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Ceramide and sphingosine-1-phosphate act as photodynamic therapy-elicited damage-associated molecular patterns: Cell surface exposure

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ABSTRACT

Molecules that appear on the surface of tumor cells after their therapy treatment may have important roles either as damage-associated molecular patterns (DAMPs) or signals for phagocytes influencing the disposal of these cells. Treatment of SCCVII and CAL27 cells, models of mouse and human squamous cell carcinoma respectively, by photodynamic therapy (PDT) resulted in the presentation of ceramide and sphingosine-1-phosphate (S1P) on the cell surface. This was documented by anti-ceramide and anti-S1P antibody staining followed by flow cytometry. The exposure of these key sphingolipid molecules on PDT-treated tumor cells was PDT dose-dependent and it varied in intensity with different photosensitizers used for PDT. The above results, together with the finding that both ceramide and S1P can activate NFκB signaling in macrophages co-incubated with PDT-treated tumor cells, establish that these two sphingolipids can act as DAMPs stimulating inflammatory/immune reactions critical for tumor therapy response.

1. Introduction

The concept of damage-associated molecular patterns (DAMPs) was conceived by Polly Matzinger as a central element of danger model of the functioning of the immune system [1,2]. This model, which is based on the idea that the immune system recognizes altered self appearing as a consequence of injury, infection or oncogenic transformation, and the perception of DAMPs has brought vital insights into innate and adaptive immunity. According to the danger model, the immune response is mobilized by DAMPs alerting the host of impending danger of disturbed local homeostasis due to the appearance of distressed and dying/dead cells [3,4]. Normally, DAMPs are molecules performing non-immunological functions within cells/tissues that acquire immunomodulatory character once exposed on the cell surface, released from stressed cells, or dislocated in other ways (e.g. extravasated). The sensors of exposed DAMPs are pattern-recognition receptors (PRRs). They are localized in the membrane or cytoplasm of immune cells, and include Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and C-type lectin receptors (CLRs) [4]. Engagement of these receptors by DAMPs triggers the danger signaling processes leading to inflammatory and immune responses.

Tumor tissue injury induced by certain types of cancer therapy was found to result in the expression of DAMPs, which emerge as pivotal mediators of host response elicited by these treatments and subsequent therapy outcome [5,6]. Treatment of solid tumors by photodynamic therapy (PDT), a clinically established modality producing oxidative stress by localized photoactivation of administrated photosensitizing drug [7], is particularly effective in generating an abundance of various DAMPs. These include cell surface-expressed calreticulin and heat shock proteins, released high-mobility group box-1 (HMGB1) protein and ATP, extracellular matrix proteins, and extravasated fibrinogen [8–10]. In this report, it is shown that ceramide and sphingosine-1-phosphate (S1P), two key members of sphingolipid family, become engaged as DAMPs after PDT treatment. This is indicated by the detected appearance of these two sphingolipids on the surface of PDT-treated tumor cells, and the participation of ceramide and S1P from these cells in signaling leading to the activation of NFκB in neighboring tumor-associated macrophages (TAMs).

2. Materials and methods

2.1. Cell culture and chemicals

The used cells were derived from squamous cell carcinoma (SCC) tumors. Cultures of SCCVII cells, which originate from a cutaneous SCC that arose spontaneously in C3H mice [11], were maintained in alpha minimal essential medium (Sigma Chemical Co., St. Louis, MO, USA)

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supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT, USA). The same medium was used for culturing CAL-27 cells (human SCC of the tongue, ATCC CRL-2095) that were kindly provided by Dr. Rajan Saini. Both these cell lines are recognized as good models of human head and neck cancer [11,12]. Mitoxantrone (M6545, Sigma) was dissolved in ethanol (0.5 mg/ml) and this initial stock was then added to cell medium for the final concentration of 1 μg/ml. Neutral sphingomyelinase from *Bacillus cereus* (S9396, Sigma) was added to serum-free cell medium for final concentration of 250 mU/ml in Petri dish containing 1×10^6 cells. The same conditions were applied for exposing cells to ceramidase, using the enzyme from *Pseudomonas aeruginosa* (a homolog of mammalian neutral ceramidases) that was cloned and expressed in *Escherichia coli* as described previously [13,14].

2.2. PDT treatment

Cells growing in 35-mm diameter Petri dishes were incubated with either Photofrin (20 μg/ml), Temoporfin (0.1 μg/ml, 0.15 μM), Pc4 (1 μg/ml, 1.4 μM) or ce6 (1.5 μg/ml, 2.5 μM). Cell exposure to Photofrin, Temoporfin and Pc4 was for 18 h in complete growth medium, while ce6 exposure was in serum-free medium for 30 min. Photofrin was obtained from Axcan Pharma (Mont-Saint-Hilaire, QC, Canada), Temoporfin (m-tetrahydrophenylchlorin, mTHPC, marketed as Foscan) was from Biolitec Research GmbH (Jena, Germany), ce6 (chlorin e6) was purchased from Frontier Scientific Inc. (Logan, UT, USA), while Pc4 was provided by Dr. Malcolm Kenney (Case Western Reserve University). After photosensitizer exposure, the medium was removed, the dishes (containing around 1 million cells) were washed, and cold PBS left during illumination. The light was produced by an integrated ellipsoidal reflector-equipped FB-QTH high throughput illuminator (Sciencetech, London ON, Canada) based on a 150 W QTH lamp and was delivered through an 8-mm core diameter liquid light guide (Oriel Instruments, Stratford, CT, USA). Interference filters 630 ± 10 and 650 ± 10 nm were used for Photofrin and Foscan, respectively, and 665 ± 10 nm for Pc4 and ce6. The fluence rate ranged from 30 mW/cm^2 for ce6- and Pc4-PDT to 50 mW/cm^2 for Photofrin-PDT.

2.3. Survival assay

Survival of PDT-treated SCCVII cells was determined by the conventional survival assay [15]. After photosensitizer exposure, cells were washed, trypsinized, and exposed to light while suspended in PBS. Immediately after illumination, the cells were counted and plated for colony growth. The colonies were stained with malachite green six days later and counted. The surviving fraction was calculated as a fraction of plating efficiency of PDT-treated cells.

2.4. Flow cytometry analysis

After PDT treatment, the Petri dishes with cells were returned to the incubator and kept in culture conditions with complete growth medium until they were collected for antibody staining. The exception was the exposure to sphingomyelinase or ceramidase, which was done for 15 min in serum-free medium. Intracellular staining for ceramide and S1P was performed as described previously [16]. Briefly, fixed and permeabilized cells were first incubated with anti-ceramide monoclonal antibody 15B4 (Enzo Life Sciences, Plymouth Meeting, PA, USA), anti-S1P monoclonal antibody NHS1P (Cosmo Bio USA, Carlsbad, CA, USA) or with normal mouse IgM (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) as their isotype control. This was followed by exposure of the cells to phycoerythrin (PE)-conjugated goat anti-mouse IgM antibody conjugated with (Santa Cruz Biotechnology). The same antibodies were used for detecting cell surface exposed ceramide and S1P, but the procedure was with non-fixed cells. Staining of calreticulin on cell surface was done with PE-conjugated polyclonal antibody to calreticulin (US Biological, Swampscott, MA, USA) with PE-conjugated normal rabbit IgG (Santa Cruz Biotechnology) used as the isotype control. To identify viable and nonviable cells, they were also stained with either 7-aminoactinomycin D (7-AAD, BD Biosciences, San Jose CA, USA) or SYTOX AADvanced (Molecular Probes, Eugene, OR, USA). Flow cytometry was performed using a Coulter Epics Elite ESP (Coulter Electronics, Hialeah, FL, USA). Ceramide, S1P or calreticulin levels were identified in viable cells based on antibody-associated fluorescence (mean fluorescence intensity in arbitrary units per cell), which was corrected by values obtained with the isotype controls. It should be emphasized that the fluorescence level measured by flow cytometry is in arbitrary units (entirely dependent on the instrument voltage settings used in taking the measurement) that should not be considered comparable between different experiments.

2.5. NFκB ELISA

For preparing primary cultures of TAMs, cells obtained by enzymatic disaggregation of SCCVII tumors and suspended in cell growth medium containing 20% FBS and 1% dispase II (Roche Diagnostics GmbH, Mannheim, Germany) were placed in 35-mm Petri dishes and left in a 37 °C incubator. After 30 min, non-attached cells were washed away leaving highly enriched populations of attached TAMs [17] as confirmed by flow cytometry documenting their positive staining with anti-mouse FITC-conjugated GR1 and PE-conjugated F480 antibodies (both from eBioscience Inc., San Diego, CA, USA). Thirty-mm culture inserts with growing SCCVII cells that were treated by Photofrin-PDT (Photofrin 20 μg/ml 18 h followed by 1 J/cm^2) were immediately transferred to Petri dishes containing freshly-selected TAMs for a 3-h co-incubation at 37 °C in complete growth medium. In control samples, TAMs were co-incubated with untreated SCCVII cells. In some samples, anti-ceramide antibody (15B4) or anti-S1P antibody (NHS1P) was present at 20 μg/ml (azide-free) in the co-incubation medium. After the co-incubation, the inserts and culture medium were removed; ice-cold lysis buffer (Cell Signaling Technology, Danvers, MA, USA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF, obtained from Sigma) was added to the dishes; and TAMs were collected by cell scraper to be stored at −80 °C until further use. The activity of NFκB in the TAMs was determined using ELISA kit PathScan® Phospho-NF-κB p65 (Ser536) manufactured by Cell Signaling. Briefly, TAM lysate samples were added to the wells coated with Phospho-NF-κB p65 (Ser536) mouse monoclonal antibody. The Phospho-NF-κB p65 protein captured by the antibody was detected by a rabbit NF-κB p65 detection antibody and recognized by peroxide-linked anti-rabbit IgG antibody through 3,3′,5,5′-tetramethylbenzydine-based colorimetric reaction at 450 nm.

2.6. Statistical analysis

Mann–Whitney test was used for data evaluation and the significance level threshold of 5% (two-tailed test) was set for determining whether the groups were statistically different.

3. Results

3.1. Mitoxantrone and PDT induce cell surface exposure of calreticulin, ceramide and S1P

Calreticulin is a protein residing primarily in endoplasmic reticulum but it can be found exposed on the cell surface in response to specific stress stimuli, which include the exposure to anthracyclines and similar drugs such as mitoxantrone or PDT treatment [9,18]. Hence, surface staining with calreticulin-specific antibody of mouse tumor SCCVII cells 30 min after their treatment by either Photofrin-PDT or 16-h exposure to 1 μg/ml mitoxantrone confirmed the appearance of calreticulin exposed on the exterior of these cells that was not detectable on