Photodynamic Therapy–Induced Cell Surface Expression and Release of Heat Shock Proteins: Relevance for Tumor Response

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Abstract

Almost instantaneously after the treatment of mouse SCCVII tumor cells with Photofrin-based photodynamic therapy (PDT), a fraction (15-25%) of total cellular heat shock protein 70 (HSP70) became exposed at the cell surface. The level of this surface-expressed HSP70 then remained unchanged for the next 6 hours and persisted at lower levels even at 18 hours after PDT. A similar induction of surface HSP70 expression was found with PDT-treated human umbilical vein endothelial cells. The same analysis for several other HSPs revealed the induced surface expression of HSP60 and GRP94, but not GRP78, on PDT-treated SCCVII cells. A fraction of total HSP70 existing in SCCVII cells at the time of PDT treatment was promptly (within 1 hour) released from cells after high treatment doses, whereas even lower PDT doses induced a substantial HSP70 release at later time intervals. Macrophages coincubated with PDT-treated SCCVII cells displayed elevated levels of both HSP70 and GRP94 on their surface and were stimulated to produce tumor necrosis factor α, whose production was inhibited by the presence of antibodies against either HSP70, Toll-like receptors 2 and 4, or specific NF-κB inhibitor in the coincubation medium. The induction of cell surface expression and release of HSPs by PDT may represent an important event in the response of tumors to this treatment modality with a critical role in the induced inflammatory and immune responses that contribute to the therapeutic outcome. (Cancer Res 2005; 65(3): 1018-26)

Introduction

Heat shock proteins (HSP) are highly conserved, abundantly expressed proteins with diverse functions (1–5). They were first characterized as intracellular molecular chaperones of nascent proteins (during their synthesis, folding, transport, assembly, and stabilization), and of degradation of naïve, aberrantly folded, damaged, or mutated proteins (2). In addition to these housekeeping and cytoprotective roles, other major functions of HSPs have been recently revealed. These molecules are now recognized as participants in signal transduction pathways (5, 6) and important regulators of inflammatory and immune response (3, 7–9). A substantial increase in the synthesis of HSPs is induced by a wide variety of stimuli including physiologic (cell growth and differentiation and tissue development), pathologic (infections, inflammation, malignancy, or autoimmunity), or environmental stress (heat shock, heavy metals, and oxygen radicals; refs. 1, 9).

Although HSPs were until recently been assumed to localize exclusively at various intracellular sites (cytoplasm, mitochondria, endoplasmic reticulum, and nucleus), it is now clear that these molecules can be also expressed on outer cellular membranes and even released from damaged and viable cells (4, 10, 11). Cell surface localization of HSP70, a major inducible member of HSPs, has been verified by immunofluorescence, cell surface biotinylation, electron microscopy, and proteomic profiling (12–14). In a recent study, 54 of 74 examined primary human tumors (head-and-neck cancers) were found positive for membrane-expressed HSP70, whereas none of the examined corresponding normal tissues tested positive (11). A similar finding was reported with biopsy material of leukemic patients (15). Moreover, chemotherapy, radiotherapy, and hyperthermia were reported to induce or increase surface HSP70 expression on treated cancer cells (11, 16). The function of HSPs localized in the outer cellular membrane is not well understood. It has been suggested to be associated with the capacity of these molecules to stabilize lipid membranes and preserve their integrity during thermal fluctuations and other insults (17), as well that HSPs participate in the cation channel formation in plasma membranes (18–20). Another possibility, suggested by Kurucz et al. (13) based on their electron microscopy study, is that HSP70 is transported to and anchored at the cell surface as part of a larger molecular complex and may be involved in functioning of certain surface receptors.

Photodynamic therapy (PDT) is a regulatory-approved modality used for the treatment of various oncologic and nononcologic lesions (21, 22). The destruction of targeted tissue by PDT is initiated by the generation of highly reactive oxygen radicals formed by the transfer to molecular oxygen of energy absorbed by photosensitizing drugs during light treatment (21). We have proposed that photooxidative lesions produced in PDT-treated tumors are recognized by the host as "altered self" (23), prompting a strong inflammatory and immune response that includes the development of adaptive immune response against the treated cancerous lesion, which contributes to the therapeutic outcome of PDT (21). Because the basic insult inflicted by PDT is a form of oxidative stress, it is not surprising that this treatment was found to induce the expression of various HSPs and this response is at the level of transcription (24, 25).

The present study shows that PDT induces cell surface expression and release of HSPs from treated cells. It also shows that HSP70 released from PDT-treated tumor cells is captured by macrophages triggering in these cells Toll-like receptor (TLR)–based signal transduction activity resulting in the production of inflammatory cytokine tumor necrosis factor α (TNFα). These findings strongly support the pivotal role of HSPs and innate

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immune responses mediated by these molecules in PDT-induced host response that is known to influence clinical efficacy of cancer treatment with this modality (21).

Materials and Methods

Cell Cultures and Tumors. Mouse SCCVII squamous cell carcinoma cells (26) were cultured in alpha minimal essential medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT). Human umbilical vein endothelial cells were grown on 0.8 mg/mL fibronectin-coated dishes in medium 199 (Sigma Chemical) containing 10% horse serum (HyClone Laboratories) and glutamine at 292 μg/mL and they were used within the first four passages of the primary culture. The experimental tumors were initiated by injecting s.c. 30 μL of suspension containing 1×10^6 SCCVII cells into lower dorsal region of syngeneic 7- to 9-week-old female C3H/HeN mice. The tumors were treated by PDT when they reached 7-8 mm in largest diameter. The experimental procedures involving mice were approved by the Animal Care Committee of the University of British Columbia.

Photodynamic Therapy *In vitro* and *In vivo*. The photosensitizer Photofrin used for PDT was provided by Axcan Pharma, Inc. (Mont Saint-Hilaire, Quebec, Canada). For *in vitro* PDT, cells growing in 30-mm-diameter Petri dishes were incubated with Photofrin (5-50 μg/mL in complete growth medium), and 24 hours later the Petri dishes were rinsed with cold PBS, placed on ice and exposed to a beam of 630 ± 10 nm light for the exposure of 1 J/cm^2 (15 mW/cm^2). For *in vivo* PDT, mice were injected Photofrin (10 mg/kg i.v.), and 24 hours later the tumors were treated with 630 ± 10 nm light (150 J/cm^2; 110-120 mW/cm^2) while the mice were restrained unanesthetized in holders exposing their backs. The chosen PDT dose would cure SCCVII tumors in 5 of 8 mice. The light was generated by a high throughput fiber illuminator (Sciencetech, Inc., London, Ontario, Canada) equipped with 150 W QTH lamp with integrated ellipsoidal reflector and 630 ± 10 nm interference filter. A liquid light guide, model 77638 (8-mm core diameter, Oriel Instruments, Stratford, CT), was used for delivering light for monodirectional superficial illumination. The effect of PDT *in vitro* was compared with that of edelfosine (Calbiochem, Merk KGaA, Darmstadt, Germany) added at 25 μg/mL to the growth medium for 1 hour at 37°C.

Flow Cytometry Analysis. After *in vitro* PDT, the cells were incubated for different time intervals in complete growth medium at 37°C and then detached using rubber policeman. For staining of cell surface–expressed HSPs, the samples were incubated 20 minutes on ice with the following antibodies: mouse anti-HSP60 (SPA-829) monoclonal antibody or goat anti-HSP60 polyclonal antibody (K-19), chicken anti-HSP70 polyclonal antibody (K-20) or FITC-conjugated mouse anti-HSP70 monoclonal antibody (SPA-810FI), goat anti-GRP78 polyclonal antibody (C-20), and rabbit anti-GRP94 polyclonal antibody (H-212). All these antibodies obtained after immunization with human HSP (or recombinant peptides mapping its segments) are cross-reactive with mouse equivalents. They recognize epitopes localized near the COOH terminus of HSP molecules, except for SPA-829 which recognizes a HSP60 region (amino acids 288-366) nearer to the NH_2 terminus (27). The antibodies SPA-829 and SPA-810FI were obtained form Stressgen Biotechnologies (Victoria, British Columbia, Canada), whereas the other anti-HSP antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Secondary antibodies conjugated with FITC used for the visualization of HSP staining were donkey anti-goat IgG (705-095-147) and donkey anti-rabbit IgG (711-095-152) from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) as well as goat anti-chicken IgY (Gallus Immunotech, Inc. Fergus, Ontario, Canada). In all cases, isotype control staining was also done and if necessary the fluorescence values obtained were used for the background subtraction. ChromPure whole molecule chicken IgY, mouse IgG, goat IgG and rabbit IgG (Jackson ImmunoResearch Laboratories) were used for isotype controls, except for FITC-conjugated ChromPure Mouse IgG (also from Jackson ImmunoResearch Laboratories) used as a control for FITC-conjugated mouse anti-HSP70. For intracellular staining of HSP70, the cells were first fixed and permeabilized using Cytofix/Cytoperm solution (PharMingen, BD Biosciences, Mississauga, Ontario, Canada). Phycoerythrin-conjugated monoclonal active caspase-3 antibody apoptosis kit (PharMingen) was used for flow cytometry-based determination of apoptosis associated with PDT treatment following the protocol recommended by the manufacturer (28). Briefly, after staining for surface HSP70, the cells were fixed and permeabilized using Cytofix/Cytoperm solution and then stained with PE-conjugated monoclonal rabbit antibody against active caspase-3 (a marker for cells undergoing apoptosis) diluted in Perm/Wash buffer provided in the kit. Flow cytometry analysis of all stained samples including appropriate staining controls was done with a Coulter Epics Elite ESP (Coulter Electronics, Hialeah, FL). Cell in advanced necrosis and cell fragments were excluded based on their light scatter signals, whereas cell aggregates were gated out using the "time of flight" variable.

For the analysis of surface-expressed HSPs in tumor cell populations, SCCVII tumors were excised 16 hours after being treated by Photofrin-PDT and dissociated into single cell suspensions (29). The cell yield/g tumor tissue was $5.2 \pm 1.4 \times 10^7$ with nontreated and $2.0 \pm 0.6 \times 10^7$ with PDT-treated tumors. In addition to staining for surface HSPs as described above, the cells were stained either with PE-conjugated rat anti-mouse CD45 or PE-conjugated rat anti-mouse Gr1 (both from PharMingen). In the case of staining for panleukocyte marker CD45, cell populations were distinguished as $CD45^{bright}$ (tumor-associated leukocytes) and $CD45^{dim}$ (malignant cells). Following staining for myeloid differentiation antigen GR1 (Ly-6G), cell populations were separated into $GR1^{++}$ (neutrophils), $GR1^{+}$ (macrophages) and $GR1^{--}$ (malignant cells).

Coincubation of Macrophages with Photodynamic Therapy–Treated SCCVII Cells. Cultures with macrophages from nontreated SCCVII tumors were prepared using a differential attachment procedure employed in our earlier studies (30). Briefly, single cell suspension obtained from SCCVII tumors that was resuspended in serum-free cell growth medium was plated into Petri dishes and placed into a 37°C incubator. After 15 minutes, nonattached cells were washed away leaving the attached cells that consisted almost exclusively of macrophages. Meanwhile, *in vitro* cultured SCCVII tumor cells were plated into 25-mm tissue culture inserts with a 0.02-μm anapore membrane base (Nalge Nunc International, Naperville, IL). Following the exposure of these SCCVII cells to Photofrin (20 μg/mL) for 24 hours and then to light (1 J/cm^2), the inserts were transferred to Petri dishes with nontreated tumor-derived macrophages. After 16 hours of coincubation, during which the cells were kept in serum and protein-free medium (S8284, Sigma Chemical), the inserts were removed and macrophages harvested using rubber policeman. The macrophages were then analyzed either for HSP surface expression (as described above) or for the levels of TNFα produced in these cells. The intracellular staining with PE-conjugated rat anti-mouse TNFα (PharMingen) was done with paraformaldehyde-fixed and saponin-permeabilized cells as in the above-described intracellular staining protocols. Matching isotype control and blocking control antibodies produced by PharMingen were also used. GolgiPlug, protein transporter inhibitor based on brefeldin A (PharMingen), was added at a final concentration 1 μg/mL to the media for the last 12 hours of macrophage-tumor cell coincubation in order to allow TNFα accumulation in the producing cells. In addition, chicken anti-HSP70 polyclonal antibody K-20, rat anti-mouse TLR2 or rat anti-mouse TLR4/MD2 (both blocking antibodies, obtained from eBioscience, San Diego, CA), or TIRAP inhibitor peptide (Calbiochem 613570) or selective NF-κB inhibitor SN50 (Calbiochem 481480) were present in some samples during the 16 hours coincubation period to block or inhibit the target molecules analyzed for their role in the induction of macrophage production of TNFα. The antibodies were used in this experiment at 10 μg/mL, whereas cell permeable TIRAP inhibitor peptide and SN50 peptide were tested at 110 and 50 μg/mL, respectively.

HSP70 ELISA. The amount of HSP70 released from PDT-treated SCCVII cells was determined by HSP70 ELISA using a commercially available kit

from Stressgen Biotechnologies. The supernatants of cultures in which SCCVII cells were kept at 37°C for different post-PDT time intervals were collected and stored at −70°C until used for ELISA by adding to the microtiter wells coated with mouse anti-HSP70 monoclonal antibody (SPA-810). The captured HSP70 was detected with a HSP70-specific biotinylated rabbit polyclonal antibody that was subsequently bound by an avidin-horseradish peroxidase conjugate.

Results

Treatment of mouse SCCVII tumor cells with Photofrin-based PDT *in vitro* results in a change in the localization of HSP70. This was determined using flow cytometry analysis following intracellular and surface staining of SCCVII cells with chicken anti-HSP70 polyclonal antibody (K-20). Whereas nontreated SCCVII cells showed a strong HSP70 fluorescence following intracellular staining protocol, no significant HSP70 levels were detectable on the cell surface. In contrast, SCCVII cells analyzed after PDT treatment showed a significant staining for surface HSP70 (about 15-25% of the levels detected by intracellular staining) that was during the first 2 hours post-PDT associated with a corresponding decrease in the intracellular levels of this HSP (Fig. 1*A*). A drop in intracellular HSP70 levels was evident at 6 hours post-PDT that was followed by their increase 6 hours later.

Further analysis of surface expressed HSP70 on SCCVII treated with the same PDT dose (which kills >80% of these cells) is depicted in Fig. 1*B*. The results show that surface HSP70 localization on SCCVII cells became evident practically immediately after photodynamic light exposure and persisted at a similar level between 15 and 120 minutes after PDT. A comparable level of surface HSP70 was induced on SCCVII cells collected 2 hours after exposure to a noncytotoxic concentration of edelfosine, synthetic alkyllysophospholipid known to induce surface expression of HSP70 (31). At 18 hours post-PDT, many treated cells were detached from the Petri dish substrate (evidently due to lethal effects of PDT) and the level of HSP70 on their surface was dramatically elevated. The surface HSP70 levels on the cells that were still attached at this time point were lower than those registered during the first 2 hours post-PDT.

The cell surface expression of HSP70 was PDT dose dependent and was not detectable in control samples with cells exposed to Photofrin only or treated with light without exposure to the photosensitizer (Fig. 1*C*). Following PDT, higher levels of surface HSP70 were found on apoptotic cells (identified in flow cytometry analysis by positive staining to the active form of caspase 3) than in nonapoptotic cells (Fig. 1*C*, *inset*). The percentage of apoptotic cells determined at 18 hours after PDT has not significantly increased compared with 1 hour after PDT (data not shown). Even a small fraction (0.6%) of cells undergoing spontaneous apoptosis present in nontreated SCCVII cell culture were found to contain surface HSP70. The PDT treatment–induced surface HSP70 expression is not restricted to tumor cells but can also be detected on normal cells, as shown with PDT-treated human endothelial cell culture (Fig. 1*D*).

To examine the possibility that the PDT-induced effect on HSPs is further manifested as a release of these proteins from the treated cells, we used HSP70 ELISA to determine its levels in culture supernatants of PDT-treated cells. The results reveal that there was a significant HSP70 release from PDT-treated SCCVII cells at 1 hour after photodynamic light treatment (Fig. 2). This was evident with highly cytotoxic PDT doses (50 μg/mL Photofrin for 24 hours followed by 1 J/cm^2). A lower PDT dose with Photofrin concentration reduced to 20 μg/mL, which induced surface HSP70 expression (Fig. 1*B*), produced no detectable HSP70 release from the treated cells during the first two hours post treatment. However, at 18 hours post-PDT, there was a strong HSP70 release from the treated SCCVII cells even with this lower PDT dose (Fig. 2).

Next experiments were focused on determining whether the PDT-induced cell surface expression is exhibited exclusively with HSP70 or shared by other types of HSPs. The detection of HSP70 was in this case based on the use of a different antibody. The results show that at 1 hour post-PDT, surface expression was detectable not only for HSP70 but also for HSP60 and GRP94 (Fig. 3). The effect was most pronounced with GRP94. There was no evidence of the surface expression of GRP78 on these PDT-treated cells. Only HSP70 but not other HSPs were detected on the surface of nontreated cells. Two antibodies, SPA-829 and K-19, were assayed for detection of surface HSP60. The results show that surface HSP60 was detected on PDT-treated cells with the antibodies raised against an epitope at the COOH terminus of HSP60 (K-19), whereas the antibody recognizing a peptide segment near the NH_2 terminus of the molecule (SPA-829) was not detecting surface-expressed HSP60 (Fig. 3). The antibodies used for identifying other HSPs were specific for epitopes located near the COOH terminus of these molecules. The antibody SPA-810 (mouse monoclonal IgG1) used in this experiment for detecting surface HSP70 seems more effective than K-20 employed in the experiments depicted in Fig. 1, because it revealed the presence of surface HSP70 on nontreated SCCVII cells. However, SPA 810 seems not suitable for intracellular staining protocols, which prevents its use for detecting cytoplasmic HSP70. Both SPA-810 and K-20 are not cross-reactive with the constitutively expressed isoform, 70-kDa heat shock cognate protein (HSC70).

Following these *in vitro* studies, the surface expression of HSPs was also examined with cells contained in tumors treated *in vivo* by Photofrin-based PDT. The tumors were excised at 16 hours after photodynamic light treatment and cell suspensions obtained by enzymatic dissociation of tumor tissues were stained with antibodies against HSPs and leukocyte membrane markers. About 10% of these cells stained positively for the apoptotic marker (active caspase 3), whereas the rest seemed viable. The cells stained for surface HSP70 were also stained for panleukocyte marker CD45, whereas for the analysis of HSP60, GRP78, and GRP94, tumor leukocyte population was further differentiated as neutrophils and macrophages. Even in nontreated control lesions, tumor microenvironment caused surface expression of HSP70 on both cancer cells and leukocytes, whereas elevated surface HSP60 and GRP94 were found on tumor-associated neutrophils. A significant PDT-induced increase in surface expression of HSP70 was detected with CD45-negative cells (cancer cells), whereas there was no significant change with CD45-positive cells (i.e., tumor-associated leukocytes; Fig. 4*A*). Surface HSP60, which was not significantly expressed on cancer cells either before or after PDT, was markedly elevated on neutrophils and macrophages in PDT-treated tumors (Fig. 4*B*). A similar situation was found with surface GRP78 (Fig. 4*C*). With respect to GRP94, PDT-induced changes were dominated by an increase in surface expression of this molecule in macrophage populations (Fig. 4*D*).

Further experiments were designed to explore the possibility that HSPs released from PDT-treated cancer cells can be captured by tumor-localized leukocytes and elicit a TLR-mediated signaling leading to cytokine production by these cells. Cultures with

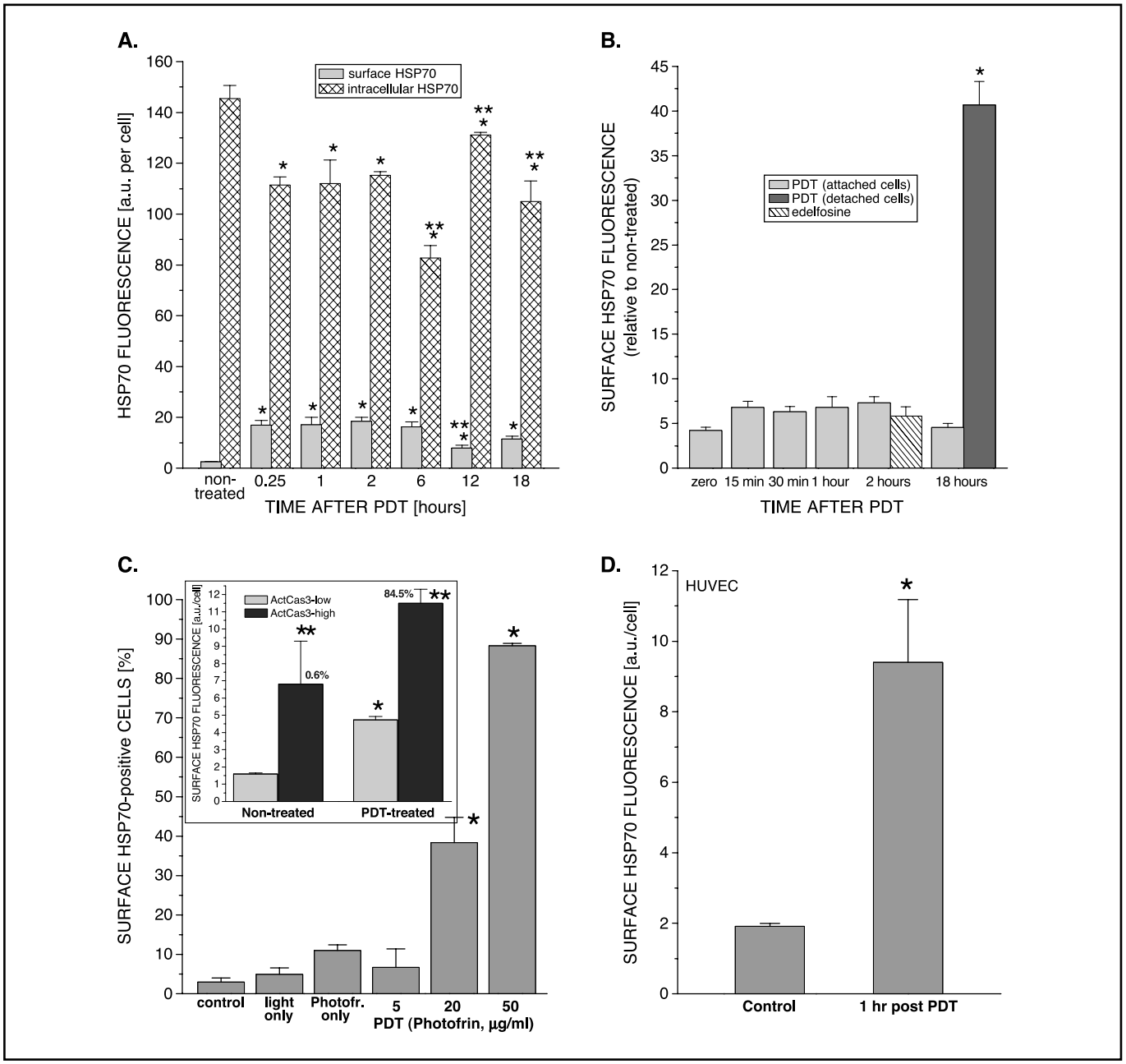

Figure 1. Expression of HSP70 on the surface of tumor and endothelial cells treated by Photofrin-based PDT. *In vitro* cultured SCCVII tumor cells or HUVEC endothelial cells were exposed to indicated Photofrin concentrations for 24 hours, washed in PBS, and treated by 630 ± 10 nm light (1 J/cm^2). The cells were then further incubated in growth medium at 37°C for different time intervals before they were harvested (attached cells only except where noted), stained using either surface or intracellular staining protocol with chicken anti-HSP70 polyclonal antibody (K-20) followed by FITC-conjugated goat anti-chicken IgY antibody, and analyzed by flow cytometry. *A,* surface and intracellular HSP70 levels for SCCVII cells exposed to 20 μg/mL Photofrin followed by 1 J/cm^2 light treatment, or sham-treated, and left in culture for different time intervals. *B,* relative surface HSP70 levels (ratio to control) on SCCVII cells exposed to 20 μg/mL Photofrin plus 1 J/cm^2 light and left in culture for different time intervals, or to edelfosine (25 μg/mL for 1 hour) and collected 2 hours later. *C,* percentage of cells positively stained for surface HSP70 in SCCVII cultures exposed to 0, 5, 20, or 50 μg/mL Photofrin plus 1 J/cm^2 light, light only, or sham-treated, and left in culture for 1 hour. *Inset,* surface HSP70 levels on SCCVII cell populations discriminated by staining with anti-active caspase 3 apoptotic marker antibody for control and PDT-treated (20 μg/mL Photofrin plus 1 J/cm^2) cells (% apoptotic cells). *D*, surface HSP70 levels on control and PDT-treated (20 μg/mL Photofrin plus 1 J/cm^2) *HUVEC*. *Bars,* SD (n = 4). *, $P < 0.05$, statistically significant difference compared with the respective control (*A*, *C*, *C inset*, and *D*), or to other time points (*B*). **, $P < 0.05$, statistically significant difference compared with the previous time point (*A*) or to the ActCas3-low level (*C*). Statistical analysis for all Fi was based on the unpaired Student's *t* test.

tumor-associated macrophages from nontreated SCCVII tumors were prepared by selective removal of cancer cells and other leukocytes as described in Materials and Methods. *In vitro* cultured SCCVII tumor cells were plated into 25-mm culture inserts, and after they were treated with Photofrin-based PDT (or sham treated for the controls) the inserts were placed into Petri dishes containing cultures of nontreated macrophages. After 16 hours of coincubation with PDT-treated SCCVII cells, the

macrophages were harvested and after cell surface staining with antibodies raised against HSP70 or GRP94 they were analyzed by flow cytometry. The results show that these macrophages displayed elevated levels of both HSP70 and GRP94 on their surface (Fig. 5*A*). Surface HSP levels on macrophages were even greater when the coincubation with PDT-treated SCCVII cells was in the cold (4°C; data not shown), which suggests the occurrence of endocytosis of receptors with bound HSPs. To a lower extent, the increase in surface GRP94 expression was also evident on macrophages that were in contact with nontreated SCCVII cells.

Additional sets of samples were used to examine whether macrophages coincubated with PDT-treated SCCVII cells become activated to produce a chosen cytokine, TNFα. The results of flow cytometry analysis following intracellular staining show a significant increase in TNFα fluorescence in macrophages coincubated with PDT-treated SCCVII cells compared with macrophages incubated alone or with nontreated SCCVII cells (Fig. 5*B*). Obstructing the interaction of HSP70 with either TLR2 or TLR4 (by the addition of specific antibodies to the coincubation medium) abrogated in the macrophages the induction of TNFα production. The presence of a selective NF-κB inhibitor also diminished the PDT-induced production of this cytokine. In additional testing following the same protocol, the involvement of TLR4 signaling pathway was confirmed with the TIRAP inhibitor peptide which blocks the engagement of this adapter molecule that binds to the cytoplasmic tail of TLR4 and functions independently of IL1R (32). The PDT-induced TNFα fluorescence in this experiment was reduced from 3.2 ± 0.4 to 1.6 ± 0.5 a.u. per cell in the samples with TIRAP inhibitor.

Discussion

The results presented in this report suggest that PDT has a profound effect on HSPs in treated cells, which could have a significant bearing on the therapeutic outcome. This effect is far from being limited to the up-regulation of *HSP* genes established from earlier reports of various investigators (24, 25). The present study provides evidence that PDT prompts a surface expression of various HSPs on cells in treated tumors as well as on *in vitro* treated cancer and endothelial cells and shows that HSP70 is released from PDT-treated cells.

Almost instantaneously after *in vitro* PDT treatment, a fraction of total cellular HSP70 becomes exposed at the cell surface (Fig. 1*B*). This surface-localized HSP70 seems to remain then at similar levels during the first 6 hours after PDT. Although less pronounced then during the initial hours after PDT, surface expression of HSP70 persisted on attached SCCVII cells even at 18 hours after PDT. A further decrease (after the initial drop) in intracellular HSP70 levels occurred between 2 and 6 hours post-PDT (suggesting its release from cells), which was followed by its restoration at 12 hours post-PDT (Fig. 1*A*). The latter presumably correlates with the up-regulation of *HSP70* gene induced by PDT (24, 25).

The extent of the initial surface HSP70 expression is related to the PDT dose. Following the treatment that renders over 80% of apoptotic cells (20 μg/mL Photofrin plus 1 J/cm^2), about 15% to 25% of total HSP70 was detected localized at the cell surface. About the same effect had the treatment with edelfosine (Fig. 1*B*), which in this regard has an effect comparable to hyperthermia (31). Interestingly, a similar percentage of total HSP70 molecules were found to be surface expressed on untreated CX+ cells, a stably HSP70 high expressing variant of human colon carcinoma line (10). A particularly pronounced surface HSP70 expression was found on apoptotic cells (Fig. 1*C*) and even more striking on detached (dying) cells collected at 18 hours post-PDT (Fig. 1*B*). Elevated surface expression of HSPs in cells undergoing apoptosis has been reported earlier (33). However, to a lesser degree surface HSP70 was also evident on SCCVII cells remaining viable after PDT treatment, which suggests that this phenomenon is not strictly dependent of cell death. HSPs, especially HSP70, were shown to be involved in the inhibition of both apoptotic and necrotic pathways (34). This inhibitory effect may be hindered by PDT-induced translocation of a fraction of HSPs to the cell surface.

The reason for this rapid emergence of surface-expressed HSP70 (which has no signal sequence and membrane-spanning region) and other HSPs remains to be elucidated. It is possible that photooxidative lesions in the membrane of PDT-treated cells cause the inner membrane sections to flop outward to the outer surface (35, 36) and expose HSP molecules associated with proteins or lipids lining the inner leaflet of the cell membrane. On the other hand, surface localized HSPs may have a role in the efforts of cells to stabilize damaged membranes and preserve their integrity (17, 37).

Boltzer et al. (10) have shown that the COOH-terminal domain of HSP70 is exposed when this molecule is expressed at the cell surface, whereas the ATP-binding site and NH_2-terminal domain remain unexposed within the membrane. In accordance with this, we found that surface expression of HSPs can be detected with antibodies specific for epitopes localized near the COOH-terminal

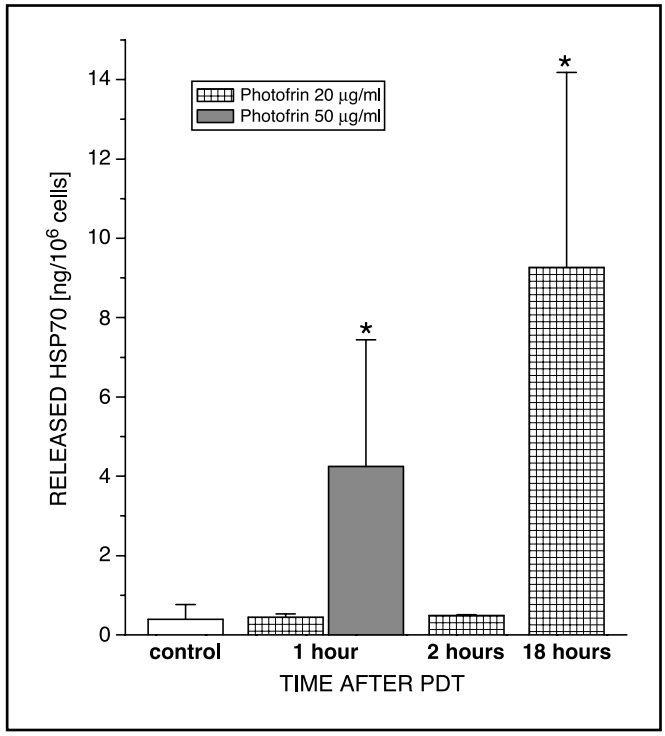

Figure 2. PDT-induced HSP70 release from SCCVII tumor cells. *In vitro* cultured SCCVII cells were treated by Photofrin-based PDT as described for Fig. 1, using the exposure to either 20 or 50 μg/mL Photofrin for 24 hours and the light dose of 1 J/cm^2. Following the indicated time of post-treatment incubation in growth medium, the cell culture supernatants were collected for the determination of HSP70 concentration based on the ELISA assay. *Bars,* SD (n = 4). *, P < 0.05, statistically significant difference compared with the control.

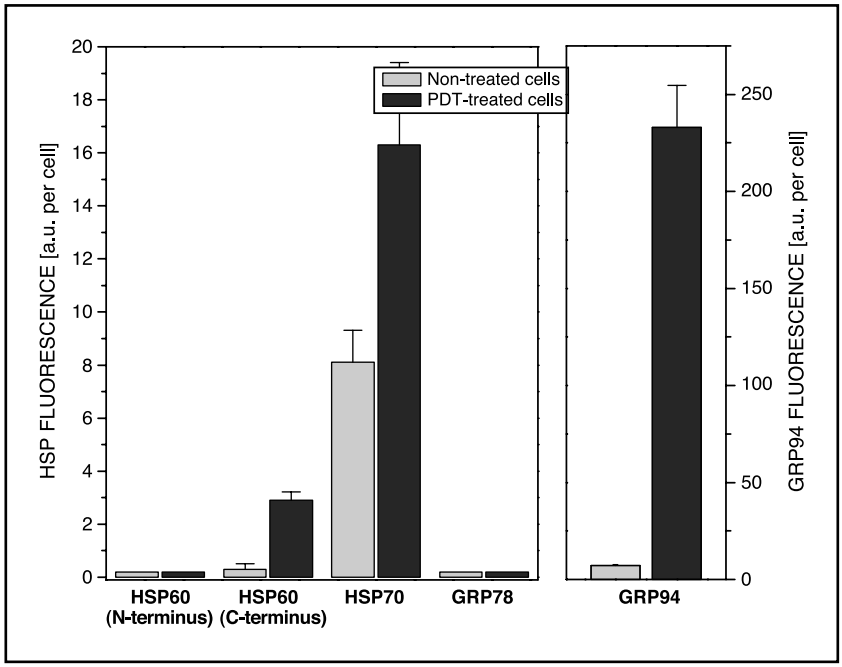

Figure 3. Surface expression of various HSPs on PDT-treated SCCVII cells. Following PDT treatment (Photofrin 20 μg/mL for 24 hours plus 1 J/cm^2), SCCVII cells were incubated for 1 hour at 37°C and then stained for surface expression of HSP60, HSP70, GRP78 and (in a separate experiment) GRP94 that was analyzed by flow cytometry as described in Materials and Methods. For HSP60 detection, the cells were stained either with K-19 (antibody recognizing an epitope near the COOH terminus) or with SPA-829 (antibody recognizing an epitope near the NH_2 terminus). All other antibodies used were raised against epitopes localized near the COOH terminus of respective HSP molecules. The antibody for HSP70 detection (SPA-810) was different than that used in the experiment described in Fig. 1. *Bars,* SD (n = 4). Except for the first pair, there was a statistically significant difference between the levels on PDT-treated and non-treated cells ($p<0.05$).

domain but not with antibodies recognizing sites near the NH_2-terminal domain (Fig. 3). Importantly, the COOH-terminal domain is involved in the interaction of HSP with its chaperoned client peptides (substrates) and endogenous peptides that may serve as a source of tumor rejection antigens (2, 38). Hence, altered/damaged cell proteins, which are more likely to be found attached to the substrate-binding domain of HSPs than normal nondamaged polypeptides (8), could become abundantly exposed to immune surveillance elements by the emergence of surface-expressed HSPs following PDT. The level of HSP70 expression in cancer cells was found to correlate with their immunogenicity (39), whereas specifically membrane-expressed HSP70 on such cells was shown to elicit CTL-mediated tumor immunity and also to correlate with sensitivity to allogeneic natural killer cells (40, 41).

In related studies, we obtained evidence that PDT-treated cells are attacked by complement, but their opsonization with complement proteins is reduced in the presence of HSP70-specific antibodies (42). It was reported that HSP70 can directly bind complement proteins (43). Hence, the opsonization of PDT-treated cells by complement proteins may occur either by direct binding to surface-expressed HSP70 or by binding to peptides associated with surface-expressed HSP70 that were recognized as altered self targets by the complement. Various types of immune cells, including macrophages, neutrophils, dendritic, natural killer, and B cells, have specific receptors for complement proteins as well as for HSPs (5) and are prone to using them to attack cells that are surface-expressing HSPs and/or are opsonized by complement proteins. Cytolytic activity of natural killer cells against tumor cells was found to be based on a specific interaction of natural killer cells with a 14-amino-acid HSP70 sequence present in the ectoplasmic domain of tumor cells (44).

In addition to inducing cell surface expression of HSPs, PDT treatment causes the release of HSP70 (Fig. 2) and probably other HSPs from tumor cells. Our results indicate that a fraction of HSP70 existing in tumor cells at the time of PDT is promptly (within 1 hour) released after very high treatment doses. Because after such doses a significant fraction of cells are known to die by necrosis, the source of detected HSP70 in this case can be its release from necrotic cells. However, our data also show that the release of substantial amounts of this HSP is induced even by lower PDT doses at later time intervals when there is an increase in HSP70 production in treated cells (24). Indirectly, the evidence for PDT-induced release of HSP70 and GRP94 is provided by the detection of elevated levels of these HSPs on macrophages that have been in contact with PDT-treated SCCVII cells (Fig. 5*A*). The capture of released HSPs by these macrophages seems more plausible than the possibility that they have produced HSPs themselves under influence of certain signals from PDT-treated cells, but this remains to be experimentally verified. It is already known that oxidative as well as thermal stress can induce HSP release. For instance, this was shown for cultured vascular smooth muscle cells exposed to oxidative stress (45), and for tumor transplanted into rat liver following laser thermotherapy (46).

The major receptor responsible for endocytosis of released HSPs is the α-macroglobulin receptor (CD91); HSP-associated peptides internalized in this way by antigen-presenting cells are represented by MHC class molecules (3). However, a number of other cell surface molecules were identified as receptors for HSPs, including TLR2 and TLR4 with their coreceptor CD14, the scavenger receptor (CD36), and the costimulatory molecule CD40 (5). Engagement of TLR2 and TLR4 driven by HSP60, HSP70, or GRP94 is now recognized as a major route of activation of dendritic cells and other antigen-presenting cells, as well as the means of up-regulation of numerous genes relevant for inflammatory and immune response (3, 47). Our finding of stimulated TNFα production in macrophages coincubated with PDT-treated tumor cells (Fig. 5*B*) reveals that the HSP70-mediated engagement of signal transduction pathways through TLR2 and TLR4 with downstream activation of NF-κB transcription factor is indeed a major mechanism involved in the induced generation of this

cytokine as a component of PDT-elicited host response. Interestingly, whereas HSP70 may stimulate in this way the production of TNFα it is also known to be capable of inhibiting the induction of cell death by this cytokine (48).

Cell surface expression of HSPs detected following PDT treatment of tumors (Fig. 4) reflects the complexity of the *in vivo* situation. In addition to cancer cells, SCCVII tumors contain significant numbers of various host cells largely dominated by the

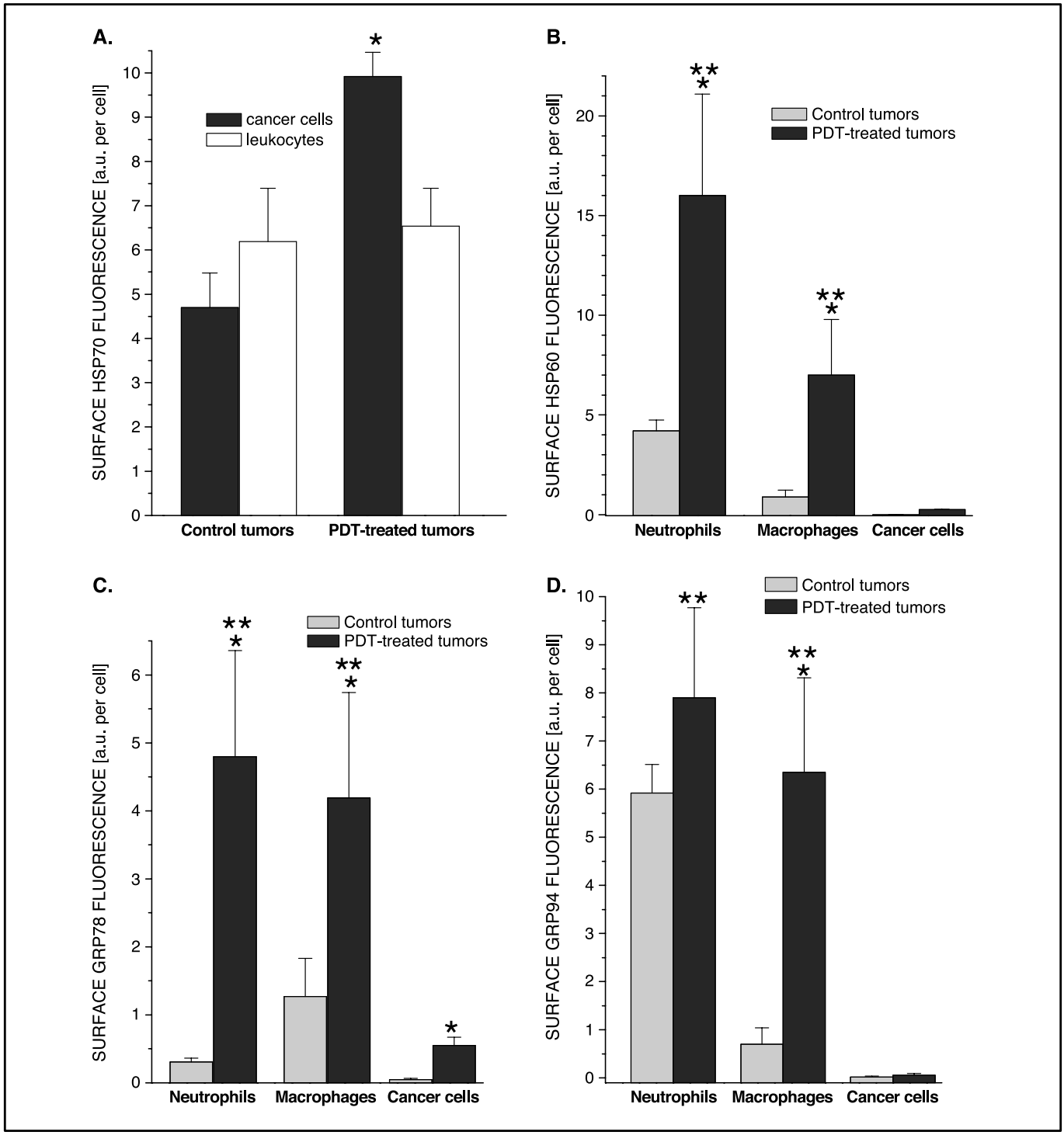

Figure 4. Surface expression of HSP60, HSP70, GRP74, and GRP94 on cells contained in PDT-treated SCCVII tumors. S.c. SCCVII tumors growing in C3H/HeN mice were treated by PDT (Photofrin 10 mg/kg i.v. followed 24 hours later by 150 J/cm^2). The tumors were excised 16 hours later and together with control tumors dissociated into single cell suspensions that were stained with antibodies for surface localized HSPs (or their isotype-matched controls) plus antibodies for identifying tumor-infiltrating leukocyte populations. Surface expression of HSP70 (*A*), HSP60 (*B*), GRP78 (*C*), and GRP94 (*D*) are shown with error bars representing SD ($n = 4$). *, $P < 0.05$, statistically significant difference compared with the level on the same cell type in control tumors. **, $P < 0.05$, statistically significant difference compared with the level on cancer cells in PDT-treated tumors.

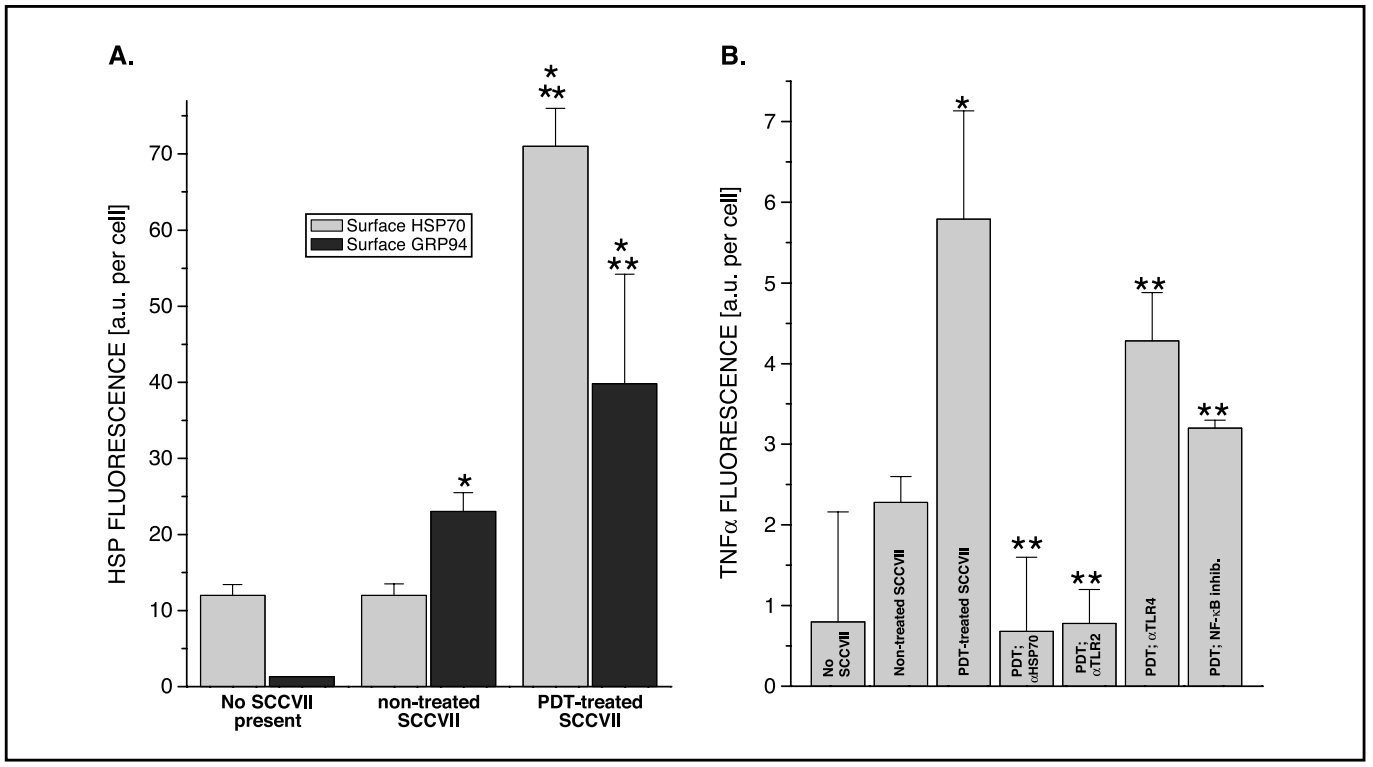

Figure 5. Surface expression of HSP70 and GRP94 on macrophages coincubated with PDT-treated SCCVII cells and their TNFα production. SCCVII cells growing in culture inserts were treated by PDT (Photofrin 20 μg/mL 24 hours plus 1 J/cm^2) and then added to Petri dishes with nontreated macrophages previously isolated from SCCVII tumors. Following 16 hours of coincubation, macrophages were harvested and stained either for surface expression of HSP70 and GRP94 (*A*), or used for intracellular staining of TNFα (*B*). The latter assay was also done with macrophages from samples that included antibodies to HSP70, TLR2, or TLR4 (10 μg/mL), or NF-κB inhibitor peptide SN50 (50 μg/mL) in the medium during the 16-hour coincubation. Results of controls obtained with macrophages incubated alone or coincubated with nontreated SCCVII cells. *Bars,* SD (n = 4). *, $P < 0.05$, statistically significant difference compared with the respective group with no SCCVII cells present. **, $P < 0.05$, statistically significant difference compared with the respective group with nontreated SCCVII cells (*A*), or between the levels with and without blocking agent (*B*).

two populations of tumor-associated leukocytes, macrophages, and neutrophils. Moreover, many of these cells are newly infiltrated, because PDT induces a massive infiltration of monocytes/macrophages and neutrophils into the treated tumors (49, 50). On the other hand, a large fraction of cells present in tumors at the time of treatment are killed and removed by phagocytes. Because tumor cells expressing surface HSPs are preferentially attacked by immune effectors and eliminated, it is likely that there is a positive selection for cells with low or no surface HSPs. Hence, surface expression of HSPs is probably induced by PDT *in vivo* (as it is *in vitro*) notwithstanding the finding of very low surface expression of HSP60, GRP78, and GRP94 on cancer cells at 16 hours post-PDT (Fig. 4). At the same time, relatively high levels of these HSPs were found on tumor-associated neutrophils and macrophages. The HSP molecules detected on these leukocytes may have been captured after being released from cells directly experiencing PDT-mediated oxidative stress. In contrast to other HSPs, cancer cells expressing elevated HSP70 were found in PDT-treated tumors and there was no dramatic increase induced by PDT in the surface expression of this molecule on tumor-associated leukocytes. Although the reasons for this difference are not clear at present, they may have to do with the kinetics of their endocytosis, differences in the PDT-induced up-regulation in the synthesis of these HSPs, or their release rate from stressed cells.

Based on the above, it can be postulated that the induction of cell surface expression and release of HSPs by PDT treatment represents an important event in the response of tumors to this modality. Liberated HSPs are now recognized to represent potent endogenous danger signals that alert the host to the presence of threat from infection or injury and orchestrate the self-protecting mechanisms mediated through a network of inflammatory and immune responses (3, 47, 51). This particularly pertains to the molecules identified in this study to have a prominent role in PDT response, HSP60, HSP70, and GRP98, which belong to the biologically most potent HSPs (3, 5, 47). Both tumor cell surface expression and extracellular manifestation of these HSPs after PDT serves as a strong stimulus for the interaction with antigen-presenting cells and other immune cells. Such interaction prompts the activation of these immune effectors and their killing of surface HSP-expressing targets. Our result with PDT-treated human umbilical vein endothelial cells (Fig. 1*D*) suggests that HSPs expressed on the endothelial surface in the blood vessels of PDT-treated tumors could be one of the elements provoking the destruction of tumor vasculature by this modality.

The presence of numerous tumor-derived peptides associated with HSPs provides the so-called antigenic fingerprint (4), and because HSPs interacting with immune cells following PDT will carry over such antigenic repertoire this can be expected to greatly facilitate the development of adaptive immunity against the PDT-treated tumor.

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