Cross-Linking of Signal Transducer and Activator of Transcription 3—A Molecular Marker for the Photodynamic Reaction in Cells and Tumors

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Abstract

Purpose: Photodynamic therapy (PDT) depends on the delivery of a photosensitizer to the target tissue that, under light exposure, produces singlet oxygen and other reactive oxygen species, which in turn cause the death of the treated cell. This study establishes a quantitative marker for the photoreaction that will predict the outcome of PDT.

Experimental Design: Cells in tissue culture, murine s.c. tumors, and endobronchial carcinomas in patients were treated with PDT, and the noncleavable cross-linking of the latent signal transducer and activator of transcription 3 (STAT3) was determined.

Results: Murine and human cancer cell lines reacted to PDT by an immediate covalent cross-linking of STAT3 to homodimeric and other complexes. The magnitude of this effect was strictly a function of the PDT reaction that is determined by the photosensitizer concentration and light dose. The cross-link reaction of STAT3 was proportional to the subsequent cytotoxic outcome of PDT. An equivalent photoreaction as detected *in vitro* occurred in tumors treated *in situ* with PDT. The light dose-dependent STAT3 cross-linking indicated the relative effectiveness of PDT as a function of the distance of the tissue to the treating laser light source. Absence of cross-links correlated with treatment failure.

Conclusions: The data suggest that the relative amount of cross-linked STAT3 predicts the probability for beneficial outcome, whereas absence of cross-links predicts treatment failure. Determination of STAT3 cross-links after PDT might be clinically useful for early assessment of PDT response.

Exposure of cells and tissues to the oxidative stress of photodynamic treatment unleashes a plethora of responses that can be classified as immediate, early, and late. The generation of reactive oxygen species, especially singlet oxygen, leads to immediate oxidative modifications of biological molecules, most importantly lipids and proteins (1, 2). This can either cause cell membrane leakage and necrosis or set in motion cell signaling events that over minutes to hours result in changes in gene expression, stress responses, repair reaction, and/or activation of cell death mechanisms, such as apoptosis (3). In the case of photodynamic therapy (PDT) of tumors, these early events set the stage for secondary local and systemic events, occurring from days to months and emanating from responses in the tumor bed, such as disturbances of the tumor vascular supply, inflammation, and antitumor immune reactions (4). The nature, extent, and time course of these responses are in part dependent on the cell/tumor type examined and the photosensitizer and light regimen used in the delivery of PDT (4).

The range of PDT-dependent modifications of cellular proteins includes oxidation, adduct formation, protein cross-linking, and degradation (5–9). Protein cross-linking, long known to occur as a consequence of PDT (2), has recently drawn new attention with the identification of highly specific substrates for the cross-linking reaction, including important cell signaling molecules, such as epidermal growth factor receptor (10, 11), Bcl-2 (3), and members of the non-activated signal transducer and activator of transcription (STAT) family (11).

PDT-mediated STAT cross-linking has the following characteristics (11): (*a*) PDT induces cross-linking of preformed homodimeric complexes of nonactivated cytoplasmic STAT3 and, to a lesser extent, of STAT1 and STAT4; (*b*) STAT cross-linking is a function of the PDT photoreaction, occurring only during the light treatment period, and correlates with PDT dose; (*c*) cross-linked STAT proteins are stable (half-life of ~18 h) and detectable immediately after completion of

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treatment; (*d*) STAT cross-linking is observed with all cell types and photosensitizers tested thus far; and (*e*) STAT cross-links can be detected in PDT-exposed tissue *in vivo*. It has to be noted that (*a*) the PDT-mediated cross-link reaction does not involve STAT3 that has been activated by phosphorylation at Tyr^{705}; (*b*) PDT cross-linked STAT3 dimer is similar in electrophoretic mobility to that generated by oxidative disulfide formation involving Cys^{259} (12) but differs in that the linking is noncleavable by reduction; and (*c*) cross-linked STAT3 is incapable of transducing cytokine and growth factor signals, but is not causative for PDT-induced cell death, as shown by PDT killing of human prostate PC3 cells that lack STAT3.

The above characteristics of STAT (particularly STAT3) cross-linking raise the possibility that it may represent a quantitative marker for the immediate, direct cellular PDT effect and may indicate the relative level of post-PDT reaction, such as cell killing. Such a marker may provide short-term information about PDT treatment delivery success or failure, information that may be highly useful to the clinician who has to decide whether potential treatment failure requires prompt retreatment of a specific tumor lesion. In this study, we tested the hypothesis that STAT3 cross-linking can act as molecular marker for the local/regional PDT reaction and thus allow early evaluation of the PDT response.

Materials and Methods

Cell and tumor systems. MCA205 (murine fibrosarcoma), Colon38 (murine colon adenocarcinoma), and A549 (human lung carcinoma) cells were grown in RPMI 1640 containing 10% FCS and antibiotics. MCA205 and Colon38 tumors were established in syngeneic C57BL/6 mice obtained pathogen-free from The Jackson Laboratory. Six- to 12-week-old animals were inoculated s.c. on the right shoulder (for tumor response) or both shoulders (for STAT3 analysis) with 3×10^5 cells harvested from exponentially growing *in vitro* cultures. Animals were housed in microisolator cages in a laminar flow unit under ambient light. Tumors were used for experimentation ~10 days after inoculation when they had reached 6 to 8 mm in diameter. The Roswell Park Cancer Institute Institutional Animal Care and Use Committee approved all procedures carried out in this study.

Photosensitizer. Clinical-grade, pyrogen-free 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH; ref. 13) was obtained from the Roswell Park Pharmacy and reconstituted to 0.4 mmol/L in pyrogen-free 5% dextrose (D5W; Baxter Corp.) in distilled water containing 2% ethanol and 0.1% Tween 80.

PDT treatment. For *in vitro* experiments, subconfluent cell monolayers in six-well plates were exposed in the dark to 0 to 2 μmol/L HPPH (exposure conditions indicated in the text). They were then illuminated with 665 nm light at a fluence rate of 14 mW/cm^2 for fluences of 1 or 3 J/cm^2.

For *in vivo* experiments, animals were injected via tail vein with 0.4 or 0.8 μmol/kg HPPH. Tumors were exposed to 665 nm light 24 h after HPPH administration. A spot diameter of 1.1 cm covering slightly more than the tumor surface was illuminated. Tumors received graded doses of 665 nm light (10-160 J/cm^2) at 10, 14, or 75 mW/cm^2. All experiments included control animals treated with either photosensitizer or light alone.

The light source for all PDT treatments consisted of a dye laser (375, Spectra Physics) pumped by an argon ion laser (2080, Spectra Physics) using DCM dye (Exciton). Output from the dye laser was passed through an 8-way beam splitter, and the power of each beam was set individually with Brewster window-type attenuators (14).

Measurement of light attenuation. Light attenuation in 4- to 5-mm-thick MCA205 tumors was measured by *in vivo* spectroscopy (15). Briefly, the diffuse fluence Φ at 665 nm was measured at increasing probe separations using a two-fiber contact probe, one fiber being the light source and the other the receiver. The total attenuation α is the slope of $\ln(r\Phi)$ plotted against r, where r is the probe separation in mm and Φ is the diffuse 665 nm fluence escaping from the tumor at r mm from the light source fiber.

Western blot analysis. Cells from *in vitro* cultures were lysed and tumor tissue was homogenized in radioimmunoprecipitation assay buffer and processed as described (11). Briefly, protein extracts (20-40 μg) were separated on 6% SDS-polyacrylamide gels. On all gels, reference protein markers for loading, molecular size detection, and cross-comparison among gels were included. After separation, proteins were transferred to nitrocellulose membranes (Hybond-P, Amersham Pharmacia Biotech). Nonspecific interactions were blocked by incubating the membranes first for 30 min at room temperature with PBS-0.1% Tween 20-5% skim milk. Membranes were then reacted overnight at 4°C with antibodies to STAT3 (Santa Cruz Biotechnology) and phosphotyrosine-STAT3 (New England Biolabs, Inc.), caspase-3, poly (ADP-ribose) polymerase (Cell Signaling Technology, Inc.), actin (Sigma-Aldrich), or leukemia inhibitory factor receptor (Santa Cruz Biotechnology). Detection of the immune complexes was done using peroxidase-coupled secondary antibodies and enhanced chemiluminescence detection (Pierce Chemical). To quantify the immunodetectable signals, enhanced chemiluminescence images were recorded on X-ray films by various lengths of exposure to ensure recovery of signals that lay in the linear range of detection by digital scanning. The net pixel values of each band were determined by integration using the ImageQuant TL program (Amersham Biosciences). The cross-comparison of separate analyses relied on normalization of the values relative to the coseparated reference markers. Due to the fact that higher-molecular-size STAT3 cross-linked complexes were not always detectable in tumor extracts, the ability of PDT to cross-link STAT3 was expressed only by the percentage conversion of monomeric STAT3 into the dimer (form I on Fig. 1). For better visibility of cross-linked proteins, the presentation of data in the figures used images that were exposed longer than those used for quantification.

In vitro ***phototoxicity assay.*** Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay 48 h after PDT or by clonogenic assay with cells plated immediately following PDT.

Assessment of tumor response. Following treatment, orthogonal diameters of tumors were measured once every 2 days with calipers. The tumor volume, V, was calculated with the formula $V = (lw^2 / 2)$, where l is the longest axis of the tumor and w is the axis perpendicular

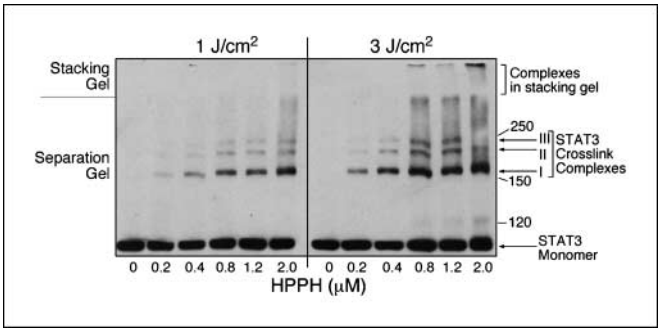

Fig. 1. PDT dose-dependent STAT3 cross-linking in MCA205 cells. Subconfluent monolayers of MCA205 cells in six-well culture plates were incubated for 4 h in medium containing the indicated concentrations of HPPH. After changing to HPPH-free culture medium, the cells were illuminated at 14 mW/cm^2 to the fluence of 1 or 3 J/cm^2. Cells were immediately extracted after PDT reaction, and the extracts were analyzed by immunoblotting for STAT3. Positions of stacking and separation gels, monomeric and cross-linked STAT3, as well as molecular size markers (size in kDa), are indicated at the left and right side.

to *1*. The tumors were monitored until they reached a volume of >400 mm^3, at which time the mice were sacrificed. Regrowing tumors reached the 400 mm^3 volume within ~10 days. No tumor regrowth was ever observed later than day ~50, and therefore, animals were considered cured if they remained tumor-free for at least 60 days after PDT.

Patient samples. Tumor tissue was obtained from patients undergoing bronchoscopic PDT for obstructive endobronchial tumors (non–small cell lung cancer). Treatment and biopsy were approved by the Roswell Park Cancer Institute Institutional Review Board, and informed consent was obtained from all patients. Forty-eight hours before light treatment, the patients had received HPPH (5.3 or 4 mg/m^2) by infusion. Light (665 nm) was delivered interstitially at 400 mW/linear cm with a 2-cm diffusing fiber for a total fluence of 130 or 140 J/linear cm. One biopsy was obtained before light exposure, and two biopsies were obtained immediately after completion of treatment; one of these was taken from an area proximal to the light source, the other from an area distal (~10 mm, estimated) from the light source and close to the bronchial wall. Samples were immediately processed for Western blotting. Three days after treatment, patients underwent a second bronchoscopy to remove necrotic tissue. At that time, the local PDT response was visually assessed and recorded.

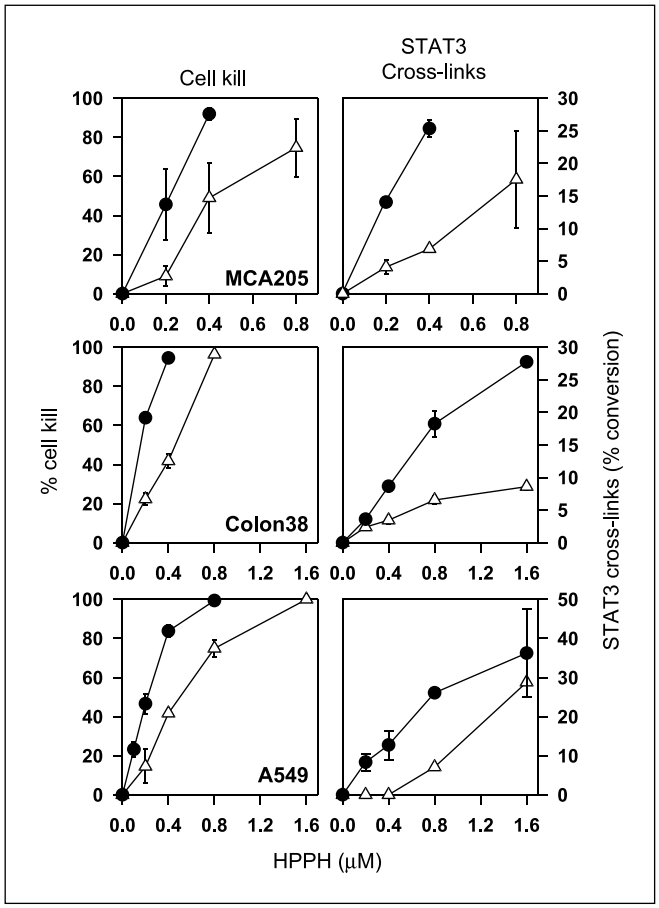

Fig. 2. STAT3 cross-linking correlates with cell kill *in vitro*. MCA205, Colon38, and A549 cultures were incubated with HPPH. Replicated cultures of MCA205 cells were exposed to HPPH for 24 h and supplied with fresh HPPH-free medium for another 3 h before light exposure. Colon38 and A549 cells were exposed to HPPH for 4 h, washed, and exposed to light immediately. Light fluences were 1 J/cm^2 (△) or 3 J/cm^2 (●). Identically treated cultures were divided into two groups: one group was used for cell survival assay (*left*) and the other group for STAT3 immunoblotting (*right*), respectively. MCA205 cell survival was determined by clonogenic assay, and Colon38 and A549 cell survival was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data represent the results from at least two independent experiments.

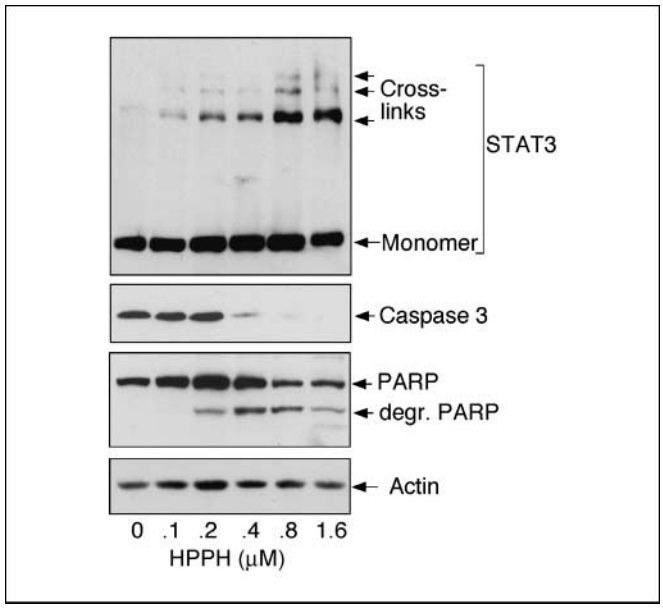

Fig. 3. PDT dose-dependent STAT3 cross-linking corresponds with apoptosis *in vitro*. MCA205 cultures were incubated with HPPH as indicated and exposed to a fluence of 3 J/cm^2. Cells were extracted after a 3-h post-PDT culture period. Replicate aliquots of the extracts containing equal amounts of protein were analyzed by immunoblotting for the indicated proteins.

Statistical evaluation. All measured values are presented as mean ± SE. The one-tailed Student's *t* test was used for comparison between groups in all experiments, except for tumor response determinations. *P* values of ≤0.05 represent statistical significance. For tumor response data analysis, hours to event (i.e., to 400 mm^3 tumor volume) was calculated for each animal by linearly interpolating between the times just before and after this volume was reached using log (tumor volume) for the calculations; both tumor volume and hours-to-event calculations were done using Excel (Microsoft). Tumor responses between groups were compared using the Kaplan-Meier analysis. Briefly, the calculated hours-to-event data for individual animals were entered in a Prism (version 3.0; GraphPad Software, Inc.) spreadsheet. Prism calculates and graphs event curves (i.e., the fraction of subjects not reaching the events as a function of time) for each group and calculates the group median time to event. Event curves were compared by the Prism program using the log-rank test, which calculated a two-tailed *P* value testing the null hypothesis that the curves were identical.

Results

***PDT induces dose-dependent cross-linking of STAT3 that correlates with cell death* in vitro.** We have shown earlier (11) that the PDT photoreaction causes cross-linking of nonphosphorylated STAT3 and, to a lesser degree, of STAT1 and STAT4, but not of STAT5A, STAT5B, and STAT6 or heterodimers of STAT1, STAT2, and STAT3.[4] A representative example of the PDT dose dependence of STAT3 cross-linking is shown in Fig. 1 for MCA205 cells treated with HPPH PDT *in vitro*. It is evident that cross-linking increases with increasing photosensitizer dose and fluence. We determined in multiple cell lines how the STAT3 cross-linking compares to cell viability

[4] Baumann H, Liu W, unpublished data.

by quantifying the percentage of cell kill and percentage of STAT3 converted to cross-linked dimer complexes as a function of PDT dose. Figure 2 shows that cell kill (*left*) and STAT3 cross-linking (*right*) have similar photosensitizer and light dose-dependent changes. In all cases, a correlation between cell kill and STAT3 cross-links was apparent, whether cell kill was assessed by clonogenic assay (MCA205) or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Colon38 and A549). It should be noted that the dose relationship of PDT and STAT3 cross-linking was maintained regardless of the chosen HPPH exposure conditions; for instance, MCA205 cells were incubated with HPPH for 24 h followed by a 3-h photosensitizer leak-off period in fresh medium, whereas Colon38 and A549 cells were incubated with HPPH for 4 h and, without leak-off, exposed to light. When the percentage of STAT3 conversion was compared with the percentage cell kill, tight correlations were found, with the highest value for MCA205 cells (r^2 = 0.9952; 3 J/cm^2) and the lowest for A549 cells (r^2 = 0.8814; 3 J/cm^2). The numerical values for STAT3 cross-links, expressed as percentage of monomeric STAT3, however, varied among cell lines, with the percentage of STAT3 conversion ranging from approximately 10% to 30% for the lower fluence and from approximately 25% to 50% for the higher fluence. The cell type–dependent differences could not be attributed to differences in STAT3 levels because each cell type yielded approximately the same immunodetectable STAT3 signal (data not shown). As expected, the extent of STAT3 cross-linking also corresponded with the extent of apoptosis that was detectable 3 h after PDT by the degradation of caspase-3 and poly(ADP-ribose) polymerase (Fig. 3).

PDT generates STAT3 cross-links in tumors* in vivo *in a dose-dependent manner. To relate the results describing the PDT effects on STAT3 in cells in culture to the *in vivo* reaction, tumors (MCA205) were grown s.c. in syngeneic C57BL/6 mice and exposed to PDT using several different light treatment regimens. Figure 4 depicts the generation of STAT3 cross-links for a range of PDT doses (0.4 μmol/kg HPPH; 12-96 J/cm^2 at 10 mW/cm^2). Increasing levels of STAT3 cross-linking were detected in tumors exposed to increasing PDT dose, whereas the contralateral, not light-exposed tumors in the same animal were devoid of detectable cross-links. The data also show good reproducibility among the three animals examined for each PDT dose. The PDT conditions chosen for this experiment resulted in a growth delay of ~15 days at the highest light dose but no long-term tumor control (data not shown).

STAT3 cross-linking within tumors depends on the position of the treated tissue with regard to the incident light. When light penetrates tissue, fluence and fluence rate decrease exponentially with distance from the light source (15, 16). Therefore, if STAT3 cross-linking is a measure of PDT dose, the level of cross-linking should differ within the treated tissue with the position of the sampled tissue relative to the light source. Thus, the relative amount of STAT3 cross-linking could inform about light distribution not only within but also adjacent to a treated tumor. To test this, MCA205 and Colon38 tumors (~3 mm thickness; Fig. 5) and adjacent normal tissue from outside the treatment field were harvested immediately following PDT and divided into sections, the position of which with regard to the incident light was recorded (Fig. 5A, *a*). These samples from the tumor and adjacent dermal layer were extracted and subjected to the Western blotting for the presence of STAT3 cross-links (Fig. 5A, *b*). Their quantification (Fig. 5A, *c*) confirmed that the tissue from a position farthest removed from the incident light (i.e., receiving the lowest fluence) contained less cross-links than the tumor samples closest to the light source and that the level was comparable with that of normal tissues harvested from outside but adjacent to the treatment field.

To further characterize the relationship between fluence and STAT3 cross-linking in tumors, the light attenuation by MCA205 tumor tissue was determined (Fig. 5B, *c*). This allowed us to estimate the range of fluences reaching the top 2 mm and bottom 2 mm of the 4-mm-deep tumors. The tumor column at the center of the light path was excised immediately after completion of illumination and divided into the top and bottom halves (as shown in Fig. 5A, *a*), and STAT3 cross-linking was determined in each. Figure 5B, *a* shows the STAT3 cross-links and percentage conversion for a tumor exposed to an incident fluence of 160 J/cm^2. STAT3 cross-linking in the top portion (Fig. 5B, *left lane*; fluence range, ~160-96 J) is twice that of the bottom portion (Fig. 5B, *right lane*; fluence range, ~96-56 J). To validate the fluence dependence observed in this tumor, another tumor was exposed to an incident fluence of 50 J/cm^2 (Fig. 5B, *b*), the fluence estimated to reach the very bottom of a tumor exposed to 160 J/cm^2. Here, the top portion of the tumor (Fig. 5B, *b*, *left lane*; fluence range, ~50-30J) showed one third of the cross-links observed in the top portion of the tumor exposed to 160 J/cm^2, and the extent of cross-linking, as expected, is less than the bottom half of the tumor exposed to 160 J/cm^2. In the bottom half of that tumor (Fig. 5B, *b*, *right lane*), STAT3 cross-links are barely detectable.

We have reported previously that PDT can not only generate STAT3 cross-links but also lead to the immediate loss of some but not all interleukin-6 family cytokine receptor proteins (7), including the leukemia inhibitory factor receptor-α. This

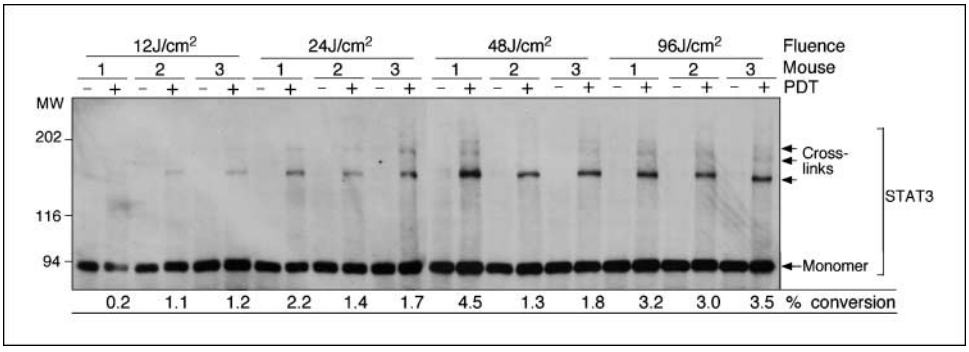

Fig. 4. STAT3 cross-linking increases with PDT dose in murine tumors. C57BL/6 mice carrying two separate MCA205 tumors were subjected to PDT (0.4 μmol/kg HPPH, graded doses of light at 10 mW/cm^2). Treated and contralateral untreated tumors from three animals were harvested for each treatment group and immediately processed for immunoblotting. Data for each individual mouse/tumor. Bottom, percentage of STAT3 cross-linked to the homodimer (% conversion) is calculated and listed.

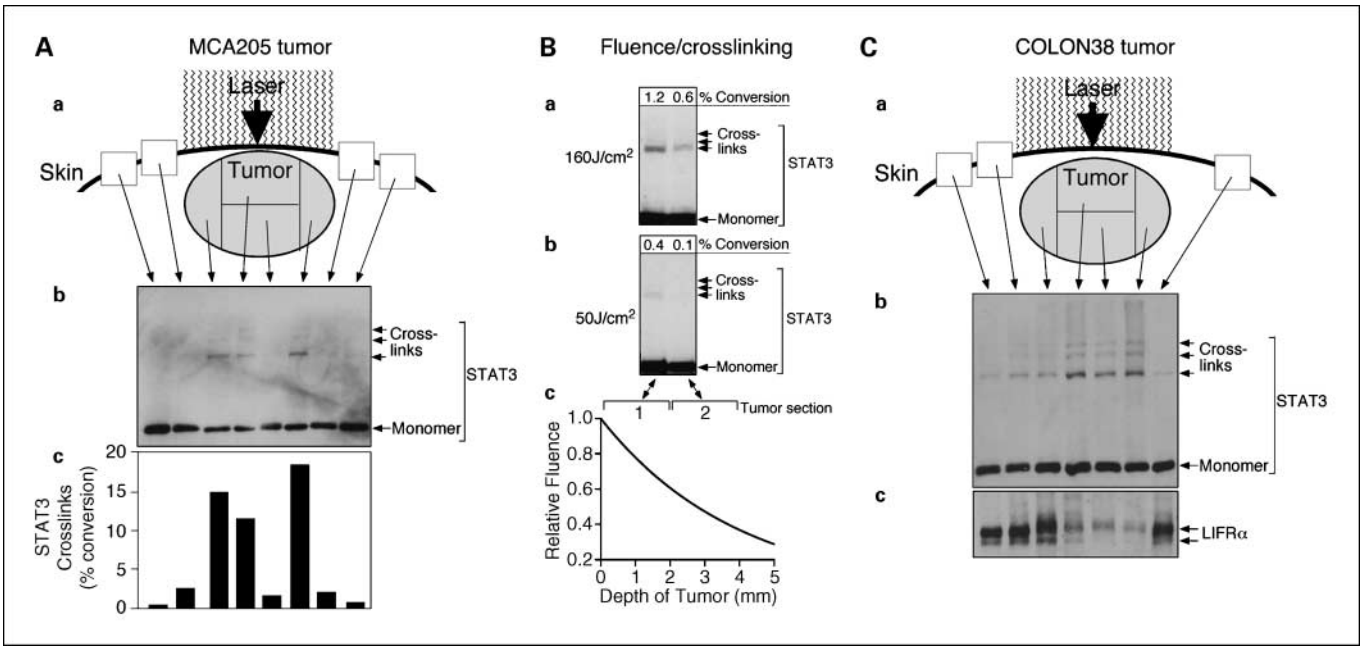

Fig. 5. STAT3 cross-linking depends on the distance from the light source and correlates with loss of cytokine receptor protein. *A,* MCA205 tumor was treated with PDT (0.4 μmol/kg HPPH, 160 J/cm^2, 14 mW/cm^2). Tumor and adjacent normal tissue was harvested and processed immediately for STAT3 immunoblotting. *a*, location of tissue samples in relation to incident light; *b*, STAT3 Western blot of selected tissue samples; *c*, quantitation of STAT3 cross-links. *B,* MCA205 tumors were treated with PDT (0.4 μmol/kg HPPH, 50 or 160 J/cm^2, 14 mW/cm^2). Immediately after illumination, the tumors were excised and the central full thickness tumor tissue was isolated, divided into top and bottom halves as shown in (*A*) and (*C, a*), and processed for STAT3 immunoblotting. *a*, Western blot of tumor samples from tumor exposed to 160 J/cm^2. Left lane, top half; right lane, bottom half. *b*, Western blot of tumor samples from tumor exposed to 50 J/cm^2. Left lane, top half; right lane, bottom half. *c*, light attenuation by MCA205 tumor tissue. Relative fluence is defined as fluence Φ/maximum fluence Φ(z). Brackets 1 and 2, top and bottom tumor halves, respectively. *C,* Colon38 tumor was treated with PDT (0.4 μmol/kg HPPH, 160 J/cm^2, 14 mW/cm^2). Tumor and adjacent normal tissue was harvested and processed immediately for STAT3 immunoblotting. *a*, location of tissue samples in relation to incident light. Western blot of selected tissue samples was analyzed for STAT3 (*b*) and leukemia inhibitory factor receptor-α (*LIFRα*; *c*). Data for representative tumors for (*A*), (*B*), and (*C*).

additional PDT-dependent event, together with STAT3 cross-linking, became particularly evident in the example of Colon38 tumors (Fig. 5C), which expresses a high basal level of leukemia inhibitory factor receptor-α. The spatial distribution (Fig. 5C, *a*) of STAT3 cross-links coincides with the reduction of leukemia inhibitory factor receptor-α (Fig. 5C, *c*), emphasizing the delivery of a biologically significant PDT dose in tumor areas where STAT3 cross-links are in evidence.

STAT3 cross-links in tumors increase with tumor cures. Having established the relationship of STAT3 cross-linking, PDT dose and, at least *in vitro*, cell death, we hypothesized that the relative level of STAT3 cross-linking *in vivo* could provide a measure for the relative cytotoxicity of the PDT treatment and tumor cure. This relationship was tested for MCA205 tumors in which the percentage of conversion of STAT3 to cross-link for individual tumors treated with a range of PDT doses was quantified immediately after treatment (Fig. 6A). Although cross-linking increased gradually up to 80 J/cm^2, it is noteworthy that the only significant increment (P = 0.0086) was between the values for 40 and 80 J/cm^2, with no further increase at 120 J/cm^2. The tumor response/cure data were obtained from animals that were treated in parallel to those used to determine STAT3 cross-linking (Fig. 6B). In agreement with the cross-linking data, a significant increase in long-term tumor control was observed between 40 and 80 J/cm^2 (P < 0.0001), with no significant further improvement at 120 J/cm^2.

STAT3 cross-links form in human tumors during PDT. Because these experimental tests showed the reproducibility and accuracy of STAT3 cross-linking as a function of PDT in experimental models, we asked whether PDT-dependent STAT3 cross-linking could also indicate the tissue reaction to clinical PDT. This question was addressed as part of a clinical trial that involved six patients who received HPPH PDT for *in situ* palliative treatment of advanced endobronchial tumors. Tumor biopsies were taken before and immediately after laser exposure and analyzed for the relative amount of STAT3 cross-links. The tissue samples from five patients (patients 2 to 6) proved to be informative (Fig. 7). Samples from patients 2 to 5 showed various degrees of cross-linking of STAT3. Although for technical reasons (variable amounts of blood contamination) the analyses of biopsy material did not yield in all cases a uniform loading of cellular protein, as evident from the variable amounts of total STAT3 proteins, the conversion of STAT3 from monomer to dimeric cross-link nevertheless provided an accurate value for level of PDT reaction. Quantitation of STAT3 conversion (indicated above the Western blots in Fig. 7) revealed the expected decrease in cross-links as a function of fluence between the proximal and distal biopsy sites in three of four cases that showed STAT3 cross-linking. The samples from patient 6 showed no detectable cross-links of STAT3 and other proteins. The samples from patient 1 could not be evaluated due to technical reasons.

Visual assessment of initial tumor response on day 3 after PDT revealed tumor responses in all patients, except patient 6. Patient 4 had a complete response, whereas all other patients showed intermediate partial responses. The study of the patients' material indicated an additional phenomenon that

needed consideration. In contrast to the *in vitro* or murine data, where cross-links were never observed without PDT light exposure, several cases of patients in Fig. 7 showed variable levels of STAT3 cross-linking in samples taken immediately before PDT. In the case of patient 3, the relative level of cross-linked STAT3 in the pre-PDT biopsy is even higher than in the biopsy taken after PDT. This seemingly basal PDT reaction probably is attributable to the white light exposure during positioning of the bronchoscope.

Discussion

The objective of our study was to determine whether STAT3 cross-linking could serve as early molecular marker and dosimeter for the PDT reaction. The rationale for our approach was based on (*a*) the characteristics of PDT-induced STAT3 cross-linking (i.e., the instantaneous and PDT dose-dependent formation that is limited to the light treatment period), suggesting to be solely a function of the light-dependent photoreaction (11); (*b*) the lack of a biomarker predictive of the PDT response; and (*c*) the clinical need for an early marker for success or failure of treatment delivery.

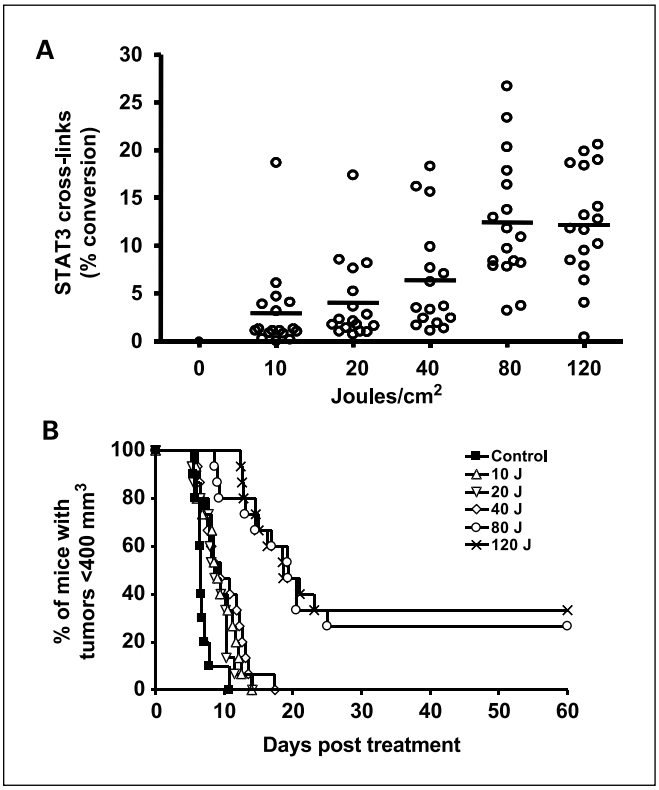

Fig. 6. STAT3 cross-links increase with increasing tumor response in murine tumors. C57BL/6 mice carrying MCA205 tumors were subjected to PDT (0.8 μmol/kg HPPH, graded doses of 665 nm light at 75 mW/cm^2). *A,* treated and control (HPPH only, no light) tumors were harvested immediately after completion of treatment and extracted. Aliquots of the extracts, containing equal amounts of protein, were analyzed for STAT3 by immunoblotting. STAT3 cross-links were quantified and the values for individual mice were plotted. Horizontal lines, median value for the groups (n = 16-17 per group). No STAT3 cross-links were observed for the unilluminated control group. *B,* Kaplan-Meier plot of the MCA205 tumor response of mice treated as described (n = 20 per group). Tumor growth was monitored for 60 d or until tumors reached a volume of 400 mm^3.

It is widely recognized that limitations to tumor cell kill by PDT exist *in vivo*, the most important of which being (*a*) inhomogeneous photosensitizer distribution within and among tumors (17), including a gradual decrease of photosensitizer concentration with distance from blood vessels (18); (*b*) insufficient light penetration through the tissue (16, 19); and (*c*) insufficient oxygen availability (20, 21). Although preclinical studies have successfully defined many of these limitations, showing the frequent regional heterogeneity of the PDT effect, detailed information about these issues in human tumors undergoing PDT is extremely scant. Several studies have searched for the presence of predictive PDT response markers. Koukourakis et al. (22) in a retrospective study of archival material from 37 patients with esophageal cancer reported that Bcl-2 expression was associated with a high complete response rate. The authors suggested that the reason for this association was the earlier observation by others that Bcl-2 is a target for PDT destruction (23, 24). On the other hand, the expression in the same material of hypoxia-inducible factor 1α and 2α, which reflects the level of tumor hypoxia, was associated with poor PDT response. The latter association is to be expected because PDT critically depends on the presence of oxygen (25). However, even if it were proven that the degree of expression of these molecules was predictive of ultimate PDT outcome, these assessments would not furnish information about the actual short-term PDT response.

These complexities taken together make it unlikely that expression of a "predictive" factor determined before PDT would be sufficient for early evaluation of PDT response. Such early evaluation, however, is a clinical need as it may inform whether a tissue has received an effective PDT exposure or whether it may need further treatment to achieve tumor control.

We have recently made the discovery that cross-linking of STAT3 showed extraordinary PDT dose dependence (11). We now present preclinical and clinical evidence that STAT3 cross-links may serve as a "dosimeter" for absorbed PDT dose and a valuable biomarker to give short-term information about the local/regional PDT response. *In vitro*, strong correlations were observed between cell kill and STAT3 cross-linking, with a tendency for increases in cross-links to diminish at very high PDT doses. The correlation was strong whether HPPH exposure of cells was brief or prolonged and whether a clonogenic or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used as survival end point, attesting to the generality of the cross-linking reaction. The upper limits in detecting STAT3 conversion at high PDT doses may be determined in part by secondary cross-linking reactions that shift immune detectable STAT3 into the high-molecular-size region of the gel (Fig. 1) and the concurrent initiation of tissue degradation with loss of protein integrity due to proteolysis. Although correlations between cell kill and STAT3 cross-linking were high, no relative cross-link values could be assigned to specific levels of cell kill across the panel of cell lines tested. However, a STAT3 conversion of >25% generally related to ~100% cell kill. Furthermore, the appearance of strong bands of cross-linked STAT3 coincided with the appearance of cleaved poly(ADP-ribose) polymerase and apoptosis. PDT dose-dependent STAT3 cross-linking occurred across the entire spectrum of cells tested, including cell types

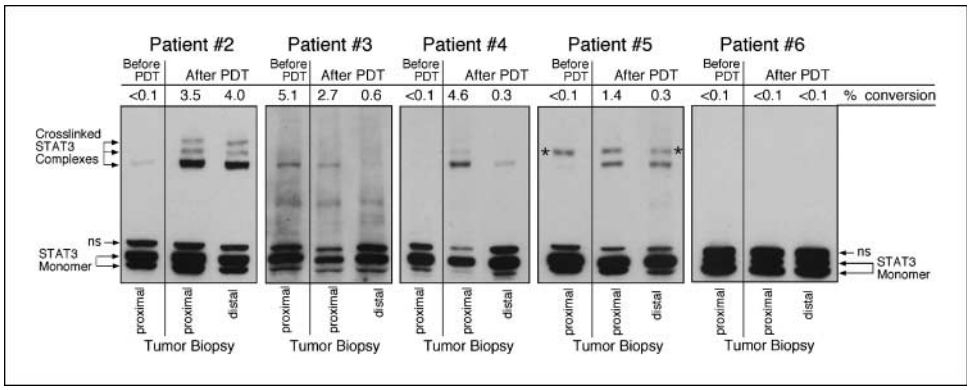

Fig. 7. STAT3 cross-links are generated in patients' endobronchial tumor tissue during PDT. Biopsies were obtained from patients with obstructive endobronchial non – small cell lung cancer tumors immediately before and after PDT. Patient 2 received 5.3 mg/m^2 HPPH, and all other patients received 4 mg/m^2 HPPH; patients 2 and 3 received 130 J/linear cm of light, and patients 4, 5, and 6 received 140 J/linear cm. Biopsy samples were extracted and aliquots of the extracts, containing equal amounts of protein, were analyzed for STAT3 by immunoblotting. The relative amount of cross-linked STAT3 in each sample was determined (% conversion) and listed at the top of the immunoblots. Immunostained bands bracketed by stars "*" in the panel for patient 5 and marked by "ns" denote nonspecific cross-reacting material.

analyzed earlier, such as FaDu, a human hypopharyngeal carcinoma – derived cell line (11). Only the human prostate cell line PC3, which lacks STAT3, did not show protein forms that reacted with anti-STAT3.

The experiments carried out in preclinical tumor models reveal two important aspects, namely the absence of any STAT3 cross-linking in photosensitizer-containing tumor tissue in the absence of light exposure (Fig. 4, contralateral tumors) and the relatively high reproducibility of results among different animals. Examination of tumor subsections suggests the expected relationship between STAT3 cross-linking and light fluence (i.e., distance from the light source). As fluence of light penetrating through tissue decreases exponentially as a function of distance from the light source (16, 19), cross-linking was least at the bottom of tumors illuminated from the top. Cross-links were also detectable, although at lower levels, in the normal tissue adjacent to the treatment field, indicating that stray light diffusing through the tissue was sufficient to cause a biological effect and also showing the sensitivity of STAT3 cross-links in reporting low PDT exposures. Although there are numerous variables that can limit the PDT reaction, it is clear that in these small and relatively homogeneous experimental tumors light dose is the dominant factor determining tissue response.

PDT has been shown *in vitro* to cause the loss/degradation of membrane receptor proteins, such as growth factor (26) and cytokine receptors (7). We now show that in regions of strong STAT3 cross-linking *in vivo* cytokine signaling can be compromised not only by the reduction of available STAT3 but also by the reduction/elimination of membrane cytokine receptors. Thus, strong STAT3 cross-linking indicates the delivery of a PDT dose sufficient to initiate other biological events, such as apoptosis and changes in signal transduction.

Side by side comparisons of STAT3 cross-linking in PDT-treated tumors and effectiveness of identical treatments in parallel experiments of tumor control also show a strong relationship between STAT3 conversion and tumor cure, with significant increases in cross-linked STAT3 corresponding to a significant increase in tumor cures. The highest percentage of cross-linked STAT3 in tumors (highest value, ~25%; median, ~12%) was less than observed in cell monolayers. This can be explained by the fact that the measured values are the average of the whole tumor, including regions that were distant from the light source and received less PDT dose.

The feasibility of using STAT3 cross-links as a "dosimeter" for the PDT response was evaluated in five of six patients undergoing PDT for endobronchial tumors. As expected, the percentage of conversion from monomeric STAT3 to STAT3 complexes in most cases decreased with distance from the light source, reflecting the fall-off in fluence. The variability in the values for STAT3 cross-links, however, was large. This may reflect differences in the light penetrance through the tumor due to bleeding or other heterogeneities, such as in tissue oxygenation. It also may in part simply be due to the lack of precision in tumor sampling inherent in the procedure. However, most importantly, the negative results obtained from patient 6, the only patient who did not respond to treatment, indicate that lack of STAT3 cross-links and other proteins detectable immediately after PDT treatment correctly predicted treatment failure. In our clinical experience with the treatment of a wide range of solid tumors, complete lack of response is seen rarely. It is thus remarkable that this nonresponse was reflected in the STAT3 cross-link assay. With photosensitizers that have relatively slow pharmacokinetics, such as Photofrin and, to a lesser degree, HPPH, it is possible to retreat without drug reinjection if regional or complete treatment failure is identified early. The described assay might help in this early detection of treatment failure. This approach also might permit evaluation of procedure-associated photoreactions, such as illumination of the treatment site with inspecting light sources causing appearance of cross-linked STAT3 in "pretreatment" samples as well as of unintended light exposure of normal tissue.

In summary, the results from our *in vitro*, *in vivo*, and preliminary clinical studies strongly support the value of STAT3 cross-linking as measure for PDT reaction. They further provide evidence for the potential value of STAT3 cross-links as a molecular marker capable of early prediction of treatment success or failure. Accurate light dosimetry is still a major challenge in the clinical application of PDT, especially when

applied in hollow organs with poor symmetry. Although systems are being developed that can directly measure *in situ* the cytotoxic agent (singlet oxygen) generated by a given treatment, these are not yet clinically available. Therefore, the identification of a surrogate molecular marker for absorbed PDT dose is of high clinical relevance.

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