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ORIGINAL ARTICLE

Involvement of damage-associated molecular patterns in tumor response to photodynamic therapy: surface expression of calreticulin and high-mobility group box-1 release

Mladen Korbelik · Wei Zhang · Soroush Merchant

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Abstract Damage-associated molecular patterns (DAMPs), danger signal molecules expressed after injury or infection, have become recognized as prerequisite for orchestrating effective anti-tumor host response. The expression of two prototypical DAMPs, calreticulin and high-mobility group box-1 (HMGB1) protein, was examined following Photofrin™-photodynamic therapy (PDT) of Lewis lung carcinoma (LLC) cells in vitro and LLC tumors growing in syngeneic mice. Cell surface expression of calreticulin was found to be highly increased at 1 h after PDT treatment both in vitro and in vivo. Increased exposure of calreticulin was also detected on the surface of macrophages from PDT-treated LLC tumors. At the same time interval, a rise in serum HMGB1 was detected in host mice. Intracellular staining of macrophages co-incubated for 16 h with PDT-treated LLC cells revealed elevated levels of HMGB1 in these cells. The knowledge of the involvement of these DAMPs uncovers important mechanistic insights into the development of host response induced by PDT.

Keywords Calreticulin · DAMPs · HMGB1 · Lewis lung carcinoma · Photodynamic therapy

Introduction

Photodynamic therapy (PDT) has become a well-established clinical modality for treatment of tumors and is in development for other non-oncologic applications [1–3]. Because of the nature of its action, inflicting trauma in the targeted lesion through oxidative stress produced by light-activated drugs [1], PDT treatment is followed by a strong host reaction, including inflammation, acute phase response, and immune response [1, 4, 5].

It is now generally recognized that cell damage and disruption of tissue homeostasis caused by trauma from various insults are countered with an evolutionary highly organized host protection response with established mechanisms for detection, containment, and repair [4, 6–8]. Its integral part is a dedicated detection component based on sensors featuring pattern recognition receptors (PRRs) that recognize warning/alarm signals whose presence alerts to the appearance of danger as a consequence of injury to endogenous structures [6, 7, 9]. The detection of these signals prompts the activation of signaling pathways that in turn secure outputs in the form of physiological responses. Such danger signals include a variety of endogenous molecules displaying damage-associated molecular patterns (DAMPs); predominantly, these are abnormally exposed or dislocated molecules or their breakdown products [8, 10, 11]. In addition to passive release from necrotic cells and pulsatile release from apoptotic cells, DAMPs can be secreted from activated leukocytes in response to feed forward signals from the same and other DAMPs or cytokines [12]. Recognition of DAMPs by PRRs leads to two major developments: (1) activation of innate immune response that can also promote adaptive immune responses and (2) restoration of homeostasis by orchestrating reconstitution (healing) of the destroyed tissue [4, 8].

In recent years, it is becoming increasingly clear that anti-tumor PDT is particularly effective in generating an abundance of danger signals [4, 13]. Well-characterized DAMPs involved in PDT response in a manner compatible

M. Korbelik (✉) · W. Zhang · S. Merchant
British Columbia Cancer Research Centre,
British Columbia Cancer Agency, 675 West 10th Avenue,
Room 6.112, Vancouver, BC V5Z 1L3, Canada
e-mail: mkorbelik@bccrc.ca

with that function include heat-shock proteins, phosphatidylserine, cell membrane degradation products, including lysophospholipids and arachidonic acid metabolites, extracellular matrix components, and fibrinogen [4, 13, 14].

This report presents evidence of the involvement in tumor response to PDT of the two best-known stress-induced DAMPs: calreticulin and high-mobility group box-1 (HMGB1). The former becomes expressed on the surface of PDT-treated cells, while the latter is released into peripheral blood of tumor hosts after PDT.

Materials and methods

Tumors and cells

Lewis lung carcinoma cells (LLC, ATCC No CRL-1642) were cultured in alpha minimal essential medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (HyClone Laboratories Inc., Logan, UT) or implanted in the lower dorsum of syngeneic C57BL/6 mice by subcutaneous injection to render cohorts of experimental tumors as described in more detail elsewhere [15]. The procedures with mice were defined by the protocol approved by the Animal Care Committee of the University of British Columbia. The medium used for LLC cells was also employed for maintaining cultures of mouse macrophage cells IC-21 (ATCC No TIB-186). For co-incubation with IC-21 cells, LLC cells were grown attached to tissue culture inserts with porous (0.4 μm) polycarbonate membrane base (Millicel-PCF inserts, Millipore Corporation, Billerica, MA).

PDT treatment

For PDT treatment of tumors, mice with LLC tumors reaching 7–8 mm in largest diameter received Photofrin™ (provided by Axcan Pharma Inc., Mont-Saint-Hilaire, QC, Canada) injected intravenously at 10 mg/kg. The tumors were treated with light 24 h later using a FB-QTH high-throughput illuminator (Sciencetech Inc., London, Ontario, Canada) furnishing a 150 W QTH lamp with integrated ellipsoidal reflector and mounted 630 ± 10 nm interference filter. Light delivery for superficial illumination was secured by an 8-mm-diameter liquid light guide (model 77638 by Oriel Instruments, Stratford, CT). The light dose used was 150 J/cm^2 with fluence rate of 80–90 mW/cm^2. For in vitro PDT treatment, LLC cells growing in 30-mm-diameter Petri dishes were incubated with Photofrin™ (20 μg/ml) for 24 h in complete growth medium and then rinsed with phosphate-buffered saline before exposure to the light dose of 1 J/cm^2 at the fluence rate 20 mW/cm^2.

Flow cytometry

Samples with cell suspension were obtained either from in vitro cultures using a cell scraper or from tumors by enzymatic disaggregation as described earlier [16]. For detection of surface expression of calreticulin, cells were stained with chicken polyclonal antibody to calreticulin (ab14234, Abcam Inc., Cambridge, MA) followed by FITC-conjugated goat anti-chicken IgY (Gallus, Immunotech Inc., Fergus, Ontario, Canada). For isotype control, the primary antibody was replaced with chicken IgY (ChromPure, Jackson ImmunoResearch Laboratories, West Grove, PA). Fluorescence values obtained with this isotype control were deducted from values obtained with the calreticulin antibody. Cell suspensions derived from LLC tumors were additionally stained with fluorophore-conjugated rat antibodies raised against mouse CD45 (PharMingen, BD Biosciences, Mississauga, Ontario, Canada) and mouse F4/80 (AbD Serotec Inc., Oxford, UK). The two largest populations by far in these tumors are parenchymal cancer cells and tumor-associated macrophages. Cells stained negatively for panleukocyte antigen CD45 are >99% cancer cells, while tumor-associated macrophages are CD45$^+$F4/80$^+$. For intracellular HMGB1 analysis, Cytofix/Cytoperm and Perm/Wash buffer (both from PharMingen) were used to fix and permeabilize IC-21 cells and for antibody staining, respectively. The staining was with rabbit anti-HMGB1 polyclonal antibodies (ab18256, Abcam) followed by FITC-conjugated donkey anti-rabbit IgG (Jackson). Rabbit IgG (ChromPure, Jackson) was used for isotype control. Flow cytometry was performed on Coulter Epics Elite ESP (Coulter Electronics, Hialeah, FL) with 20,000 cells included for each test.

HMGB1 ELISA

Determination of HMGB1 levels in mouse serum samples was performed using ELISA kit for HMGB1 obtained from IBL international GmbH (Hamburg, Germany). This sandwich enzyme immunoassay utilizes anti-HMGB1 polyclonal antibody–coated wells that bind HMGB1 from the samples, which is then recognized by peroxidase-linked anti-HMGB1 monoclonal antibody by means of 3, 3′, 5, 5′-tetramethylbenzydine-based colorimetric reaction at 450 nm. The kit measures only HMGB1 without interference from HMGB2.

Statistical analysis

Each experimental group contained at least 4 mice or in vitro samples. The evaluation of the results was based on Mann–Whitney test with the significance level threshold set

at 5% for determining whether the groups were statistically different.

Results

Surface calreticulin expression after PDT in vitro

Evidence revealing that PDT induces the appearance of calreticulin on the surface of treated cells is presented in Fig. 1. In this experiment, in vitro-cultured LLC cells were collected 1 or 3 h after their treatment with Photofrin™-based PDT and stained with antibody raised against calreticulin (followed by FITC-conjugated secondary antibody) that enabled the detection of this normally endoplasmic reticulum-resident chaperone on the surface of these cells using flow cytometry. The results demonstrate that while there was no evidence of a significant calreticulin-associated fluorescence on untreated LLC cells, a clearly manifest surface expression of calreticulin was discernible at 1 h after PDT treatment that declined but was still evident 2 h later (Fig. 1). No significant calreticulin surface exposure was detectable with light-only and Photofrin™-only controls (data not shown). The inset in the same graph shows that, among the cells collected 1 h after PDT, those conditionally designated as dying (showing distinct reduced forward light scatter values) exhibited a much more prominent surface expression of calreticulin compared to the cells that appeared alive judging from their light scatter characteristics. For in vitro cultures consisting solely of LLC cells, the identification of dying population by this light scatter signal is very reliable. The chosen PDT dose is lethal to over 80% of LLC cells with evidence of both apoptotic and necrotic cell death [17].

Surface calreticulin expression after PDT in vivo

Encouraged by the above in vitro findings, further experiments were designed to ascertain that PDT-induced surface expression of calreticulin pertains under in vivo conditions. Hence, subcutaneous LLC tumors growing in syngeneic C57BL/6 mice were treated with Photofrin™-based PDT using a dose that renders typically 10–25% cures of these lesions [17]. At 1 h after PDT (time interval suggested to be optimal from the in vitro results), the mice were killed and the excised tumors disaggregated into single-cell suspensions suitable for antibody staining and flow cytometry analysis. The surface calreticulin expression was analyzed separately on tumor parenchymal cancer cells and tumor-associated macrophages. The results show that while the surface calreticulin expression was detectable only in around 6.5% of cancer cells, it increased almost threefold after tumor PDT (Fig. 2). The increase in the same parameter

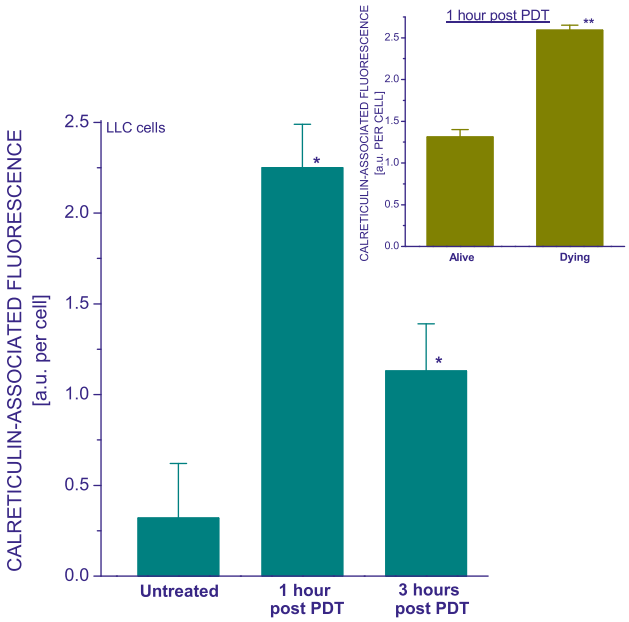

Fig. 1 Expression of calreticulin on the surface of LLC tumor cell following PDT in vitro. Cultured LLC cells were incubated with Photofrin™ (20 μg/ml) for 24 h followed by exposure to 1 J/cm^2 of 630 ± 10 nm light. The cells were then left in culture for 1 or 3 h before they were collected for flow cytometry. Expression of calreticulin was detected by surface staining with chicken polyclonal antibody to calreticulin followed by FITC-conjugated secondary antibody. Dying cells were identified by their decreased light scatter characteristics. The results show the extent of calreticulin-associated fluorescence (in arbitrary units per cell corrected by values obtained with the isotype control) in all cells, or separately in alive vs. dying cell populations (insert). The *bars* denote SE, $N = 4$; *statistically significant difference compared to the level in untreated group ($P < 0.05$); **statistically significant difference compared to the value with alive cell populations ($P < 0.05$)

in the samples from tumors treated with light only was not statistically significant. The data also revealed that the intensity of calreticulin-associated fluorescence per cancer cell rose almost fourfold when comparing untreated and PDT-treated tumors (inset to the same Figure). Since calreticulin is known to function as one of the molecules in macrophage surface receptor repertoire [18], it is not surprising that calreticulin fluorescence (even stronger than with cancer cells) was evident with macrophages from untreated LLC tumors; nonetheless, a further increase in surface calreticulin expression was found with these cells after PDT treatment (Fig. 2).

Rise in serum HMGB1 after PDT

Evidence of PDT-induced release of HMGB1 protein into the blood stream is presented in Fig. 3. Blood samples from control and PDT-treated groups of mice with subcutaneous LLC tumors were collected (only once from each mice) and stored as sera for HMGB1 determination using a commercial ELISA kit. It can be seen that serum HMGB1 levels

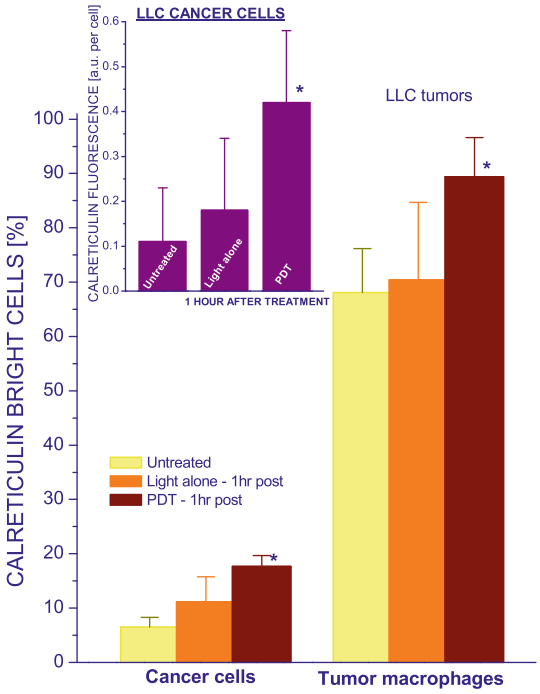

Fig. 2 Expression of calreticulin on the surface of cells from PDT-treated LLC tumors. Photofrin™ (10 mg/kg, i.v.) was administered to C57BL/6 mice bearing subcutaneous LLC tumors, and 24 h later, the tumors were exposed to light (150 J/cm^2, the same wavelength as in vitro). Illumination of tumors in mice that were not injected with photosensitizer was done for light-alone group. The mice were killed 1 h after light treatment, and single-cell suspensions prepared from the excised tumors were examined by flow cytometry. Surface calreticulin expression was determined as described for Fig. 1. Two major cell populations in LLC tumors were delineated as cancer parenchymal cells (stained negatively by panleukocyte marker CD45) and tumor-associated macrophages (positively stained for CD45 and macrophage marker F4/80). The results are shown as percentage of calreticulin-positive (*bright*) cells, and additionally for the cancer cells as the extent of calreticulin-associated fluorescence in arbitrary units per cell (insert). The *bars* denote SE, $N = 4$; *statistically significant difference compared to the level in untreated group ($P < 0.05$)

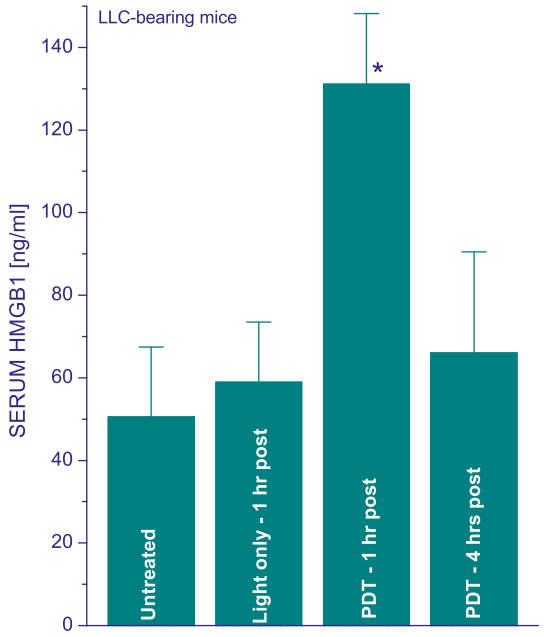

Fig. 3 Serum HMGB1 increase in mice bearing PDT-treated LLC tumors. Subcutaneous LLC tumors growing in C57BL/6 mice were treated by PDT as described for Fig. 2, and blood for serum samples was taken from host mice either at 1 or 4 h after therapy. The results show serum levels of HMGB1 determined by ELISA. The *bars* denote SE, $N = 4$; *statistically significant difference compared to the level in untreated group ($P < 0.05$)

rose significantly at 1 h after the treatment of LLC tumors by PDT (from around 50 ng/ml in untreated mice to over 130 ng/ml) while there was no significant effect detected with light-only control group. This elevation in HMGB1 levels was almost completely resolved at 4 h after PDT.

PDT signals influence HMGB1 levels in IC-21 cells

Since HMGB1 protein is an important participant in the macrophage activation process [19] known to be stimulated by PDT [20], we examined the levels of this protein in IC21 macrophages after they were co-incubated with PDT-treated LLC cells. Tissue culture inserts with PDT-treated or untreated LLC cells were added to dishes with IC-21 cells for co-incubation for 16 h at 37°C. The results, based on intracellular HMGB1 staining monitored by flow cytometry, are presented in Fig. 4. The data clearly demonstrate that the co-incubation of naïve IC21 cells with PDT-treated LLC cells produced a significant increase in intracellular levels of HMGB1 in these macrophages. No change in HMGB1 content was detected in IC21 macrophages co-incubated with PDT-untreated LLC cells. No HMGB1 was detectable after surface staining of IC-21 cells (not shown).

Discussion

This study demonstrates that PDT treatment prompts the expression of two pivotal cellular stress response proteins, calreticulin and HMGB1. The latter is also known as amphoterin. Calreticulin is highly conserved ubiquitous calcium-binding protein residing primarily in endoplasmic reticulum involved in calcium homeostasis and acting as a molecular chaperone assisting with protein assembly [21]. Within endoplasmic reticulum, calreticulin also facilitates major histocompatibility (MHC) class I assembly and loading on it antigen peptides [22]. However, calreticulin was in recent years identified as one of the key alarmins (DAMPs presented by endogenous molecular danger signals) that alert for the presence of stress-induced damage and ensure a response for eliminating the original threat [10, 23]. In response to specific stress stimuli, to which PDT can now

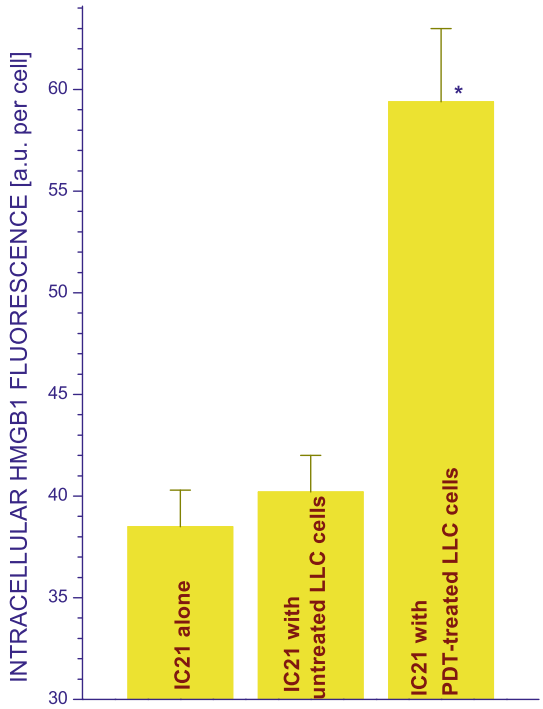

Fig. 4 Changes in intracellular HMGB1 levels in IC-21 cells co-incubated with PDT-treated LLC cells. In vitro-cultured IC-21 cells were co-incubated 16 h with LLC cells that were either untreated or treated with PDT as described for Fig. 1. Intracellular staining of IC21 cells collected after the co-incubation with antibody recognizing mouse HMGB1 was followed by flow cytometry analysis. The results show the extent of intracellular HMGB1-associated fluorescence (isotype control values deducted) in arbitrary units per cell. The *bars* denote SE, $N = 4$; *statistically significant difference compared to the level in IC-21-alone group ($P < 0.05$)

also be added, calreticulin becomes exposed on the cell surface and can even be released from cells [24, 25]. Recently, it was established that surface calreticulin translocation, exhibited after treatment of cells with anthracyclin drugs and several other immunogenic anticancer agents, functions as an "eat me" (engulfment) signal for dendritic cells and macrophages that initiates immune response [26–28]. Such immunogenic surface exposure of calreticulin appears very early in the apoptotic process and was found to be associated with the infiltration of T cells in stage IIIB colon cancer and could be linked with higher 5-year survival rate of these patients [29].

The results of this study demonstrate that surface exposure of calreticulin on tumor cells is highly elevated already at 1 h after PDT treatment both in vitro and in vivo and that it is more pronounced on dying cells. In PDT-treated LLC tumors, surface-localized calreticulin expression on tumor-associated macrophages was even more pronounced than in parenchymal cancer cells. This is in accordance with the fact that calreticulin complexed with CD91 acts as macrophage surface receptor for complement factor C1q and collectin molecules and is one of the key receptors for phagocyte binding of apoptotic cells and innate immune response development [18, 30].

Although initially identified as a nonhistone DNA-binding protein regulating gene transcription [31], HMGB1 is now best known as an important DAMP and one of the essential mediators of response to infection, injury, and inflammation [9, 10, 23]. It amplifies the inflammatory response to tissue injury by instigating and extending the production of proinflammatory cytokines, while suppressing anti-inflammatory responses. Functioning as a late pro-inflammation cytokine, HMGB1 binds to immune cell receptors like TLR2, TLR4, and RAGE. It can be passively released during cell injury (late necrosis and apoptosis, and sustained autophagy) or actively secreted by activated immune cells (including monocytes/macrophages, dendritic cells, NK cells, and B cells) [19, 32–37]. It has been shown to provoke inflammation, regulate migration of monocytes and neutrophils, contribute to DC maturation, and affect T-cell differentiation [32, 38, 39]. There is evidence that HMGB1 mediates various inflammatory and immune disorders, including sepsis, colitis, rheumatoid arthritis, and systemic lupus erythematosis, and is involved in tissue damage due to ischemia [34, 38]. The results of our study suggest that both passively released and actively secreted HMGB1 are involved in the early and later stages of PDT response, respectively. Signals from PDT-treated tumor cells can instigate production of HMGB1 in macrophages and possibly other immune cells. These signals, instrumental in orchestrating the cross talk between PDT-damaged tumor cells and macrophages, could consist of multitudinous DAMPs, including heat-shock proteins, lysolipids, and HMGB1 itself.

Demonstrating the expression of both calreticulin and HMGB1 following PDT treatment is important because the knowledge of their involvement brings forth wide-ranging mechanistic insights gained from the investigation of these two prototypical DAMPs in PDT-unrelated studies [13]. It was proposed that within tumor microenvironment, HMGB1 functions as regulator of cell death and survival, because depending on its redox status, it can trigger autophagy or apoptosis in cancer cells [40]. Thus, this DAMP released in the early time intervals such as 1 h after PDT (Fig. 3) can generate a positive feedback loop in stimulating apoptosis and autophagy of tumor cells injured by this therapy. On the other hand, HMGB1 produced by tumor-associated macrophages at later time intervals such as 16 h post-PDT (Fig. 4) can be largely of autocrine use with limited release into circulation. It is expected to act as a late pro-inflammatory cytokine sustaining anti-tumor host response of PDT and suppressing anti-inflammatory cytokines such as TGF-β [34, 41]. In recent years, DAMPs have been considered with increased interest as therapeutic targets for treating various human disorders including

cancer [12]. For instance, HMGB1 accumulation in PDT-treated tumors could be amplified by delivery of endogenous HMGB1 protein for promoting cancer cell death by either apoptosis or autophagy [40].

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Conflict of interest The authors declare to have no conflict of interest in any form with respect to this article.

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