Analytical & Bioanalytical Techniques

Korbelik et al., J Anal Bioanal Tech 2014, S1
http://dx.doi.org/10.4172/2155-9872.S1-009

Research Article Open Access

Ceramide and Sphingosine-1-Phosphate/Sphingosine act as Photodynamic Therapy-Elicited Damage-Associated Molecular Patterns: Release from Cells and Impact on Tumor-Associated Macrophages

Mladen Korbelik[1]*, Judit Banáth[1], Wei Zhang[1], Fred Wong[1], Jacek Bielawski[2] and Duska Separovic[3]

[1]*British Columbia Cancer Agency, Vancouver BC, Canada*
[2]*Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, Carolina, USA*
[3]*Department of Pharmaceutical Sciences, Eugene Applebaum College of Pharmacy and Health Sciences, Wayne State University, Detroit, USA*

Abstract

A recent finding showed that ceramide and sphingosine-1-phosphate (S1P) become exposed on the surface of cells treated by photodynamic therapy (PDT) and acquire the capacity to act as danger-associated molecular patterns (DAMPs). To explore this further, the present study examined whether ceramide and S1P can be released from PDT-treated cells and investigated changes in the levels of these sphingolipids in tumor-associated macrophages (TAMs) left in contact with PDT-treated tumor cells. Mass spectroscopy-based analysis detected increased levels of C16-ceramide and dihydroC16-ceramide in media supernatants from SCCVII cells collected three hours after they were treated by PDT, compared to untreated cell supernatants. While no release of S1P was detected, elevated levels of its precursor sphingosine were found in the supernatants of PDT-treated cells. The co-incubation of TAMs-containing primary cultures derived from mouse SCCVII tumors with PDT-treated SCCVII cells was followed by ceramide and S1P analysis in these cells based on staining with specific antibodies and flow cytometry. Levels of both ceramide and S1P as well as inflammasome protein NLRP3 were found to rise in TAMs when they were co-cultured with PDT-treated SCCVII cells, while no significant change was seen with cancer cells. Such changes were induced also in TAMs incubated with supernatants from PDT-treated cells. The findings of the present study affirm the potential of sphingolipids including ceramide, S1P, and sphingosine to act, either exposed on cell surface or released in the microenvironment, as DAMPs in the response of tumors to PDT.

Keywords: Photodynamic therapy; Ceramide; Sphingosine-1-phosphate; Sphingosine; Damage-associated molecular patterns (DAMPs); Sphingolipid metabolism-modulating drugs (SMMDs)

Introduction

Sphingolipids (SLs) comprise one of the principal groups of bioactive lipids that besides their basic role in constituting cellular membranes mediate many key processes in cell physiology, including cell proliferation/death and immune responses [1-3]. Their key members, ceramide, sphingosine, and sphingosine-1-phosphate (S1P), are critical mediators of cellular stress response [4]. Various types of stress were found to induce regulated changes in the SL metabolism altering the levels of ceramide and other key SLs that control specific pathways of cellular survival/growth or programmed death [5]. This particularly pertains to cancer cells sustaining stress from anti-tumor modalities such as chemotherapy, ionizing radiation, hyperthermia, cytokine treatment or photodynamic therapy (PDT) [6,7].

The eradication of malignant tumors by PDT is achieved by the induction of oxidative stress due to the localized activation of photoreactive drugs with light that generates reactive oxygen species (mainly singlet oxygen) in targeted lesions [8]. The resulting phototoxic lesions elicit a complex response that produces cancer cell death, damage to tumor vasculature and other stromal elements, as well as inflammatory/immune responses [9-11]. Recent findings reveal that SLs belong to biomolecules with a potential to strongly influence PDT response [7,12,13]. It was documented that PDT has a distinct signature effect on the SL profile in treated cells and tumor tissue, which is dominated by the induction of *de novo* ceramide synthesis [13,14]. This *de novo* ceramide was shown to be involved in the initiation of tumor cell apoptosis after PDT.

The levels of SLs in cells can be altered by various sphingolipid metabolism-modulating drugs (SMMDs) developed as prospective anti-cancer agents or for other therapeutic interventions [15,16]. We have identified several such agents as effective for use in combination with PDT for improving the cure-rates of treated tumors [13,17].

Our studies aimed at monitoring ceramide and S1P levels in major cellular populations of PDT-treated tumors have revealed that, compared to cancer cells considerably higher levels of these SLs can be found in tumor-associated macrophages (TAMs) both before and after PDT [18]. Interestingly, treatment of SCCVII tumor-bearing mice with LCL29 (C6-ceramide analogue and established SMMD [19]) that increased PDT-mediated cure rates resulted also in the rise in ceramide levels in TAMs but not in cancer cell population of these tumors [18]. This raises the possibility that there is an interaction between ceramide activity in cancer cells and TAMs, bestowing ceramide and perhaps other SLs the function of intercellular signals. Indeed, S1P was suggested to represent one of the "find-me" signals released from apoptotic cells to mobilize immune effector cells with S1P receptors [20]. Several types of cells were also reported to recognize ceramide as

***Corresponding authors:** Dr. Mladen Korbelik, B.C. Cancer Research Centre, 675 West 10th Avenue, Vancouver BC, V5Z 1L3 Canada, Tel: 604-675-8084; Fax: 604-675-8099; E-mail: mkorbelik@bccrc.ca

Received June 18, 2014; **Accepted** July 29, 2014; **Published** July 31, 2014

Citation: Korbelik M, Banáth J, Zhang W, Wong F, Bielawski J, et al. (2014) Ceramide and Sphingosine-1-Phosphate/Sphingosine act as Photodynamic Therapy-Elicited Damage-Associated Molecular Patterns: Release from Cells and Impact on Tumor-Associated Macrophages. J Anal Bioanal Tech S1: 009. doi:10.4172/2155-9872.S1-009

one of the agonists of their Toll-like receptor-4 (TLR4) [21]. Recently, we have demonstrated that ceramide and S1P become expressed on the surface of PDT-treated tumor cells acquiring the capacity of acting as damage-associated molecular patterns (DAMPs) [22]. As such they can serve as alarm signals that alert immune cells to the presence of endogenous damage and prompt the activation of inflammatory/immune responses [23].

The aim of the present study was to establish whether ceramide and S1P can become not only surface-expressed but also released from PDT-treated tumor cells, and how could PDT affect their levels in neighboring TAMs.

Materials and Methods

SCCVII tumor and cell culture

Murine squamous cell carcinoma SCCVII is an accepted model of head and neck cancer of spontaneous origin with established record of the absence of strong immunogenicity [24]. Subcutaneous SCCVII tumors were grown in syngeneic C3H/HeN mice. For experiments, the tumors were excised from sacrificed mice when they reached around 10 mm in largest diameter and immediately subjected to enzymatic digestion for obtaining single cell suspensions following a routine standard protocol [25]. Primary cultures formed from these tumor cell suspensions as well as long-term SCCVII cell line were maintained *in vitro* using alpha minimal essential medium (Life Technologies, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS) obtained from Life Technologies. The mouse procedures were in compliance with the protocol approved by the Animal Care Committee of the University of British Columbia.

Photodynamic treatment

Cultured SCCVII cells, growing either in 35 mm diameter Petri dishes or in 30 mm diameter Millicell cell culture inserts with 0.4 μm pore polycarbonate membrane (Millipore Ltd., Carrigtwohill, County Cork, Ireland) were first exposed to Photofrin (Axcan Pharma, Mont-Saint-Hilaire QC, Canada) at 20 μg/ml concentration for 18 hours. The cells were then washed in PBS and kept in that buffer while irradiated with 1 J/cm^2 of 630 ± 10 nm light (15 mW/cm^2). The light was produced by a FB-QTH high throughput illuminator (Sciencetech, London ON, Canada) based on a 150 W QTH lamp and equipped with integrated ellipsoidal reflector. It was delivered through an 8 mm core diameter liquid light guide (Oriel Instruments, Stratford CT, USA).

Electrospray ionization/double MS

Release of key sphingolipids from PDT-treated SCCVII cells was monitored by analysis of their supernatants after they were kept for various post-treatment time intervals in culture conditions at 37°C. In initial experiments, PDT-treated cells were maintained in their growth medium that is supplemented with 10% FBS. Since the levels of some SLs, particularly S1P, were relatively high in this medium, in additional experiments SCCVII cells were maintained for two days in serum-free medium Ex-cell NS0 (Sigma 14650C) and then kept in this medium for generating supernatants. In order to assess possible autocrine/paracrine re-internalization of S1P by binding to S1P receptors on SCCVII cells, anti-S1PR1 or anti-S1PR2 (both rabbit polyclonal antibodies) purchased from Biorbyt Limited (Cambridge, UK) were added to the cell medium in some samples at 20 μg/ml during the post-PDT incubation. Some supernatants were after collection further processed for exosome isolation using P100 PureExo® Exosome Isolation kit (101Bio, Palo Alto, CA) following manufacturer instructions. The exosomes appear as a separate fluffy layer after vortexing of cell medium sample with solutions provided by the kit and microcentrifugation. This kit isolates >95% pure exosomes with the yield of up to 100 μg exosomal material per ml of medium. After extraction, SLs from cell culture supernatants were introduced to electrospray ionization source and analyzed by double mass spectrometry using an ACCELA-TSQ Quantum Access LC-MS-MS system (Thermo-Fisher Scientific, Waltham MA, USA) as described earlier [12]. This allowed simultaneous measurement of various ceramides, dihydroceramides and sphingoid bases employing internal standards and using comparison with the calibration curves based on a linear regression model [26].

Flow cytometry-based determination of cellular ceramide, S1P and NLRP3 levels

Cellular levels of ceramide and S1P were determined by intracellular staining with specific antibodies followed by flow cytometry as described in detail in an earlier report [18]. Briefly, cells detached using a cell scraper were first exposed to surface staining with either FITC-conjugated anti-mouse F4/80 or FITC anti-Ly-6G known as GR1 (eBioscience Inc., San Diego CA, USA). As repeatedly proven in our previous studies [7,18] staining intensity of SCCVII tumor cell suspensions for myeloid marker GR1 allows gating them into two major populations, TAMs (GR1$^+$) and cancer cells (GR1$^-$). In addition to these two populations, SCCVII tumors usually contain less than 1% of other cell types; among them neutrophils and myeloid-derived suppressor cells can be gated out as GR1^{++}. Hence, TAMs were routinely identified as GR1$^+$ and F4/80$^+$ cells. Surface staining was followed by intracellular staining of fixed and permeabilized cells with mouse anti-ceramide monoclonal antibody 15B4 (Enzo Life Sciences, Plymouth Meeting PA, USA) or mouse anti-S1P monoclonal antibody (clone NHS1P) (Cosmo Bio USA, Carlsbad CA, USA) used as primary antibodies. Goat anti-mouse IgM antibody conjugated with phycoerythrin (Santa Cruz Biotechnology Inc., Santa Cruz Ca, USA) served as the secondary antibody. Staining with normal mouse IgM (Santa Cruz) was employed as the isotype control. Under physiological conditions the used anti-ceramide antibody is highly specific for C16- and C24-ceramide and does not cross-react with sphingomyelin, cholesterol or other phospholipids [27], while anti-S1P antibody shows no-cross-reactivity with ceramide, sphingosine, sphingomyelin or other phospholipids [28]. The inflammasome activity in TAMs was assessed by monitoring levels of the prominent protein of this complex, NLRP3, based on intracellular staining with rabbit anti-NLRP3 (Boster Biological Technology, Pleasanton, CA) followed by PE-conjugated chicken anti-rabbit IgG (Santa Cruz) with non-specific rabbit IgG as isotype control. Flow cytometry was performed by a Coulter Epics Elite ESP (Coulter Electronics, Hialeah FL, USA) with at least 2×10^4 cells included for each test.

Statistical analysis

Experimental groups contained quadruplicate samples (N=4). The results were evaluated based on Mann-Whitney test and the significance level threshold of 5% (two-tailed test) was set for determining whether the groups were statistically different.

Results

Levels of SLs in cell supernatants

Cultures of SCCVII cells (2×10^6 per sample) were exposed to the standard dose of Photofrin-PDT that resulted in 80-90% cell kill [22]. The cells were then left in the 37°C incubator with fresh regular growth