Photodynamic Therapy Causes Cross-linking of Signal Transducer and Activator of Transcription Proteins and Attenuation of Interleukin-6 Cytokine Responsiveness in Epithelial Cells

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ABSTRACT

Photodynamic therapy (PDT) is a local treatment of cancers. The principle of PDT is the production of reactive oxygen species, in particular singlet oxygen, by light activation of a photosensitizer introduced into the target cells. The direct photochemical and subsequent redox reactions can lead to cell death. This study sought to identify effects occurring during PDT and some of their consequences in surviving cells. Using epithelial cells in tissue culture and in tumors, several distinct PDT-mediated reactions were found, including global dephosphorylation of proteins, induced phosphorylation of a 71-kDa protein, initiation of cellular stress responses, structural modification and loss of epidermal growth factor receptor, and cross-linking of proteins. Specific covalent cross-linking of nonactivated signal transducer and activator of transcription (STAT)-3, and to a lesser extent of STAT1 and STAT4, correlated with PDT dose. Cross-linked STAT3 was primarily localized to the cytoplasm and failed to bind to DNA. The combination of STAT cross-linking and inactivation of receptor functions rendered PDT-treated cells refractory for at least 24 hours to interleukin-6 and oncostatin M, cytokines known to be elevated at sites of tissue damage and inflammation. It is suggested that the loss of responsiveness to these inflammatory cytokines in the PDT-treated field assists tumor cells in evading the growth-suppressive activity of these mediators expected to be present at tissue sites after PDT.

INTRODUCTION

Photodynamic therapy (PDT) is designed to eliminate abnormal tissue lesions, including tumors (1). Although it is desired to kill target cells either directly by the photochemical reactions or indirectly by PDT-induced secondary responses, invariably, a fraction of the malignant and normal cells within or adjacent to the treatment field escape the lethal PDT effects. In the case of cancer treatment, the surviving tumor cell population contributes to the recurrence of the cancer. A survival process demands that the cells exposed to nonlethal PDT are able to repair cellular damages and eventually to resume proliferation.

Molecular studies of PDT damage have focused largely on the mechanisms that explain necrosis and apoptosis of treated cells (2–5). The relative levels of PDT effects may determine the difference between lethal and nonlethal outcome, however, the biochemical nature of these effects is not well understood. We and others have identified PDT-dependent modifications of cellular proteins and have evaluated the consequence of these modifications on the functions of these proteins. The range of modifications includes oxidation, adduct formation, protein cross-linking, and degradation (6–11). Particularly noted was the loss of enzymatic activities due to redox reactions such as found for phosphatases (12) and kinases (6, 13–15). The PDT-dependent loss of immunodetectable epidermal growth factor (EGF) receptor (EGFR) appeared as one of the prominent effects of PDT in various epithelial cell types (6, 14). To gain a more comprehensive picture of the processes initiated by PDT and to identify potential quantitative markers for immediate PDT damage, we determined in cells in culture and growing as tumors in vivo the effects of PDT on profiles of protein modification and signaling functions.

The study indicates several novel responses that define PDT action: (1) increased dephosphorylation of tyrosine-phosphorylated proteins, (2) stimulation of a PDT-sensitive protein tyrosine kinase activity that is detectable after inhibition of tyrosine phosphatases, and (3) a highly specific cross-linking of nonactivated signal transducer and activator of transcription (STAT)-3 and, to a lesser extent, STAT1 and STAT4. The modification of STAT proteins, together with the attenuated signaling by the cytokine receptors, correlates with reduced cellular responsiveness to interleukin (IL)-6 cytokines.

MATERIALS AND METHODS

Cells. The following cell lines were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and antibiotics: FaDu (human hypopharyngeal carcinoma), A549 (human lung carcinoma), H-35 (rat hepatoma), Colo26 (mouse colon carcinoma), and COS-1 (green monkey kidney epithelial). Human prostate carcinoma PC3 cells were maintained in RPMI 1640 containing 10% fetal calf serum. Human pulmonary macrophages were isolated from residual lung tissue obtained from Roswell Park Cancer Institute Tissue Procurement Service under the approved Institutional Review Board protocol CIC 00-17. Macrophages were mechanically extracted from minced lung tissue and purified by Histopaque gradient centrifugation (Sigma Chemical Co., St. Louis, MO). The cells (1 x 106 per mL) were suspended in RPMI 1640 containing 10% fetal calf serum and 1 µg/mL lipopolysaccharides. Conditioned medium was collected after 12 hours of incubation.

PDT Treatment. Cells at ~90% confluence were incubated for 4 hours at 37°C with growth medium containing 0 to 1 mmol/L aminolevulinic acid (16) or 0 to 1.2 µmol/L 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH; ref. 17), or for 15 minutes with PBS containing 0 to 1.5 µmol/L 2-carboxy-3,4,5,6-tetrachlorophenol)-2,4,5,7-tetraodio-3,6-dihydroxyxanthylum-dipotassium salt [Rose Bengal (RB); Sigma Chemical Co.; ref. 18]. Where necessary, advance treatment for 2 hours with 1 mmol/L sodium vanadate or for 15 minutes with 100 ng/mL oncostatin M (Amgen Inc., Seattle, WA) or EGF (Invtrogen Corp., Carlsbad, CA) was initiated by adding these reagents to the medium containing the photosensitizer. Medium was replaced with fresh, photosensitizer-free medium containing 10% fetal calf serum and the cytokines or EGF necessary to maintain signaling during the light-induced photoreactions. Light treatments were carried at room temperature by applying the following conditions: aminolevulinic acid-PDT used a halogen light at 400 to 700 nm and was delivered at a fluence rate of 18 mW/cm² to a fluence ranging from 1 to 10 J/cm²; RB-PDT used the same light delivered to a fluence ranging from 0.5 to 1.5 J/cm²; and HPPH-PDT used a red-filtered arc light at 590 to 700 nm with a fluence rate of 14 mW/cm² and fluence ranging from 0.5 to 8 J/cm². All experimental series included controls consisting of cultures that were incubated with photosensitizer but not exposed to light. Immediately after PDT or after various length of time in growth medium and treatments with cytokines or EGF, the cells were washed with PBS and processed one of two ways. The cells were lysed in the culture dish with radioimmunoprecipitation assay buffer for analysis of protein expression by immunoblotting, or the cells were extracted with high-salt buffer [0.4 mol/L NaCl, 20 mmol/L 4-(2-

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hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.6), 20 mmol/L NaF, 1 mmol/L Na3PO4, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L diethylo-
tiol, 20% glycerol, 1 mmol/L sodium orthovanadate, 1 μmol/L leupeptin and
aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride for measuring DNA
binding activity of STATs by electrophoretic shift assay. Most PDT exper-
iments also included cultures that were subjected to the treatment and were used
24 hours later to determine the effect of PDT on cell survival. Adherent cells
were washed free of material derived from dead cells with PBS and then were
released by trypsination. Viable cells were counted based on trypan blue dye
exclusion in a hemocytometer.

Electrophoretic Mobility Shift Assay. To determine DNA-binding activ-
ity of STAT1 and STAT3, aliquots of whole-cell or nuclear extracts containing 5
to 12 μg of proteins were reacted with a [32P]-labeled double-stranded
oligosaccharide representing the m67 sis-inducible element and analyzed for
protein interaction by electrophoresis as described previously (19, 20). Incu-
bation of extracts with anti-STAT3 (C20; Santa Cruz Biotechnology) before
electrophoresis identified the presence of STAT3 in the sis-inducible element
complexes by supershifting.

PDT-MEDIATED MODIFICATION OF STATS

RESULTS

PDT Causes Several Immediate Biochemical Reactions. Be-
cause biological consequences of PDT often are only evident hours
after treatment (22), we asked what cell reactions describe the initial
PDT effects in epithelial cells. To broaden the assessment, we also
determined the effects of PDT on signaling by the receptors for
oncostatin M and EGF, two factors that prominently act on normal
and on many transformed epithelial cells. We used the human hypop-
opharyngeal cell line FaDu as an experimental system because this line
expresses abundant amounts of receptors for oncostatin M, IL-6,
leukemia inhibitory factor, and EGF and responds to these factors
comparably with normal epithelial cells (6). We examined two sec-
donerationphotosensitizers that currently are in clinical trials: the
drug aminolevulinic acid that is converted into the endogenous
photosensitizer protoporphyrin IX (16), and the exogenous photosen-
sitizer HPPH (17). Both agents are believed to target primarily mito-
chondria. To evaluate the relationship of subcellular distribution of the
photochemical reaction and pattern of outcome, we also treated cells
with RB, a more hydrophilic cationic photosensitizer that shows a
broad cytoplasmic and membrane distribution (18).

The PDT effects were assessed by determining the level of phos-
phorylation of total cell proteins, expression of receptors, and modi-
fication of downstream signaling proteins. Key PDT responses are
summarized by the representative experiment shown in Fig. 1. Data
from more than 30 independent series of PDT treatments of FaDu
cells support the stated findings. The results, in part, confirmed
reactions reported previously (6, 14). Using an aminolevulinic acid–
PDT dose that led to ~60% killing of the cells during a 24-hour
post-treatment period, five distinct immediate PDT effects were con-
sistently observed. (1) EGFR changed to a slower electrophoretic
mobility. (2) The level of EGFR protein was reduced. (3) Tyrosine
phosphorylation of many cellular proteins was decreased. Because the
basal level of protein tyrosine phosphorylation in FaDu cells was low,
a longer ECL exposure of the immunoblot was necessary to reveal this
PDT effect (Fig. 1, left panel at the top). The loss of phosphorylation
also was associated with a lower action of EGFR and oncostatin M
receptor, as was evident by the reduced phosphorylation of EGFR,
PDT-MEDIATED MODIFICATION OF STATS

The analysis of different STAT proteins in FaDu and other epithelial cell types, including A549 lung epithelial cells that have a particularly high level expression of STAT1 and STAT3, indicated that PDT-mediated STAT cross-linking was not restricted to FaDu cells. Most effectively cross-linked was STAT3, less was STAT1, and not appreciably was STAT5 (Fig. 2C, left panel). When cells were pretreated with oncostatin M to increase tyrosine phosphorylation of STAT3 and STAT1, only a minimal amount of phosphorylated STAT3 and none of phosphorylated STAT1 were detectably associated with cross-linked STAT proteins (Fig. 1; data not shown). PDT treatment of the human prostate cell line PC3, which lacks STAT3, did not result in the appearance of protein forms that reacted with anti-STAT3 (data not presented). This finding ruled out the possibility that an epitope was generated by the PDT reaction that cross-reacted with anti-STAT3 but was unrelated to STAT3.

Fig. 1. PDT mediates several types of immediate cell reactions. FaDu cells were treated with 0.8 mmol/L aminolevulinic acid for 4 hours. Where indicated at the top, pretreatments with 1 mmol/L sodium vanadate (2 hours) and EGF or oncostatin M (OSM, 15 minutes) were carried out. Cells were subjected to light treatment (10 J/cm²) and lysed immediately after PDT. A duplicate set of control cells, with and without PDT treatment, was maintained for an additional 24 hours in culture to determine the percentage of viable cells (40% in this example). Equal amounts of cell lysates were analyzed by immunoblotting for the antigens indicated at the left. The ECL exposure of the film was limited by the patterns with highest signal. Longer exposures permitted identification of changes in samples with low immune signals. PDT-mediated changes in basel level tyrosine-phosphorylated proteins are demonstrated by the 45-minute ECL exposure of the control samples at the left of the top panel (WB: PY). Conversely, the reduction in immunodetectable monomeric STAT3 due to cross-linking is shown by a 1-second ECL exposure of the control samples at the left of the third panel (WB: STAT3). Position of the nonmodified antigens, the cross-linked complexes, and the molecular weight markers are indicated at the right. The open arrow at the top panel points to the position of the 71-kDa tyrosine-phosphorylated protein detected in PDT-treated cells, the phosphaese activities of which were inhibited by vanadate. Circles in the phosphotyrosine-STAT3 (PY-STAT3) patterns indicate the position of cross-linked STAT3 complexes containing phosphorylated STAT3.

The analysis of stress mitogen-activated protein kinases was restricted to JNK. Under condition of vanadate-inhibited phosphatase activities, a PDT-dependent tyrosine phosphorylation of a 71-kDa protein was detected. This protein, the identity of which remains to be established, was also a target for phosphorylation by EGFR signaling. The vanadate treatment also helped to maintain the receptor-mediated phosphorylation of many, but not all, signaling proteins during PDT. (5) Specific cross-linked products were evident in addition to a low level “nonspecific” cross-linking of proteins that appeared as smears of immunodetectable proteins at the higher molecular size region. Of note was the shift of the nonphosphorylated, but not the oncostatin M–activated (phosphorylated), STAT3 to discrete slower mobility forms (labeled as I, II, and III). Due to the strong immunoblot signal for STAT3, the proportional reduction of non-cross-linked, monomeric STAT3 proteins was only evident on short ECL exposures of immunoblots (Fig. 1, panel at the left of the third immunoblot). A cross-linking of the EGFR dimer was also detectable in EGF-treated and PDT-exposed cells. Because of the critical role of STAT3 in mediating the signaling of cytokines and growth factors, the specificity of STAT3 cross-linking prompted an additional characterization of its process and its consequence for cellular receptor functions in surviving cells that rely on STAT3.

Cross-linking of STAT Proteins Is a Function of the PDT Photoreaction. Cross-linking of STAT3 was proportional to the photosensitizer dose (Fig. 2A) and fluence (Fig. 2B, two left panels) for aminolevulinic acid–PDT and HPPH-PDT. Both PDT regimens generated qualitatively similar conversion into forms migrating with apparent mass of 178 kDa (form I), 190 kDa (form II), and 200 kDa (form III). Maximal conversion of monomeric to cross-linked STAT3 was with aminolevulinic acid–PDT ~15%. HPPH-PDT resulted in a consistently higher conversion of ~30%. Although aminolevulinic acid–PDT and HPPH-PDT were targeted to mitochondria, the reactive intermediates seemed to reach the cytoplasmic sites where STAT3 resides. By using RB, a cross-linking was obtained that on average was 1.5-fold higher than with HPPH-PDT (Fig. 2B, right panel). PDT reactions, which produced maximal STAT3 cross-linking, led to killing of FaDu cells ranging from 80 to 90% (aminolevulinic acid–PDT) to ~95% (HPPH) and 100% (RB) when checked for viable cells 24 hours after PDT (see also ref. 6). Interestingly, although somewhat variable from experiment to experiment and among the photosensitizers used, half-maximal cross-linking of STAT3 by the PDT reaction correlated with killing 40 to 90% of the cultures. Thus, the relative level of STAT3 cross-linking appeared to be an indicator of the severity of the photoreaction. Moreover, PDT conditions could be chosen that yielded a sufficient number of surviving cells that permitted determination of the relationship of STAT cross-linking and STAT function.

(4) The level of phosphorylated (activated) c-jun-NH₂-terminal kinase (JNK) was increased. Activation of the p38 stress mitogen-activated protein kinase was also observed in FaDu cells (see ref. 6), however, the available antibodies yielded only a weak and often close to nondetectable immunoblot signal for p38. Thus, the analysis of stress mitogen-activated protein kinases was restricted to JNK. Under condition of vanadate-inhibited phosphatase activities, a PDT-dependent tyrosine phosphorylation of a 71-kDa protein was detected. This protein, the identity of which remains to be established, was also a target for phosphorylation by EGFR signaling. The vanadate treatment also helped to maintain the receptor-mediated phosphorylation of many, but not all, signaling proteins during PDT. (5) Specific cross-linked products were evident in addition to a low level “nonspecific” cross-linking of proteins that appeared as smears of immunodetectable proteins at the higher molecular size region. Of note was the shift of the nonphosphorylated, but not the oncostatin M–activated (phosphorylated), STAT3 to discrete slower mobility forms (labeled as I, II, and III). Due to the strong immunoblot signal for STAT3, the proportional reduction of non-cross-linked, monomeric STAT3 proteins was only evident on short ECL exposures of immunoblots (Fig. 1, panel at the left of the third immunoblot). A cross-linking of the EGFR dimer was also detectable in EGF-treated and PDT-exposed cells. Because of the critical role of STAT3 in mediating the signaling of cytokines and growth factors, the specificity of STAT3 cross-linking prompted an additional characterization of its process and its consequence for cellular receptor functions in surviving cells that rely on STAT3.

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Cross-linked STAT Complexes Are Primarily Cytoplasmic. In FaDu cells treated with aminolevulinic acid–PDT, HPPH-PDT, or RB-PDT, only trace amounts of cross-linked STAT3 and none for STAT1 were detected in the nuclear fraction. In cell lines with high levels of STAT proteins, such as A549, the PDT reaction caused a conversion of up to 70% of STAT3 epitopes appearing in cross-linked complexes (Fig. 2C, center panel). In RB-PDT–treated A549 cells, cross-linked STAT3 was also more abundant in the nuclear fraction (Fig. 2C, right panel). Of note is that the amount of cross-linked STAT3 relative to monomeric STAT3 in nuclei was 2–5-fold lower than in the cytoplasmic fraction from the same cells. The cross-linked nuclear STAT3 failed to react with antibodies against tyrosine phosphorylated STAT3. These results suggest that cross-linking of STAT3 is mainly a cytoplasmic event with the cross-linked STAT proteins remaining largely localized to the cytoplasm and/or that reactive oxygen species, which are responsible for cross-linking, did not efficiently reach the nuclear compartment (23).

The relatively high level of cross-linked STAT3 complexes as found in RB-PDT–treated A549 cells allowed us also to determine whether cross-linked STAT3 gained DNA-binding activity, such as to the high-affinity substrate sis-inducible element. Such a DNA-binding activity was found with STAT3 dimers generated by oncogen transactivation through phosphorylation or with nonphosphorylated dimers formed by the A661C and N663C mutations in STAT3-C (24). However, electrophoretic mobility shift assay analysis with extracts from RB-PDT–treated A549 cells indicated no PDT-induced sis-inducible element-binding by STAT3 (Fig. 2D). In fact, PDT lowered the basal activity of STAT3 binding probably in part by PDT-dependent dephosphorylation of STAT3 (Fig. 1). From these findings, we concluded that PDT- dependent cross-linking inactivated STAT3 and thus removed it from the pool of signal-transducing proteins.

Cross-linked STAT Proteins Are Stable. Time-course analysis of the post-PDT reactions indicated that protein cross-linking by either aminolevulinic acid–PDT or HPPH-PDT was limited to the light treatment period (Fig. 3A), suggesting that it was solely a function of the light-dependent photoreaction. In contrast, other PDT-initiated reactions continued to increase during post-PDT period, such as activation (phosphorylation) of JNK, dephosphorylation of ERK, loss of EGFR, and appearance of markers for apoptosis, such as the delayed degradation of phosphorylated JNK and poly(ADP-ribose) polymerase. Cross-linked STAT3 protein forms were detectable in the surviving cell population for several days, indicating an apparent half-life of 18 hours (Fig. 3B).

Nature of the Cross-linked STAT Proteins. PDT-dependent cross-linking of STAT3 was primarily through formation of covalent linkages that were noncleavable by reducing agents (Fig. 4A). In three separate experiments, we determined that consistently less than 25% of the cross-linked STAT3 contained within forms I, II, and III was susceptible to reduction by 2-mercaptoethanol. This was in contrast to PDT-cross–linked EGFR dimers, of which ~50% were cleaved by reduction (Fig. 4B).

The molecular size of 177 kDa for the major cross-linked form I is compatible with a homodimer of STAT3. It has been shown previously that noncovalently linked homodimers of nonactivated STAT1 and STAT3 are present in cytoplasmic cell extracts (25–27). Thus, we hypothesized that the same preformed dimers of STAT3 also exist in intact cells and that PDT covalently cross-linked these to yield form I. Forms II and III may include additional accessory proteins. Prior
Fig. 3. PDT induces cellular stress reactions. A. FaDu cultures were incubated with 0.4 mmol/L aminolevulinic acid or 0.6 μmol/L HPPH and then exposed to light to the indicated fluence. Note, the light treatment period was 26 minutes for aminolevulinic acid-PDT and 3 minutes for HPPH-PDT. This time difference is reflected in the time course of the cellular stress response in the two series. Both PDT treatments yielded 40% of viable cells at the 24-hour time point. Cells from the entire cultures (adherent and floating) were collected at the times in hours indicated at the bottom and extracted. Replicate aliquots of the extracts containing equal amounts of protein were analyzed by immunoblotting for the indicated proteins. A long exposure (2 hours) of the chemiluminescence image of phosphorylated ERK was chosen to demonstrate basal level phosphorylation and PDT-induced changes. B. In three separate experimental series, FaDu cells were treated with aminolevulinic acid-PDT as indicated in A. During a 96-hour post-treatment period, the adherent (viable) cells were collected and extracted. The levels of immunodetectable cross-linked STAT3 complexes I, II, and III in equal number of viable cells were determined and expressed relative to the value for complex I in the extract from the 0-hour post-PDT culture of each series (defined as 100%). The numerical values for each time point from the three experimental series are indicated. 0SMRβ, oncostatin M receptor β. n.s., non-specific.

Fig. 4. Nature of PDT cross-linked proteins. A and B. Duplicate cultures of FaDu cells were incubated with 0.8 μmol/L HPPH. Fifteen minutes before light treatment, the set of cultures in B was treated with EGF. The cells were exposed for 3 minutes to light yielding a fluence of 3 J/cm². Cells were immediately extracted. Aliquots were subjected to SDS-PAGE under nonreducing [-MeOH] and reducing [MeOH] condition and immunoblotted for STAT3 (A) and EGFR (B). C. COS-1 cells were transfected with the expression vectors for Myc-STAT3, STAT3D55C (STAT3), or the combination of the two as indicated at the bottom. The cells were subjected to HPPH-PDT (0.8 μmol/L; 3 J/cm²) and immediately extracted. Equal amounts of extracts were immunoblotted for STAT3 and Myc epitopes. Open circles to the right of the PDT lanes indicate the position of heteromers formed by the STAT3 isoforms. D. Wild-type STAT3 was expressed in COS-1 cells. The cells were extracted in isotonic buffer, and the cytosolic fraction was prepared. Five-μL aliquots (containing 5 μg of protein) were placed on a paraffin sheet on an ice-cooled support. To two samples, 40 mmol/L N-acetyl-cysteine (NAC) was added. Then, each sample received 0.75 μL of 6.73 μmol/L HPPH and was immediately illuminated to a fluence of 3 J/cm². Aliquots of 0.25 μL of the samples were immunoblotted for STAT3.
analysis of STAT3 contained in subcellular fractions had suggested a coassociation of STAT3 with caveolin-1 and stress proteins (28). Therefore, by immunoprecipitation and immunoblotting, we assessed in PDT-treated FaDu cells whether HSP90, HSP70, glucose-regulated protein-58, or caveolin-1 was integrated into the cross-linked STAT complexes. Data not shown indicated that none of the accessory proteins could be immunoprecipitated with any of the PDT-cross-linked STAT3 forms.

Due to technical limitations, we were unable to recover by immunoprecipitation and SDS gelelectrophoresis sufficient quantity of the cross-linked STAT3 forms to identify by mass spectrometry proteins contained in these complexes. Therefore we applied an alternative approach to test whether the cross-linked STAT3 form I could represent STAT3 homodimers. This approach used recombinant STAT proteins that were specifically tagged. To obtain recombinant proteins, we used COS-1 cells that yielded high expression of transfected genes under the control of the SV40 promoter/enhancer. We overexpressed in COS-1 cells two electrophoretically distinct forms of STAT3, Myc-STAT3 and the truncated STAT3Δ55C (labeled as STAT3C in Fig. 4C), alone or in combination. These cells were treated with HPPH-PDT. Each STAT3 form produced a cross-linked component that migrated with a size of a dimer. Cells coexpressing both Myc-STAT3 and STAT3Δ55C also indicated two similarly sized bands with intermediate mobility consistent with that predicted for a cross-linked heterodimer of the two STAT3 species. The biochemical nature for the two size forms is, however, still unknown.

Based on the fact that STAT3 dimers are stable in cell extract (27), we reasoned that PDT in cell-free solution should also cross-link STAT3. The cytosolic fraction of COS-1 cells overexpressing wild-type STAT3 was prepared and subjected to HPPH-PDT. The same cross-linked STAT3 forms I to III were generated as found in PDT-type STAT3 was prepared and subjected to HPPH-PDT. The same STAT3, Myc-STAT3 and the truncated STAT3Δ55C were subjected to aminolevulinic acid PDT (0.8 mmol/L; 10 J/cm²). This treatment resulted in a survival of 40% cells when determined after 24 hours. Immediately after PDT, or at successive 24-hour intervals, the adherent cells were treated with EGF or oncostatin M (OSM) for 15 minutes and then extracted. The extracts were analyzed by Western blotting for the proteins indicated at the left. B. Cultures of H-35 cells were treated with HPPH-PDT (0.6 μmol/L HPPH; 3 J/cm²). Immediately after PDT and at subsequent 24-hour intervals, replicate cultures were treated for 24 hours with IL-6 [induction of thiostatin (TST)] or conditioned medium from lipopolysaccharide-activated lung macrophages [induction of α1-acid glycoprotein (AGP)]. The number of viable cells after cytokine treatment was determined for each culture. Aliquots of conditioned medium were analyzed by immunoelectrophoresis for the amount of α1-acid glycoprotein and thiostatin. The level of plasma protein production was normalized to the number of viable cells in these cultures. Values represent mean and SD of four separate measurements. *values that are significantly different from those of the control cultures (P < 0.01). C. A549 cells, like the cells in B, were exposed to HPPH PDT. Immediately after PDT, or at successive 24-hour post-treatment intervals, the adherent cells were treated for 24 hours with oncostatin M. The number of viable cells present at the end of oncostatin M treatment was determined and indicated a survival rate of 25%. Aliquots of cell lysates, representing equivalent amounts of viable cells at the end of treatment, were used for immunoblot detection of fibrinogen.

Functional Consequence of PDT on Cytokine- and STAT-Dependent Processes. Besides cross-linking of STAT3, PDT also altered levels of membrane receptors and their responses to ligands (Fig. 1; refs. 6, 13, and 14). In three separate experimental series, we consistently observed that in the surviving FaDu cell population, STAT3 activation by oncostatin M was drastically reduced immediately after PDT, partially recovered by 24 hours, and fully restored by 48 hours (Fig. 5A). This recovery process coincided temporarily with the disappearance and restoration of EGFR protein expression and responsiveness to EGF (Fig. 5A).
cells regained full cytokine inducibility of 24 hours after PDT. The surviving cells showed strongly attenuated in the presence of the IL-6 cytokines. We applied to H-35 cells an because the expression of the plasma protein genes is detectable only (fibrinogen; Fig. 5C). These gene regulatory systems were chosen because the expression of the plasma protein genes is detectable only in the presence of the IL-6 cytokines. We applied to H-35 cells an HPPH-PDT treatment that led to 50 to 60% killing of the cells within 24 hours after PDT. The surviving cells showed strongly attenuated induction of the plasma protein production. By 48 to 72 hours, the cells regained full cytokine inducibility of α1-acid glycoprotein and thiostatin. However by 72 hours after PDT, the cellular proliferation rate had not yet returned to the normal 24-hour doubling time (Fig. 5B). A549 cells showed a slower recovery of oncogen M-inducible fibrinogen production (Fig. 5C), with no sign of cell proliferation over 72 hours (not shown).

Cross-linking of STAT3 is a Maker for PDT Action in Tumors In vivo. To relate the data describing PDT action in FaDu cells in tissue culture with corresponding events in tumors, FaDu cells were grown as xenografts in scid BALB/c mice. HPPH-PDT of the tumors led to reactions (Fig. 6A) that were very similar to those seen in vitro (see Fig. 1): STAT3 was cross-linked with detectable forms I to III; the electrophoretic mobility of EGFR was decreased; and the immunodetectable amount of EGFR was reduced, as was phospho- rylated STAT3. An equivalent PDT effect on STAT3 cross-linking was also observed in HPPH-PDT–treated Colo26 tumors grown in BALB/c mice (Fig. 6B).

From these data, we concluded that cells in a tumor were subject to the same modifications that we have detected in tissue culture. This suggested that surviving cells in PDT-treated tumors would be transiently insensitive to IL-6 cytokines, the mediators that are prominent during the post-PDT inflammatory reaction at the site of treatment (30).

DISCUSSION

This study demonstrates several mechanistic aspects of PDT activity in cells. The data indicate that PDT causes a set of immediate modifications of cellular proteins that includes loss of EGFR, dephosphorylation of many cellular proteins, increased kinase action leading to the phosphorylation of a 71-kDa protein, activation of cellular stress responses, and cross-linking of nonactivated STAT3. The last process has proven to be a new and sensitive marker for PDT action in cultured cells and in tumors. Cross-linking of STATs and PDT-mediated reduction in signaling by cytokine receptors result in a 24-hour and longer period of reduced responsiveness to IL-6 cytokines in surviving cells.

Three aspects regarding the consequences of PDT demand discussion: (1) the biochemical reactions that alter cellular proteins and, at the same time, inform us about the biology of the affected proteins; (2) the biological responses to PDT; and (3) the potential role of the PDT reactions in the physiology of tumor tissues.

Fundamental to the biochemistry of PDT is the photosensitizer-dependent and subcellular site–restricted activity of singlet oxygen that has an approximately 10–to 100-nanosecond life span and thus a very restricted range of action (~10–20 nm; ref. 2). Although aminolevulinic acid–PDT and HPPH-PDT are believed primarily to act within the mitochondria, it is unlikely that the direct photochemical oxidation and cross-linking of STAT3 at cytoplasmic sites and EGFR at the plasma membrane is due to singlet oxygen emanating from mitochondria. However, the photosensitizers are very lipophilic, and low levels of broadly distributed protoporphyrin IX exported from mitochondria in aminolevulinic acid–treated cells or low levels of HPPH maintained in cytoplasmic and plasma membrane structures could contribute to the generation of singlet oxygen throughout the cells, particularly near nonpolar sites. Moreover, generation of longer lived reactive oxygen species by singlet oxygen (31) at the mitochondria and other subcellular sites may account for some of the cell-wide molecular modifications. The ensuing peroxidation reactions may lead to adduct formation involving, among others, lipids and proteins (31), which could result in the low level of cross-linked molecules with broad size distributions that were found.

More interesting is that PDT-initiated reactions effectively cross-link defined substrates, such as STATs and EGFR with nonreducible and reducible bonds (Fig. 4A). Previous studies (25–29) have indicated that nonactivated STAT1, STAT3, and STAT4, but not STAT5, are found as noncovalently bound homodimeric forms in cell extracts. The PDT results show that dimers for these STAT isoforms are also formed in cells, probably in or near a nonpolar environment that would contain photosensitizers. The dimeric complexes of nonactivated STAT1, STAT3, or STAT4 must have residues that are able to form covalent linkages (cysteine, methionine, tryptophane, tyros ine, and histidine) in close proximity (11, 32). The fact that fully tyrosine phosphorylated and SH-2 domain-interacted STAT1 or STAT3 dimers cannot be cross-linked by PDT suggests that the activated STAT dimers differ structurally from the nonactivated dimers. The low level of cross-linked STAT complexes reacting with

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anti-phosphoSTAT3 (Fig. 1) may represent cross-linked STAT dimers with one of the STAT3 molecules phosphorylated.

A multimolecular structure that incorporates the nonactivated cytoplasmatic STAT3 has been proposed (28, 33). Analysis of that complex suggested an interaction of STAT3 with caveolin-1, HSP90, and other chaperone proteins (28). However, we could not demonstrate PDT-mediated cross-linking of those accessory proteins to STAT3 as part of the pattern of covalent STAT3 complexes. Thus, the biochemical nature of the larger cross-linked complexes for wild-type STAT3 (forms II and III) remains to be explained. It is conceivable that they contain interacting proteins that are not associated with the cell membrane (both forms are also found in the PDT-treated cytosol fraction; Fig. 4D) but may still represent part of the preformed statosome.

Although our study has highlighted the cross-linking reaction of PDT, a number of other biochemical reactions accompany the PDT response, some of which are mediated by singlet oxygen and reactive oxygen species. A general loss of tyrosine phosphorylation from cellular proteins is evident (Fig. 1). Loss of phosphates can reflect an increased action of phosphatases and/or decreased action of kinases. The fact that vanadate treatment prevents in part the PDT-dependent dephosphorylation of cellular proteins (Fig. 1) suggests that PDT enhances the activity of vanadate-sensitive phosphatase(s). As suggested by Kochevar et al. (14), PDT-resistant and even reactive oxygen species-inducible phosphatases may account for the net dephosphorylation evident in PDT-treated cells. Although PDT action is associated with reactive oxygen species, the PDT effects on protein phosphorylation and oxidation differ drastically from those found in cells subjected to strong redox reactions, such as treatment with hydrogen peroxide (34). In H2O2-treated cells, cysteine residues at the active site of phosphatases are oxidized, and the enzymes are reversibly inactivated (12). The resulting loss of phosphatase activity shifts the balance of phosphorylation/dephosphorylation reactions and raises the level of tyrosine-phosphorylated proteins in cells, the opposite of what is found with PDT.

Besides activation of phosphatases, an immediate loss of protein tyrosine kinase activities can also contribute to the change in phosphorylation status of the cellular proteins (13, 15, 35). The complexity of PDT-dependent changes of enzymatic activities is further evident by the PDT effects in vanadate-inhibited cells (Fig. 1). Although the responsible enzymes still remain to be defined, a prominent PDT-stimulated tyrosine kinase action is detected by the phosphorylation of a 71-kDa protein. This activity is efficiently quenched in cells not exposed to vanadate. This suggests that vanadate-sensitive tyrosine phosphatases are effective in reversing the effects of the PDT-stimulated protein kinase activity. Surveying data derived from different cell types and PDT treatments, we have to conclude, however, that dephosphorylation prevails. The enhanced phosphatase activity in PDT-treated cells may also account in part for the attenuated, or even lost signaling function of cytokine and growth factor receptors.

One of the striking biological consequences of PDT is that surviving cells transiently lose responsiveness to cytokines such as oncostatin M. Oncostatin M is one of the cytokines that are expected to be high at site of tissue injury as caused by PDT. Although in vivo PDT may injure or eliminate host cytokine-producing cells that are located within the treatment field, immigrating leukocytes will be activated by the milieu of the damaged tissue (30, 36). Cytokines from these cells including, among others, tumor necrosis factor, IL-1, IL-6, and oncostatin M act in a paracrine fashion on cells within and adjacent to the PDT-treated area. These cytokines are known to suppress epithelial cell growth (37). Thus, those cells surviving PDT, whether normal or tumor cells, are refractory to these components and temporarily sheltered from the inhibitory cytokine actions. This scenario appears advantageous for normal cells in that it would favor their survival and the contribution of the cells to subsequent tissue repair. In the context of cancer, however, the same benefit that the loss of cytokine responsiveness provides to tumor cells will assist in tumor survival and recurrence.

The covalent protein modification in cells surviving the PDT treatment may conceivably have far reaching physiologic consequences. PDT-cross–linked or otherwise modified proteins at the treatment site may introduce neoantigens in the midst of an inflammatory reaction and may thereby facilitate an antitumor immune response. Cross-linked STAT3 appears to be a stable compound that is detectable in PDT-surviving cells for more than 24 hours and thus is available for presentation to the immune cells. Considering that STAT3 under normal conditions has a half-life of only few hours (38, 39), it seems that cross-linking has enhanced the stability of the STAT proteins or that cross-linked STATs are subjected to a different degradation mechanism than normal STATs. A positive immune response may become relevant for patients that are subjected to multiple rounds of PDT treatments. Regardless of the site of treatment, the ubiquitous STAT3 (or other analogous proteins) will be cross-linked and may provide the necessary immunogen for a host antitumor response. Although we do not yet have evidence that cross-linked proteins are able to elicit a specific and effective immune response (40), if effective, one could envision that it assists in eliminating cells bearing or presenting the neoantigen during the post-PDT period.

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Photodynamic Therapy Causes Cross-linking of Signal Transducer and Activator of Transcription Proteins and Attenuation of Interleukin-6 Cytokine Responsiveness in Epithelial Cells

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