ACCELERATED MIGRATION AND INVASION OF PROSTATE CANCER CELLS AFTER A PHOTODYNAMIC THERAPY-LIKE CHALLENGE: ROLE OF NITRIC OXIDE

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Abstract

Employing an in vitro model for 5-aminolevulinic acid (ALA)-based photodynamic therapy (PDT), we recently reported that human prostate cancer PC3 cells rapidly and persistently overexpressed inducible nitric oxide synthase (iNOS) and nitric oxide (NO) after a moderate ALA/light challenge. The upregulated iNOS/NO was shown to play a key role in cell resistance to apoptotic photokilling and also in the dramatic growth spurt observed in surviving cells. In the present study, we found that PC3 cells surviving an ALA/light insult not only proliferated faster than non-stressed controls, but migrated and invaded faster as well, these effects being abrogated by an iNOS inhibitor or NO scavenger. Photostressed prostate DU145 cells exhibited similar behavior. Using in-gel zymography, we showed that PC3 extracellular matrix metalloproteinase-9 (MMP-9) was strongly activated 24 h after ALA/light treatment and that MMP-9 inhibitor TIMP-1 was downregulated, consistent with MMP-9 involvement in enhanced invasiveness. We also observed a photostress-induced upregulation of α6 and β1 integrins, implying their involvement as well. The MMP-9, TIMP-1, and integrin effects were strongly attenuated by iNOS inhibition, confirming NO’s role in photostress-enhanced migration/invasion. This study reveals novel, potentially tumor-promoting, side-effects of prostate cancer PDT which may be averted through use of iNOS inhibitors as PDT adjuvants.

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Conflict of interest statement
The authors have no conflicts of interest to declare.

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Keywords
prostate cancer; photodynamic therapy; nitric oxide; cell migration/invasion

Introduction

Photodynamic therapy (PDT) is a unique anti-tumor modality involving a light-absorbing dye or pigment (sensitizer), sensitizer-exciting light (usually in the visible-near red range), and molecular oxygen [1,2]. Energy transfer from triplet excited state sensitizer to ground state oxygen typically results in conversion of the latter to highly reactive singlet molecular oxygen ($^{1}\text{O}_2$), which can irreversibly damage many different target molecules, including nucleic acids, proteins, and unsaturated lipids [2]. A major advantage of PDT over other therapeutic interventions such as chemotherapy and radiotherapy is that photodamage is localized to the solid tumor area itself and does not occur until all three elements (sensitizer, light, $\text{O}_2$) are concurrently engaged. Thus, light alone or sensitizer alone is usually ineffective, nor does it exhibit any significant toxicity to normal tissue. The first FDA approval for PDT in the USA (1996) was for treatment of esophageal cancer, using Photofrin® (porphimer sodium) as the sensitizer. The rapid development of laser and LED light sources along with fiber optics for selective light delivery to solid tumors allowed successful application of PDT for a variety of other cancers, including head-and-neck, bladder, pancreas, and prostate [2]. Steady progress has been made in the case of PDT for prostate cancer, clinical trials achieving significant tumor attenuation with mTHPC (Foscan) as sensitizer [3,4] or 5-aminolevulinic acid (ALA) as pro-sensitizer [5]. PDT is seen as a promising alternative or follow-up to radiotherapy, which often falls short due to tumor resistance or recurrence [3,4]. Another clear advantage of PDT is that it can cause tumor ablation in a focal manner with minimal collateral damage [6].

Cancer cells in many different tumors produce nitric oxide (NO) at sub-micromolar levels to activate signaling pathways leading to evasion of apoptosis on the one hand and promotion of cell proliferation, migration, and metastatic invasion on the other [7–10]. However, NO at relatively high (micromolar range) levels, e.g. produced by resident inflammatory macrophages, can be deleterious to tumor cells after conversion to toxic pro-oxidants such
as peroxynitrite (ONOO⁻) and nitrogen dioxide (NO₂⁻) [11]. In addition to steady state concentration, type of tumor and site of NO generation within a tumor play important roles in pro-tumoral vs. anti-tumoral effects of NO [11,12]. There is growing awareness that endogenous NO can also play a key role in tumor resistance to various therapeutic interventions, including radiotherapy, chemotherapy, and PDT [13–15]. How tumor NO might affect PDT in vivo was first investigated about 15 years ago in studies involving Photofrin®-sensitized PDT in various mouse tumor models [16,17]. It was shown that tumor cure rate could be substantially improved by administering nitric oxide synthase (NOS) inhibitors, the extent of improvement correlating with NO output, tumors with highest constitutive output responding best [17]. The proffered explanation was that NO-mediated dilation of tumor blood vessels acted in opposition to PDT’s known vasoconstrictive effects, and NOS inhibitors suppressed the vasodilation [16,17]. The question of whether other effects of endogenous NO besides vasodilation might play an anti-PDT role was first addressed in the authors’ laboratory about 5 years ago [18,19]. We found that exposure of two breast cancer lines to an ALA-PDT-like challenge caused a rapid and prolonged upregulation of inducible nitric oxide synthase (iNOS) and NO. Moreover, apoptotic photokilling of these cells was strongly enhanced by an iNOS inhibitor, iNOS knockdown, or NO scavenger, implying that iNOS/NO was acting cytoprotectively [18–20]. More recent work showed that prostate cancer PC3 cells responded similarly to ALA/light stress, but with a more profound post-irradiation induction of iNOS/NO, which not only increased photokilling resistance, but stimulated surviving cell proliferation [21]. We now report that ALA/light stress in PC3 cells results in MMP-9 activation, TIMP-1 down-regulation, and accelerated migration/invasion, iNOS/NO playing a key role in each of these responses. These findings raise a serious concern about therapy-enhanced tumor aggressiveness in the PDT setting and point to the importance of considering pharmacologic use of iNOS inhibitors as PDT adjuvants.

Materials and methods

Chemicals, reagents, and antibodies

The following compounds were obtained from Cayman Chemicals (Ann Arbor, MI): (i) N-[3-(aminomethyl)benzyl]acetamide (1400W), a specific inhibitor of iNOS activity; (ii) 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), a NO scavenger; (iii) DETA-NONOate (DETANO), a slow release NO donor (t½ ~20 h at 37 °C); and (iv) a monoclonal antibody against human iNOS. Monoclonal antibodies against human MMP-9, TIMP-1, and TIMP-2 were obtained from EMD Millipore (Bellerica, MA). Cell signaling Technology (Danvers, MA) supplied the monoclonal antibodies against human α6-integrin and β-actin. The antibody against human β1 integrin was from BD Biosciences (San Jose, CA). All other reagents, including ALA, 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), fetal bovine serum (FBS), growth medium, and other cell culture materials were obtained from Sigma-Aldrich (St. Louis, MO).

Cell culture conditions

Human prostate cancer PC3 and DU145 cells were obtained from the ATCC repository (Manassas, VA). Cells were grown under conventional culture conditions, using Dulbecco’s
Modified Eagles’/Ham’s Nutrient F-12 (DME/F12) medium containing 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/ml). Proliferating cells received fresh medium every third day and were passaged fewer than 6 times for all experiments. Additional details were as described previously [21].

Cell sensitization and irradiation

For most experiments, PC3 or DU145 cells at ~40% confluency in 35-mm or 100-mm culture dishes, and in phenol red- and serum-free DME/F12 medium, were metabolically sensitized with protoporphyrin IX (PpIX) by incubating in the presence of 1.0 mM ALA for 30 min in the dark at 37 °C. When an iNOS inhibitor (1400W) or NO scavenger (cPTIO) was used, it was introduced 30 min before ALA and maintained at the same concentration throughout irradiation and post-irradiation incubation. Immediately after ALA treatment, cells were switched to fresh medium either lacking or containing iNOS inhibitor or NO scavenger, and then irradiated on a translucent plastic platform over a bank of four 40-W cool-white fluorescent tubes [22]. The intensity (fluence rate) of light reaching the bottom surface of culture dishes was ~11 W/m², as measured with a YSI radiometer (Yellow Springs, OH). After a pre-determined irradiation period (e.g. 15 min, corresponding to a delivered light fluence of ~1 J/cm²), the medium was replaced with 1% serum-containing DME/F12 (without or with NOS inhibitor or NO scavenger), after which cells were returned to the incubator. At various time points, dishes were removed for determination of parameters such as growth rate (MTT assay), migration rate (gap closure assay), and invasiveness (basement membrane traversal assay). A dark control (ALA without irradiation) was analyzed alongside.

Immunoblot procedures

The effects of ALA/light stress on expression of iNOS, two MMP inhibitors (TIMP-1, TIMP-2), and two integrin subunits (α6, β1) in PC3 cells were assessed by Western blotting. Cells at ~60% confluency in 10-cm culture dishes were treated with ALA as described in Sect. 2.3 and then either not irradiated (dark control) or irradiated (~1 J/cm²). Cell lysates were prepared as described [21,22], analyzed for total protein, then subjected to Laemmli SDS-PAGE, using 15% acrylamide/bis-acrylamide. Separated proteins were transferred to a polyvinylidene difluoride membrane and, after blocking, the membrane was incubated overnight at 4 °C with an iNOS, TIMP1, TIMP-2, integrin α6, or integrin β1 antibody at a supplier-recommended dilution. After washing, the blots were treated with a peroxidase-conjugated secondary antibody and analyzed, using Super Signal West Pico chemiluminescence detection (Thermo Scientific, Rockford, IL). Blots were also probed for β-actin as a loading standard. Other details were as described previously [21].

Analysis of post-irradiation cell proliferation

Proliferation rate of cells surviving a photodynamic challenge was assessed by MTT (thiazolyl blue) assay. PC3 or DU145 cells in 35-mm dishes were treated with ALA alone or ALA and light as described in Sect. 2.3. After irradiation, the cells were washed once, overlaid with 10% FBS-containing DME/F12 medium, and returned to the incubator. At increasing time intervals up to 72 h, the medium was removed and replaced with 1.0 ml of DME/F12 medium containing 0.5 mg MTT/ml. After a 4 h incubation period, cells were
solubilized in 1.0 ml of acidified isopropanol, and formazan absorbance at 563 nm was measured as a numerical indicator of live, proliferating cells.

**Evaluation of post-irradiation cell migration**

The effects of ALA/light stress on migration properties of PC3 and DU145 cells was assessed by a gap closure (also known as “wound healing”) assay, which measures departure of cells from the general population on a flat surface [23]. Cells were seeded in 35-mm dishes and allowed to grow to at least 90% confluency, then sensitized with ALA-generated PpIX as specified in Sect. 2.3. After sensitization, a linear scratch was produced midway across the monolayer, using a sterile 200 μl pipette tip. The cells were then irradiated for 15 min (~1 J/cm² light fluence) in the absence vs. presence of an iNOS inhibitor and placed back in the incubator. At various times up to 48 h, cells in and around the gap region were observed and photographed using a Nikon Eclipse TS100 microscope with CoolSnap ES Photometrics camera and MetaVue software from Molecular Devices (Sunnyvale, CA). Extent of gap closure relative to a dark control and how iNOS inhibition affected this was determined by analysis of representative images at each post-irradiation time point [23]. For each reaction condition, data were obtained from at least six separate experiments.

**Analysis of post-irradiation cell invasiveness**

The invasiveness of PC3 cells before and after a photodynamic challenge was determined using CytoSelect® cell invasion assay units (Cell Biolabs Inc., San Diego, CA), which function similarly to Boyden chambers [24]. Approximately 7.5 × 10⁵ cells were plated in 35-mm dishes and incubated overnight at 37 °C, after which the cells were treated with ALA in serum-free DME/F12 medium, washed, and irradiated (light fluence ~1 J/cm²).

Immediately after irradiation, the cells were recovered by gentle scraping into 1 ml of serum-free medium. After preparation of the invasion chambers according to supplier recommendations, a 0.3 ml portion (~3 × 10⁵ cells) of each cell suspension was added to the upper chamber of an assay unit over a membrane insert with 8 μm pores, and 0.5 ml of 10% FBS-containing medium was added to the lower chamber. After incubation at 37 °C for 48 h, non-invasive cells were removed from the membrane insert of the upper chamber, using a cotton-tipped swab. The chamber with residual cells that had traversed or invaded across the membrane was then placed into 0.4 ml of a cell staining solution and held there for 10 min at room temperature. After rinsing several times with water and drying, the insert was placed into 0.2 ml of CytoSelect® extraction solution and agitated for 10 min. Absorbance of the extract at 560 nm (recorded on a plate reader) indicated the extent of invasiveness.

**Evaluation of matrix metalloproteinase status in photostressed cells**

The activity of matrix metalloproteinase-9 (MMP-9) in PC3 cells before and after an ALA/light insult was determined by gelatin zymography, i.e. an in-gel assay based on separation of the 92 kDa pro-enzyme (zymogen) from ~82 kDa active enzyme and examining the ability of the latter to degrade gelatin in its locale, thus generating a void band in the zymograph [25]. Cells were grown to ~90% confluency in 100-mm dishes, then exposed to ALA and light in the absence or presence of an iNOS inhibitor (1400W) or NO trap (cPTIO), as indicated. Irradiated cells and dark controls were returned to the incubator for...
24 h, after which each conditioned medium was collected, concentrated using a 0.5 ml ultracentrifugal filter (Amicon, Billerica, MA), and examined for total protein using the BCA assay (Pierce Chemical Co., Rockford, IL). A cell sample (~20 μg or protein) was mixed with 2x-sample buffer (Life Technologies, Madison, WI) and subjected to SDS-PAGE, using 10% acrylamide co-polymerized with gelatin (0.1 mg/ml), 175 V, and 90 min running time. After development, the gel was placed in renaturing buffer (50 ml of 2.5% Triton-X100) for 1 h with gentle agitation. After removal of renaturing buffer, the gel was equilibrated with 50 ml of developing buffer (Life Technologies) for 30 min with gentle agitation, then incubated with 50 ml of fresh developing buffer for 48 h at 37 °C. The gel was stained with SimplyBlue Safestain according to supplier instructions (Life Technologies, Carlsbad, CA), then de-stained, photographed, and quantified using ImageLab 4.0 software.

Data analysis

The two-tailed Student’s t-test was used for determining the significance of perceived differences between numerical data, P values <0.05 being considered statistically significant.

Results

Upregulation of iNOS in photostressed PC3 and DU145 cells

In an initial experiment, two androgen-independent human prostate cancer cell lines, PC3 and DU145, were compared for levels of pre-existing iNOS protein and also for ability to upregulate iNOS after treatment with 1 mM ALA for 30 min in the dark, followed by irradiation with broad-band visible light for 15 min (fluence ~1 J/cm²). In a previous study with similarly sensitized PC3 cells [21], ALA-induced PpIX was detected mainly in mitochondria (where it is synthesized), indicating that photodynamic stress originated in the mitochondrial compartment. As shown by the Western blots in Fig. 1A, iNOS was barely detectable in ALA-treated PC-3 cells kept in the dark, but its level increased steadily during post-irradiation incubation, reaching 5-times the control level after 2 h and 8-times after 20 h. Similarly sensitized DU145 cells exhibited a higher constitutive level of iNOS than PC3 cells, but also upregulated the enzyme in response to photostress, although to a lesser extent, the level reaching ~3.6-times that of the dark control after 24 h. Substantial and prolonged iNOS upregulation after an ALA/light challenge has also been observed in COH-BR1 and MDA-MB-231 breast cancer cells [20] and U87 glioblastoma cells (unpublished results), suggesting that this may be a general response of malignant cells to a PDT-like oxidative insult.

It is important to note that highly elevated iNOS observed in PC3 cells 24 h after an ALA/light challenge decayed during additional post-irradiation incubation out to 72 h (Supplementary Fig. 1), suggesting that enzyme upregulation after photostress was of limited duration or was obscured by continuing proliferation, the non-stressed descendant cells expressing very little iNOS.
Induction of a growth spurt in photostressed cells: NO involvement

One of the earliest observed consequences of stress-upregulated iNOS in PC3 cells was increased resistance to photokilling. As shown in Fig. 2A, the viable fraction of ALA/light-treated cells decreased progressively during post-irradiation dark incubation, reaching ~0.6 after 24 h. When 1400W, a high affinity competitive inhibitor of iNOS [26], was present before and after irradiation, the viability loss after 24 h was ~38% greater. Moreover, when cPTIO (a nitronyl nitroxide that scavenges NO with high specificity [27]) was present throughout, the loss of viability after 24 h was even greater, i.e. ~50% more than with ALA/light alone (Fig. 2A). The more extensive post-irradiation cell kill over ~24 h when 1400W or cPTIO was present indicated that iNOS-derived NO was acting cytoprotectivey, most likely by inhibiting apoptosis in some fashion, as observed previously [21]. This NO would have been generated for the most part by stress-induced iNOS rather than pre-existing iNOS (Fig. 1A), which accounted for only ~10% of the total enzyme observed at 20 h after ALA/light. When viable PC3 count was monitored beyond the one day post-irradiation point, a striking observation was made, viz. that surviving cells exhibited a significant growth spurt over at least one additional day. As shown in Fig. 2A, photostressed cells grew ~2.7-times faster than dark (ALA-only) controls from 24 to 48 h. The stressed cell growth rate was reduced to ~1.5-times and ~1.8-times the control rate by 1400W and cPTIO, respectively (Fig. 2A), which implies that iNOS/NO not only increased resistance to photokilling, but stimulated surviving cell proliferation as well. We asked whether the indicated higher growth rates of photostress survivors ± 1400W relative to dark controls might be underestimates because live cell counts of the former at 24 h post-irradiation would have been significantly lower than those of the latter. To correct for this, we adjusted the starting ALA/light and ALA/1400W/light cell titers such that they would coincide with the control titer at 24 h. When cells were sensitized and irradiated under these conditions, their viable levels at 24 h post-irradiation were essentially the same as those of dark controls (as planned), and the ALA/light-treated cells grew 3.5-times faster than controls over the next 24 h, whereas the ALA/1400W/light-treated cells grew only 1.6-times faster (Supplementary Fig. 2). These findings based on “normalized” cell counts confirmed that the growth spurt observed in photostressed PC3 cells was, in fact, mainly dependent on stress-upregulated iNOS and NO.

We found that treating non-stressed PC3 cells with an exogenous source of low level NO could also result in accelerated growth. For this experiment, we used the chemical donor DETA-NONOate (DETA/NO), which decomposes with a half-life of ~20 h at 37 °C, releasing 2 NO per DETA/NO [28]. As shown in Supplementary Fig. 3, cells incubated continuously in the presence of 10 μM DETA/NO exhibited a growth rate enhancement of ~25% relative to non-DETA/NO controls over the first 24 h, and thereafter proceeded at or near the control rate. Completely decomposed DETA/NO had no effect on cell growth (results not shown), confirming that NO was the pro-growth agent. At increasingly higher levels of active donor, growth stimulation eventually subsided, and at 100 μM DETA/NO, growth arrest was observed (Supplementary Fig. 3), suggesting that NO at this higher level elicited anti-proliferative effects, possibly after conversion to reactive nitrogen-oxide species such as peroxynitrite and nitrogen dioxide [29].
As shown in Fig. 2B, DU145 cells that survived ALA/light stress also grew faster than dark controls from 24 to 48 h post-irradiation, the average rate enhancement for this experiment being ~3.2-fold. As with PC3 counterparts, this response was attenuated by 1400W or cPTIO, consistent with an underlying iNOS/NO requirement. In this case, as with PC3 cells, stress-induced iNOS probably played a dominant role, although pre-existing iNOS (Fig. 1B, DC) may have made a larger contribution than in PC3 cells (Fig. 1A, DC).

**Accelerated migration of photostressed PC3 and DU145 cells: role of NO**

Knowing that PC3 and DU145 cells are more aggressive than other prostate cancer lines (e.g. LNCaP) in terms of metastatic properties [30], we asked whether photostress-enhanced PC3 and DU145 proliferation might be accompanied by more rapid cell migration. To test this, we used a gap-closure or “wound-healing” assay [23] in which the migration of ALA/light-stressed cells into a scratch-voided area on a culture dish was monitored as a function of post-irradiation incubation time. As shown by the timed photomicrographs in Fig. 3A with corresponding gap-closure quantification, beginning immediately after irradiation (0 h) and extending to 24 h and 48 h, ALA/light-stressed PC3 cells migrated significantly more rapidly than dark (ALA-only) controls. Individual cells well away from the background populations were observed in the voided area (e.g. ALA/hv at 24 h in Fig. 3A), confirming that this was true migration rather than merely cell proliferation. The accelerated migration (~100% and 55% faster at 24 h and 48 h, respectively) was reduced to nearly the dark control level by 25 μM 1400W, which on its own, had no effect on migration rate. Non-stressed cells treated with 10 μM DETA/NO also exhibited a migration spurt, which slightly exceeded that elicited by photostress-induced iNOS/NO at 24 h and 48 h (Fig. 3A). As shown in Fig. 3B, ALA/light-stressed DU145 cells also migrated more rapidly that dark controls. This response, like that of PC3 counterparts, was nearly abolished by 1400W or cPTIO, thus establishing once again that it was iNOS/NO-dependent.

**Accelerated invasion of photostressed PC3 cells: role of NO**

We asked whether photostress-stimulated mobilization of PC3 cells, as evidenced by greater migration, would also be reflected in greater invasiveness. To assess this, we used relatively simple and improved versions of the originally developed Boyden chamber [24]. Cells in serum-free medium were recovered after an ALA/light challenge and plated onto a membrane insert of an upper chamber, which was inserted into a lower chamber holding serum-containing medium (Fig. 4A). After a 48 h incubation period, cells that had invaded through 8 μm pores in the basement membrane were washed, stained and quantified. As shown in Fig. 4B, PC3 cells treated with ALA, but not irradiated, exhibited a significant degree of background invasiveness over a 2-day period. Non-ALA-treated cells behaved identically (results not shown), in agreement with the constitutive invasiveness of PC3 cells observed by other investigators [31]. After exposure to ALA and 1 J/cm² light fluence, the cells invaded much more aggressively, increasing to ~150% of the control level over a 48 h period (Fig. 4B). When present before and after the photochallenge, 1400W resulted in a significant reduction in invasiveness to ~112% of the control value. Note that the plotted values represent live cells under the different reaction conditions, i.e. after correcting for proportion of dead cells at 48 h. It is clear from these results that photostress-induced iNOS/NO significantly enhanced the invasion aggressiveness of PC3 cells. This raises the
concern that a similar escalated breach of the extracellular matrix (ECM) during in vivo PDT could foster metastatic tumor progression.

**NO-dependent MMP-9 activation and TIMP-1 down-regulation in photostressed cells**

Matrix metalloproteinases (MMPs) are Zn$^{2+}$-containing endopeptidases that play key roles in cell mobilization and migration by catalyzing the degradation of basement membranes and ECMs. It is well known that certain MMPs are intimately associated with the invasive and metastatic properties of many different human cancers, including prostate cancers [32,33]. For example, MMP-2 and MMP-9 (also known as gelatinases) have been observed in prostate tumors, high levels of expression/activity correlating with poor prognosis [34]. Focusing on MMP-9, the major MMP of PC3 cells [34], we examined its activity status 24 h after an ALA/light challenge in the absence vs. presence of 1400W or cPTIO, using a gel zymographic assay. As shown in Fig. 5A, representing a light fluence of 1 J/cm$^2$, two voided “bands” were observed for each condition, the upper reflecting pro-MMP-9 (~92 kDa) and the lower, active MMP-9 (~82 kDa). Note that the lower band was more intense in the ALA/light sample than in the ALA-only control, quantification indicating a 2.2-fold greater activity in the former (Fig. 5A, lower panel). When 1400W or cPTIO was present during and after photochallenge, MMP-9 activity was reduced to nearly the control level, clearly implicating stress-induced iNOS/NO in the observed MMP-9 hyper-activation. A NO-dependent increase in MMP-9 activation was also observed after using a 2 J/cm$^2$ fluence (Fig. 5C), although this was only ~60% of that produced by 1 J/cm$^2$. A small increase was also seen when 0.5 J/cm$^2$ was used (Fig. 5B), but this was barely significant. Clearly, therefore, the maximal response occurred at or near 1 J/cm$^2$, which was used in all other experiments in this study. There is evidence that exogenous low level NO can activate MMP-9 in certain cells, possibly by disrupting the Zn$^{2+}$-cysteine thiolate bond in the pro-enzyme, thus liberating Zn$^{2+}$ for catalytic activity [35–37]. This might account for the apparently greater activity observed for the upper (pro-enzyme) zone in the Fig. 5A zymograph (ALA/hv lane). However, most of the NO-enhanced activation that we determined was associated with the lower MMP-9 zone, i.e. the proteolytically cleaved pro-enzyme. It is important to point out that Western analysis revealed that the total level of MMP-9 in PC3 cells remained the same as that of a dark control over a 24 h post-irradiation period (results not shown), ruling out any protein induction as a contributing factor in hyper-activation.

Activities of various MMPs are known to be regulated by endogenous polypeptide inhibitors known as tissue inhibitors of metalloproteinases (TIMPs), TIMP-1 having the highest affinity for MMP-9 [38]. We used Western blotting to determine whether ALA/light stress might alter the expression status of TIMP-1 in PC3 cells and if so, how NO might impact this. As shown in Fig. 6A, cells treated with ALA, but not irradiated, exhibited a strong TIMP-1 band as well as TIMP-2 band. However, after ALA/light treatment, there was a dramatic decline in TIMP-1 protein level, which reached 15–20% of the control level after 2 h and remained there for at least 22 h longer. TIMP-2 expression was reduced after photostress, but not as dramatically as TIMP-1. A striking reversal of the TIMP-1 decline after photostress was observed when 1400W was present in the photoreaction system, nearly 80% of the protein now persisting throughout post-irradiation incubation; 1400W also
inhibited the decline in TIMP-2 expression (Fig. 6B). Thus, in similar fashion to macrophages exposed to exogenous NO at low fluxes, as reported previously [36], stress-upregulated NO in our system reciprocally modulated MMP-9 activity and inhibitory TIMP-1 expression. Whether an indirect NO effect (TIMP-1/2 downregulation) or a direct effect (disruption of the Zn$^{2+}$-cysteine bond in the pro-enzyme; see above) was more important in photostress activation of MMP-9 is not clear at this point, nor is it clear why more than one NO-based mechanism might be necessary.

**NO-dependent integrin upregulation in photostressed cells**

Integrins play an additional crucial role in the mobilization, migration, and vascular system invasion of many cancer cells [39,40]. In the case of aggressive prostate cancers, many integrin subtypes are downregulated, but α6 and β1 integrins (the subunits of functional α6-β1 dimer) are unique in being upregulated, suggesting a crucial role in invasion/metastasis [31,41]. Consequently, we asked how ALA/light stress might affect the expression status of α6 and β1. As shown by the Western blots in Fig. 7A (upper panel), there was a low constitutive level of α6 subunit in dark control cells (ALA-only), but after ALA-treatment and irradiation, the level rose dramatically during post-irradiation incubation, reaching >8-times the control after 24 h. Likewise, the level of β1 subunit increased steadily in the post-irradiation period, but to a much lesser extent than that of α6 subunit. Importantly, overexpression of α6 and β1 in stressed cells, like TIMP-1/2 overexpression and MMP-9 activation, exhibited NO-dependency, as demonstrated by the strong suppressive effects of cPTIO (Fig. 7A and 7B, lower panels). Highly relevant to these findings is recent evidence that exogenous NO or transfected iNOS caused site-specific S-nitrosation of α6 integrin in PC3 cells, which increased α6 association with β1, leading to greater migration/invasion [42].

**Discussion**

This is the first study to demonstrate that tumor cells which survive a PDT-like oxidative insult become more aggressive in terms of accelerated migration and invasion through an ECM-simulating basement membrane. Our findings are not only novel in the area of PDT, but appear to be so for any type of anti-tumor treatment involving oxidative stress. Two human prostate cancer lines, PC3 and DU145, exhibited increased motility after moderate ALA/light-induced photostress, and both responded with greater proliferation rates as well, which were observed 24–48 h after the stress was incurred. iNOS and NO were shown to play a major role in each of these responses by virtue of the strong suppressive effects of iNOS inhibition and NO scavenging. The effects with PC3 cells are particularly notable because they depended almost entirely on stress-induced iNOS, its constitutive level being barely detectable in these cells, as observed previously [21]. The induced enzyme persisted for at least 20 h after ALA-treated cells were irradiated. DU145 cells also upregulated iNOS under photostress, but less so relative to controls than PC3 cells due to more pre-existing enzyme in DU145 cells. We found that the NO-dependent growth and migration spurt in photostressed PC3 cells could be recapitulated by exposing naïve cells to a very low flux of NO from an exogenous source, 10 μM DETA/NO (Fig. 3, Supplementary Fig. 2), suggesting that low flux NO generated in tumor surroundings, e.g. by vascular endothelial cells, might
promote basal motility of tumor cells or possibly augment the effects of photostress. Interestingly, however, 100 μM DETA/NO did not further stimulate PC3 growth/migration, but rather strongly repressed it, which illustrates the dichotomy in tumor cell responsiveness to NO concentration (29) and why PC3 cells exploit only low level NO for a survival advantage.

In examining some of the possible participating proteins in accelerated migration and invasion, we found that gelatinase MMP-9 was substantially more active in ALA/light-stressed PC3 cells than in non-stressed controls. This hyper-activation maximized at a light fluence of ~1 J/cm² and applied exclusively to pro-MMP-9/MMP-9 found in the extracellular compartment, i.e. in the post-hv or control conditioned medium collected and concentrated for gel zymography. Western analysis revealed that cellular pro-MMP-9 level remained unchanged over 24 h after ALA/light stress (results not shown), ruling out any protein upregulation as a possible contributing factor in increased MMP-9 activity. Photostress-elevated MMP-9 activity was strongly suppressed by 1400W or cPTIO, consistent with iNOS/NO involvement. Moreover, 1400W also inhibited stress-induced down-regulation of TIMP-1 and TIMP-2, the known natural inhibitors of MMP-9 and other MMPs [38]. Latent MMP-9 contains Zn²⁺ in its active site domain which interacts with a cysteine thiolate in the pro-domain, the so-called “cysteine switch” [43]. Disruption of this interaction by proteolytic release of the pro-domain gives active enzyme, but disruption by NO (possibly via N₂O₃-induced S-nitrosation) has been reported as an alternate mechanism for macrophages and other NO-generating cells [36]. Regarding NO’s role in photostress activation of MMP-9, it is unclear whether direct action on the pro-MMP-9 cysteine switch or indirect action via down-regulation of TIMP-1/2 might have been more important. A similar question arose in a study dealing with the effects of exogenous NO on MMP-9 activation and TIMP-1 expression in bone marrow macrophages [36]. It was found that loss of TIMP-1 expression occurred at far lower NO levels than MMP-9 activation, suggesting that the indirect effect is a more sensitive means of up-regulating MMP-9 activity. Whether this might also apply to the system we describe remains to be investigated, and also whether the TIMP-1 down-regulation that we observed was cGMP-dependent, as reported for macrophages [36].

In addition to MMP-9 hyperactivation and TIMP-1/2 down-regulation, we observed an up-regulation of integrin α6 and β1 subunits in photostressed PC3 cells, and once again the response was iNOS/NO-dependent. It has been proposed that integrins such as α6-β1 dimer may interact with other migration-facilitating partners such as MMP-9, urokinase-plasminogen activator and its receptor (uPA/uPAR), and other plasma membrane proteins to form a dynamic complex termed an “invadosome” [33]. It has been postulated that this complex is short-lived and assembles only when tumor cells under a migration/invasion stimulus signal for interaction with and dismantling of ECM proteins [33]. While photostress-induced NO somehow stimulated integrin expression, it might also have activated integrin(s) for migration/invasion, e.g. via S-nitrosation of crucial cysteine residue(s). The latter possibility is suggested by recent evidence that migration and invasion of PC3 and DU145 cells could be enhanced by iNOS transfection or treatment with GSNO, a transnitrosating agent [42]. The observed motility enhancement, along with greater α6 and

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β1 dimerization, and weakened cell adhesion to a laminin-1 matrix was attributed specifically to S-nitrosation of Cys86 on integrin α6 [42].

In summary, PC3 and DU145 cells that survived a PDT-like challenge were found to be more aggressive in terms of accelerated proliferation, migration, and invasion. This transformation was accompanied by greater activation or decreased/increased expression of key regulatory proteins such as MMP-9, TIMP-1, and α6 integrin. It should be stressed that in identifying iNOS/NO involvement in these photostress responses, we focused almost exclusively on natural intracellular iNOS/NO as opposed to exogenous NO or transfected iNOS employed in many other recent studies dealing with NO’s tumor-supporting properties. Thus, our findings are more realistic with regard to iNOS/NO involvement, particularly in the context of PDT as an anti-tumor modality. That conventional PDT (including ALA-PDT) for prostate and other malignancies might promote NO-dependent residual tumor aggressiveness is an alarming prospect, and suggests that iNOS inhibitors should be seriously considered as pharmacologic PDT adjuvants. At least two such inhibitors have the advantage of having already been tested in clinical trials, albeit unrelated to cancer or PDT [44,45].

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**References**

iNOS is strongly induced in ALA-PDT-stressed prostate cancer PC3 and DU145 cells.

Photostress stimulates growth, migration and invasion of surviving cells.

iNOS inhibition or NO scavenging suppresses stimulated growth, migration and invasion.

Enhanced cell aggressiveness can compromise clinical PDT effectiveness.

iNOS inhibitors are proposed as pharmacologic PDT adjuvants.
Fig. 1.
Effect of ALA/light-induced stress on iNOS protein level in prostate cancer cells. (A) PC3 cells were dark-incubated with 1 mM ALA for 30 min, switched to ALA-free medium, exposed to a 1 J/cm² light fluence, and then recovered for analysis – either immediately after irradiation (0 h) or after increasing periods of dark incubation up to 20 h. ALA-treated cells kept in the dark throughout were used as controls (DC). Cell lysates were prepared, analyzed for total protein concentration, then probed for iNOS and β-actin levels by Western blotting, the latter protein serving as a loading standard. Numbers below lanes represent iNOS band intensities relative to β-actin and normalized to DC. (B) DU145 cells were treated with ALA, irradiated, and analyzed similarly to PC3 cells. Total protein per lane: 100 μg (A, B).
Fig. 2.
Post-photostress survival and proliferation characteristics of two prostate cancer cell lines: effects of an iNOS inhibitor and NO trap. (A) PC3 cells at ~40% confluence were subjected to an ALA/light challenge (1 J/cm²) in the absence or presence of 25 μM 1400W (W) or 25 μM cPTIO (cP). An ALA-only dark control (DC) was studied alongside. At various post-irradiation times up to 72 h, the viable cell content relative to that at time-0 was determined by MTT assay. (B) DU145 cells were treated with ALA and either kept in the dark (DC) or irradiated (1 J/cm²) in the absence vs. presence of 25 μM 1400W or 25 μM cPTIO, and then analyzed for viable cell content at various post-irradiation times. Means ± SE of values from at least 3 separate experiments are plotted in (A) and (B).
Fig. 3. Migration of photostressed prostate cancer cells: effects of an iNOS inhibitor and NO scavenger. (A) PC3 cells at >90% confluency in 35-mm dishes were pre-incubated with ALA and then either not irradiated (ALA) or irradiated in the absence (ALA/hv) or presence of 25 μM 1400W (ALA/W/hv). Immediately after irradiation, a straight scratch was made across each cell layer using a 200 μl pipette tip, the dark control being treated likewise. After microscopic images of the initial gap area were recorded, the plates were returned to the incubator; subsequent images were recorded 24 h and 48 h later (upper panel). The extent of gap closure at each time point was determined using the following relationship: (time-0 gap - time-t gap)/time-0 gap (lower panel). The effects of NO from an exogenous source (10 μM DETA/NO) on cell migration in the absence of photostress were examined similarly. (B) DU145 cells were examined for ALA/light-enhanced migration and iNOS/NO dependency thereof similarly to PC3 cells. Effects of cPTIO (25 μM) as well as 1400W (25 μM) on gap closure rate were assessed. Plotted values in (A) and (B) are means ± SD of measurements from at least 6 replicate experiments.
Fig. 4.
Invasiveness of photostressed PC3 cells: effects of iNOS inhibition. Immediately after an ALA/light challenge (1 J/cm\(^2\)) in the absence vs. presence of 25 μM 1400W, cells were recovered into serum-free medium and tested for invasiveness, using CytoSelect® assay modules with basement membrane inserts. A dark control (ALA) was studied alongside as a reference. Invasive cells (i.e. those degrading matrix proteins in the inserts and traversing 8 μm pores over a 48 h incubation period in response to 10% serum-containing medium in lower chambers) were recovered, stained, and quantified. (A) Scheme showing steps used in invasion assays. (B) Extent of invasion −/+ 1400W relative to the dark control. Plotted values are means ± SE of data from 3 replicate experiments, and were corrected for non-viable cells under each reaction condition. *P<0.01 relative to ALA; **P<0.05 relative to ALA/hv.
Fig. 5. MMP-9 activation in photostressed cells: effects of an iNOS inhibitor and NO trap. PC3 cells at ~90% confluence in 10-cm dishes were pre-incubated with ALA in the absence or presence of 1400W or cPTIO. When used, 1400W and cPTIO were maintained at 25 μM throughout, i.e. before, during, and after irradiation (24 h). Light fluences of 0.5, 1.0, and 2.0 J/cm² were used. An ALA-only dark control served as a reference. After 24 h of post-irradiation incubation, conditioned media were collected, concentrated, analyzed for protein content, and then subjected to SDS/gelatin zymography. After protein re-naturation, the gel was stained and voided zones representing sites of proteolysis by MMP-9 and pro-MMP-9 were quantified. (A) Gel zymograph and corresponding MMP-9 activity profile for a 1 J/cm² fluence. Plotted values are means ± SE (n = 3). *P<0.01 relative to ALA; #P<0.005 relative to ALA/hv. (B) MMP-9 activities for 0.5 J/cm². (C) MMP-9 activities for 2.0 J/cm². *P<0.05 relative to ALA; #P<0.05 relative to ALA/hv.
TIMP-1 and TIMP-2 down-regulation by ALA/light stress: role of iNOS/NO. ALA-treated PC3 cells were irradiated (1 J/cm²) in the absence or presence of 25 μM 1400W. Immediately thereafter (0 h) or after increasing periods of dark incubation from 2 h to 24 h, cells were recovered, lysed, and homogenized. After protein determination, samples were subjected to Western analysis, using antibodies against TIMP-1, TIMP-2, and β-actin. Dark controls (DC), i.e. ALA-treated cells kept in the dark for 24 h, are also represented. Total protein per lane: 75 μg. Number below each lane represents band intensity normalized to β-actin and relative to DC. Blots are from one experiment representative of three for each condition.

Fig. 6.

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Induction of α6 and β1 integrins by ALA/light stress: effects of an NO scavenger. ALA-treated cells were either kept in the dark for 24 h (DC) or irradiated (1 J/cm²) in the absence (ALA/hv) or presence (ALA/cP/hv) of 25 μM cPTIO. After 0, 2, 5, 18, and 20 h of post-irradiation dark incubation, cell lysates were prepared and analyzed for iNOS by Western blotting, using β-actin as an internal standard; total protein per lane: 75 μg. Band intensities normalized to β-actin and relative to DC are indicated below the various time points. Data are from one experiment representative of two with similar results.

Fig. 7.