, we return to our discussion of light scattering and the PSF shown in Fig. 5. It is possible that, even when the laser focus is positioned $10 \mu\text{m}$ away from the cell, scattered photons will excite a sufficient number of PD molecules that are much closer to the cell membrane. Although we have inferred that scattered light is not a major problem in our system, this is nevertheless the most likely explanation for this phenomenon. Of course, because the density of scattered photons near the cell membrane will decrease as the distance between the laser focus and the cell is increased, one would still see a correlation between cell death and the distance between the cell membrane and the laser focus. Second, singlet oxygen generated upon irradiation of PD may react with components in the medium and, in turn, produce a different and longer-lived cytotoxic agent that could diffuse over a greater distance. However, given the components abundant in our medium (36), which either do not react with singlet oxygen or physically deactivate singlet oxygen to the ground state (*i.e.*, inorganic salts and glucose), this scenario

is not probable. Finally, despite a large quantum yield of PD-sensitized singlet oxygen production, singlet oxygen may not be the cytotoxic agent. Rather, irradiation of PD may directly produce another compound, longer-lived than singlet oxygen, which causes cell death.

With respect to the latter two points, if singlet oxygen is not the cytotoxic agent, it is reasonable to consider that H_2O_2 , a hydroperoxy-substituted compound or a free radical could be the pertinent species responsible for cell death in our experiments. To test these possibilities, we performed independent PD-sensitized experiments in the presence of catalase, sodium citrate and sodium pyruvate, respectively. Catalase is an enzyme that should protect against H_2O_2 -mediated cell death (60), and sodium citrate and sodium pyruvate should mitigate a variety of oxidation reactions, including some mediated by radicals (60–62). In all cases, for experiments performed over a range of distances ($\sim 3\text{--}9\ \mu\text{m}$) between the laser focus and the cell membrane, the addition of these antioxidants did not inhibit or mitigate cell damage in D_2O -based systems (representative data are shown in the Supporting Information).

In an earlier section, we presented the pertinent control experiment that complements the catalase, citrate and pyruvate experiments: upon the addition of the singlet oxygen quencher NaN_3 to the surrounding medium, we inhibited PD-sensitized cell death (Fig. 9). Collectively, these results support our thesis that, in these extracellular PD-sensitized experiments, cell death is a result of a direct reaction with singlet oxygen produced in the extracellular medium.

The effect of replacing H_2O with D_2O on the cell response—Because there is a large solvent isotope effect on the lifetime of singlet oxygen (63), a control experiment that complements the NaN_3 experiment discussed above can be made by performing experiments in D_2O -based ABM and comparing the resultant data to those recorded using H_2O -based ABM. It is well established that the lifetime of singlet oxygen in neat D_2O , $\sim 67\ \mu\text{s}$ (64), is significantly longer than that in neat H_2O , $\sim 3.5\ \mu\text{s}$ (65). This inequality is generally maintained in solutions such as ABM that do not contain solutes capable of efficiently quenching singlet oxygen (36). As such, in the context of our present spatially-resolved experiments on cell death, singlet oxygen should have a larger sphere of activity in D_2O -based ABM than in H_2O -based ABM (3). Of course, if this solvent isotope effect is indeed manifested in our data, then we have also provided even more substantive evidence to indicate that the cell death we observe occurs as a result of a process that, in one way or another, involves singlet oxygen.

When cells are immersed in a D_2O -based medium, a substantial fraction of the intracellular H_2O will also be replaced by D_2O . However, we have independently shown that, at least over a period of several hours, such a change in solvent, in itself, is not toxic to the cell (36). This was confirmed in the present study upon irradiation of samples exposed to a D_2O -based ABM lacking PD.

With a light dose of $40\ \text{kJ}/\text{cm}^2$ delivered $\sim 3\ \mu\text{m}$ away from the cell membrane, vacuole formation characteristic of a perturbed cell appeared within $\sim 7\ \text{min}$ for a system in D_2O -based ABM (Fig. 12). In contrast, in a similar experiment performed in H_2O -based ABM, and where the light dose was delivered at an even shorter distance from the cell membrane ($d \sim 2\ \mu\text{m}$), evidence for vacuole formation first appeared only after waiting for $\sim 15\ \text{min}$ (Fig. 13). These data are representative of data reported from experiments performed over a range of distances d between the laser focus and the cell membrane, and indicate that there is indeed a solvent isotope effect on the cytotoxic event in our system. In turn, this supports the notion that the pertinent reactive intermediate is singlet oxygen.

The role of singlet oxygen

We have thus far provided evidence to indicate that, under a variety of readily-controlled conditions, the extracellular photosensitized production of singlet oxygen can lead to cell death. Future studies must now focus on (a) elucidating the exact role played by singlet oxygen in initiating cell death, and (b) identifying the site(s) in or on the cell where the pertinent adverse reactions with singlet oxygen occur. With these points in mind, it is useful to recall two key experimental results.

First, we have independently shown that, after having been produced on one side of the plasma membrane, an appreciable amount of singlet oxygen can diffuse across the membrane and react with a substrate on the other side of the membrane (28, 29). This was demonstrated with the intracellular photosensitized production of singlet oxygen, noting a change in the intensity and decay kinetics of the singlet oxygen phosphorescence signal upon the addition of an extracellular quencher. By extension, we assume that extracellular singlet oxygen can likewise diffuse across the plasma membrane and react with an intracellular substrate. On the basis of our intracellular lifetime studies (3, 18), we can estimate that the pertinent distance from the membrane into the cell over which this diffusing population of singlet oxygen could react is ~ 155 nm in a H₂O-equilibrated cell and ~ 550 nm in a D₂O-equilibrated cell. Thus, the pertinent singlet oxygen targets and/or substrates are not necessarily localized in the membrane itself. Second, recall that Sies and colleagues have been able to document that cell response to an external population of singlet oxygen can indeed be different from the response to an internal population of singlet oxygen (22, 23).

CONCLUSIONS

The response of a single cell to the extracellular photosensitized production of singlet oxygen was examined using a membrane-impermeable porphyrin-dendrimer as a sensitizer in combination with a focused laser to define the spatial domain of singlet oxygen production. Cells in close proximity to the domain of singlet oxygen production showed morphological changes associated with cell death. The post-irradiation “waiting time” required before the appearance of morphological features of cell death depended on (a) the distance between the cell membrane and the domain irradiated, (b) the incident laser power used to irradiate the photosensitizer, and (c) the lifetime of singlet oxygen as determined by the solvent and added quenchers used in the experiment. The data presented herein are thus consistent with a model in which the response of a cell to singlet oxygen, and the waiting time before this response becomes apparent, are proportional to the local concentration of singlet oxygen.

Perhaps the most important ramification of this study is that the amount of singlet oxygen produced by this extracellular photosensitized approach is more readily controlled and quantified than the amount produced when using an intracellular photosensitizer. In the extracellular approach, problems associated with light scattering by the cell and cell-dependent changes in the singlet oxygen quantum yield are precluded. In this way, we provide one solution to a problem that currently limits many mechanistic studies of singlet-oxygen-mediated cell death.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Chemical structure of PdP-AG¹-PEG750, denoted PD, used as a hydrophilic and cell-impermeable singlet oxygen sensitizer in this study.

Figure 2.

(A) Normalized absorbance (solid line) and emission (dashed line) spectra of 5.7 μM PD in ABM (pH = 7.2). (B) Plots of the integrated intensity of singlet oxygen phosphorescence against incident laser power used for the determination of the PD-sensitized quantum yield of singlet oxygen production, ϕ_{Δ} . The lines are linear fits to the data, with slopes that are proportional to ϕ_{Δ} (43).

Figure 3.

Representative example of data used to verify that PD does not associate with or penetrate into a cell. (A) DIC image of the HeLa cell used for this experiment. The circle marks the area from which emitted light was collected. (B) Emission spectra obtained upon PD perfusion and ABM reperfusion.

Figure 4.

DIC images of HeLa cells perfused with (A) PD solution or (C) ABM solution lacking PD. These images were recorded prior to irradiation at 425 nm. The circles mark the area irradiated. Panels B and D show the same cells, respectively, 10 min after a 1.5 min period of 425 nm irradiation (0.07 kJ/cm^2). In the system containing PD (panels A and B), the cell is clearly damaged after irradiation, as indicated by the formation of several large vacuoles at the outer cell membrane (indicated by the arrows). Cells outside the irradiated zone did not show morphological changes. In the system lacking PD (panels C and D), we were unable to see obvious morphological changes to distinguish the non-irradiated and irradiated cells.

Figure 5.

Cross sectional intensity profiles of scattered light upon 420 nm irradiation of a quartz plate and, independently, light emitted at $\lambda > 740$ nm upon 420 nm irradiation of a solution of PD in ABM on a polylysine-coated coverslip. The abscissa represents the distance along a line in the xy -plane orthogonal to the direction of beam propagation through the sample (z axis). The data from the PD-containing solution (*i.e.*, trace with the higher level of noise) show a broader profile that is principally due to the increased thickness of the sample through which light propagates (*i.e.*, we are collecting light from multiple xy -planes).

Figure 6.

Bright field images of a HeLa cell exposed to extracellular singlet oxygen produced by focused 420 nm laser irradiation of PD. The domain irradiated, represented by the white spot in panel A, is $\sim 2.4 \mu\text{m}$ away from the cell membrane. Upon irradiation with a fluence of 215 kJ/cm^2 , the cell showed immediate morphological changes characteristic of cell death (arrow, panel B), and these changes became more pronounced after a waiting period of 60 min (panel C).

Figure 7.

Bright field images of a HeLa cell exposed to extracellular singlet oxygen produced by focused 420 nm laser irradiation of PD. The domain irradiated, represented by the white spot in panel A, is $\sim 2.9 \mu\text{m}$ away from the cell membrane. Upon irradiation with a fluence of 40 kJ/cm^2 , the cell does not show immediate signs of an adverse response. Only in the image recorded after a waiting period of 41 min do we see the first signs of cell death (i.e., apparent condensation of nuclear chromatin along the nuclear membrane, arrow in panel C) and, after 109 min, vacuole formation (arrows in panel D).

Figure 8.

Bright field images of a HeLa cell exposed to extracellular singlet oxygen produced by focused 420 nm laser irradiation of PD. The domain irradiated, represented by the white spot in panel A, is $\sim 1.9 \mu\text{m}$ away from the cell membrane. Upon irradiation with a fluence of 40 kJ/cm^2 , the cell does not show immediate signs of an adverse response. Only in the image recorded after a waiting period of 69 min do we see evidence of cell death (vacuole seen in panel C). In panel D, we show the corresponding image created using the fluorescence of PI.

Figure 9.

Bright field images of a HeLa cell before and after focused 420 nm laser irradiation of PD. In these experiments, the D₂O-based medium contained 10 mM NaN₃. The domain irradiated, represented by the white spot in the top panel, is ~ 3 μm away from the membrane of the cell. Upon irradiation with a fluence of 40 kJ/cm², the cell does not show signs of an adverse response over a period of up to 55 min after the irradiation. Similar observations were made in experiments performed using longer distances between the laser focus and the cell membrane.

Figure 10.

Bright field images of HeLa cells exposed to extracellular singlet oxygen produced by focused 420 nm laser irradiation of PD. The domain irradiated, represented by the white spot in panel A, is $\sim 8 \mu\text{m}$ away from the membrane of the top cell. Upon irradiation with a fluence of 40 kJ/cm^2 , the cells do not show immediate signs of an adverse response. Only in the image recorded after a waiting period of 90 min do we see evidence of death in the top cell (vacuoles seen in panel E).

Figure 11.

Bright field images of HeLa cells exposed to extracellular singlet oxygen produced by focused 420 nm laser irradiation of PD. The domain irradiated, represented by the white spot in panel A, is $\sim 1.7 \mu\text{m}$ away from the membrane of the cell on the left, whereas it is $\sim 20 \mu\text{m}$ away from the membrane of the cell on the right. Upon irradiation with a fluence of 40 kJ/cm^2 , vacuoles formed on the left cell within 19 min of light dose delivery (arrows in panels C and E). No vacuole formation was observed with the cell on the right over the 248 min period of post-irradiation observation.

Figure 12.

Bright field images of HeLa cells exposed to extracellular singlet oxygen produced by focused 420 nm laser irradiation of PD in D₂O based ABM. The domain irradiated, represented by the white spot in panel A, is ~3.1 μm away from the membrane of the cell at the bottom of the image. Upon irradiation with a fluence of 40 kJ/cm², vacuoles appear to be formed within ~7–8 min of light dose delivery. The images shown have been selected from a sequence recorded using video microscopy.

Figure 13.

Bright field images of HeLa cells exposed to extracellular singlet oxygen produced by focused 420 nm laser irradiation of PD in H₂O based ABM. The domain irradiated, represented by the white spot in panel A, is ~ 2 μm away from the membrane of the cell. Upon irradiation with a fluence of 40 kJ/cm², a small vacuole appears near the cell within ~ 15 min of light dose delivery (arrow in panel C). The images shown have been selected from a sequence recorded using video microscopy.