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Gemcitabine kills proliferating endothelial cells exclusively via acid sphingomyelinase activation

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ABSTRACT

Gemcitabine is a widely-used anti-cancer drug with a well-defined mechanism of action in normal and transformed epithelial cells. However, its effect on endothelial cells is largely unknown. Acid sphingomyelinase (ASMase) is highly expressed in endothelial cells, converting plasma membrane sphingomyelin to pro-apoptotic ceramide upon activation by diverse stresses. In the current study, we investigated gemcitabine impact in primary cultures of endothelial cells. We find baseline ASMase increases markedly in bovine aortic endothelial cells (BAEC) as they transit from a proliferative to a confluent growth-arrested state. Further, gemcitabine activates ASMase and induces release of a secretory ASMase form into the media only in proliferating endothelial cells. Additionally, proliferative, but not growth-arrested BAEC, are sensitive to gemcitabine-induced apoptotic death, an effect blocked by inhibiting ASMase with imipramine or by binding ceramide on the cell surface with an anti-ceramide Ab. Confluent growth-arrested BAEC can be re-sensitized to gemcitabine-induced apoptosis by provision of exogenous sphingomyelinase. A highly similar phenotype was observed in primary cultures of human coronary artery endothelial cells. These findings reveal a previously-unrecognized mechanism of gemcitabine cytotoxicity in endothelium that may well contribute to its clinical benefit, and suggest the potential for further improvement of its clinical efficacy via pharmacologic modulation of ASMase/ceramide signaling in proliferative tumor endothelium.

1. Introduction

A large body of evidence supports tumor endothelial cells, while non-transformed, as being phenotypically distinguished from normal resting endothelium in healthy tissue [1,2]. Tumor cells secrete substantial amounts of endothelial mitogens, amongst which VEGF-A is predominant [3], that "educate" their neo-vasculature. These angiogenic factors induce many alterations in endothelial function of which proliferation, as it relates to response to chemotherapy, is the topic of the current studies. While the large majority of endothelial cells in normal mammalian tissues are in G_0, endothelial cell turnover is elevated in tumors (estimates range from 20 to 2000× fold of that in normal tissue [4]) and vessels remain immature, in sharp contrast to the pericyte-covered, mature endothelial monolayer of the resting normal microvasculature [5,6]. Tumor endothelium has been recognized as a target for anti-cancer therapy for over 50 years [7]. Thus far, mainstream approaches aimed at either halting angiogenesis via VEGF blockage [8], or enhancing endothelial death, have had limited clinical success [9].

Acid sphingomyelinase (ASMase), which initiates the Sphingomyelin-Ceramide Signaling Pathway, is highly enriched in endothelial cells [10,11] and known therein to be responsive to diverse cellular stresses [12]. ASMase is a sphingomyelin-specific phospholipase C that hydrolyzes sphingomyelin to generate the second messenger ceramide [13]. While there is only one *asmase* gene in mammalian cells, the primary gene product is post-translationally processed to give rise to two distinct forms: a form found in classic lysosomes that is independent of exogenous zinc having acquired it during intracellular transport; and a secreted "zinc-dependent" lysosomal form found in secretory lysosomes [11,14]. While classic lysosomal ASMase is critical for catabolism of membrane sphingomyelin during physiologic turnover of membranes, secretory ASMase generates ceramide at the outer plasma

Abbreviations: ASMase, acid sphingomyelinase; BAEC, bovine aortic endothelial cells; BrdU, 5-bromo-2′-deoxyuridine; bSMase, bacterial sphingomyelinase; CNT, concentrative nucleoside transporter; dFdC, 2′,2′-difluoro 2′-deoxycytidine; EDTA, ethylene diamine tetraacetic acid; ENT, equilibrative nucleoside transporter; HCAEC, human coronary artery endothelial cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; z-VAD-fmk, benzyloxycarbonyl Val-Ala-Asp (OMe) fluoromethylketone.

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membrane to initiate transmembrane signal transduction. Ceramide generated via secretory ASMase signals diverse cellular functions including initiation of vesicular transport [15,16], activation of NADPH-dependent vasodilatory function in the vasculature [17,18], facilitation of plasma membrane repair [19], and the often reported induction of apoptotic cell death [20,21], to list a few.

Gemcitabine (2′,2′-difluoro 2′-deoxycytidine; dFdC) is a widely-used chemotherapeutic agent employed as first-line treatment for locally-advanced pancreatic cancer, inoperable lung cancer, relapsed ovarian carcinoma, and in various instances in breast cancer and metastatic sarcoma. The drug is a fluorinated analogue of the nucleoside cytidine, which upon phosphorylation by nucleoside kinases, is incorporated into DNA, causing DNA replication fork stalling and cytotoxicity. This is considered the main mechanism of its cytotoxic activity, thus targeting malignant cells of rapid cellular division. Evidence exists, however, that gemcitabine also acts on endothelial cells, already at low drug concentrations [22–24]. In addition, ceramide has been linked to gemcitabine efficacy, and recently reported as a biomarker for response effectiveness [25]. Here, we investigate impact of gemcitabine on endothelial cells and show that it acts on proliferating, but not growth-arrested, endothelium by a mechanism distinct from DNA damage involving activation of ASMase signaling, providing an additional rationale for its therapeutic index in cancer therapy.

2. Materials and methods

2.1. Materials

Lyophilized gemcitabine (Sun Pharmaceutical Industries LTD, Gujarat, India) was reconstituted in 0.9% NaCl and stored at room temperature, according to manufacturer's instructions. Mouse IgM monoclonal anti-ceramide antibody MID 15B4 was from Enzo Life Science (Farmingdale, NY, USA). MTT cell proliferation kit was from Roche (Mannheim, Germany). Bacterial sphingomyelinase (*Bacillus cereus*) was purchased as a 371 U/ml stock in 50% glycerol from Sigma Aldrich. Unless indicated otherwise, all other reagents and chemicals were from Sigma Aldrich (Milwaukee, WI, USA).

2.2. Cell culture

Primary bovine aortic endothelial cells (BAEC) were established from the intima of bovine aorta as described [26] and maintained in 1.0 g/l glucose DMEM (HyClone™, GE Healthcare, South Logan, UT, USA) supplemented with 10% bovine calf serum (GemCell™, Gemini Bio-Products, West Sacramento, CA, USA), 50 IU/ml penicillin and 50 μg/ml streptomycin. Cells were subcultured by trypsinization up to passage 18 and were maintained at 37 °C in a water-saturated atmosphere containing 10% CO2. Human coronary artery endothelial cells (HCAEC) were obtained from Lonza (Walkersville, MD, USA) and maintained in EGM-2 full medium (Lonza) at 5% CO2. HCAEC were sub-cultured up to passage 7. For experiments, EGM-2 medium was replaced with DMEM (1.0 g/l glucose) without supplements.

2.3. ASMase activity in cells and culture medium

For ASMase activity studies, cells were placed on serum-free DMEM for at least 4 h. For measurement of secreted ASMase, culture medium was collected and the cells were washed 1× with cold PBS. The cells were then lysed in 0.5% Triton ×100 and protein content of each lysate was determined by DC protein assay (Biorad, Hercules, CA, USA). The cell lysates were used for the measurement of cellular ASMase activity. For secreted ASMase activity, the PBS wash and the collected medium were pooled, cleared by a single centrifugation step at 3200 ×g for 5 min, and concentrated in a 30,000 molecular weight cutoff centrifugal unit (Merck Millipore, Tullagreen, Ireland). Samples were concentrated at least 40-fold by volume.

ASMase activity was quantified by radioenzymatic assay, measuring conversion of bovine [^{14}C-methylcholine]sphingomyelin (Perkin Elmer, Waltham, MA, USA) according to [27]. Briefly, a film of unlabeled bovine brain sphingomyelin (Avanti Polar Lipids, Alabaster, AL, USA) and radioactive [^{14}C]sphingomyelin (39:1 M ratio) was hydrated in a 3× acidic assay buffer. 30 μl of this radiolabeled assay buffer was added to 60 μl sample (i.e. concentrated medium, or a 60 μl lysate of 25 μg protein). The final assay concentrations are: 1% triton X-100, 250 mM sodium acetate and 128 μM sphingomyelin, pH 5.0. Zinc chloride was added to 0.1 mM for total ASMase, while 5.0 mM EDTA was added to measure zinc-independent ASMase. After 2 hours incubation at 37 °C, the enzymatic reaction was terminated by adding 125 μl of a 2:1 (v/v) chloroform:methanol solution. Samples were well mixed and centrifuged for 5 min at 7000 ×*g*. The aqueous (upper) phase was transferred into scintillation vials and ^{14}C-radioactivity counted in ScintiVerse™ BD Cocktail (Fisher Scientific, Waltham, MA, USA) in a 2200CA Tri-Carb analyzer (Packard Instrument, Downers Grove, IL, USA). For each assay, a buffer only and a sample boiled for 5 min at 95 °C were included as negative controls.

2.4. Cell viability

BAEC, cultured in flat-bottom 96-well plates, were incubated with gemcitabine from serial dilutions in 0.9% NaCl. After 16 h, cells were washed and medium (10% BCS) was refreshed for an additional 6 h. MTT reagent was added and formazan was allowed to develop over 2–4 h. The formazan crystals were solubilized by adding 100 μl of 10% SDS, 0.01 M HCl to each well. Absorbance was measured at 570 nm with the reference wavelength at 680 nm. As controls, wells were supplemented with vehicle (0.9% NaCl; set to 100%), or 1.0 mM arsenic oxide to kill all cells (0%).

2.5. Apoptosis quantification

Cells were harvested by trypsinization 16 h after start of treatment. Detached cells and medium were pooled, and cells were collected by centrifugation. After a PBS wash to remove remaining serum and trypsin, cells were fixed in 3% paraformaldehyde and stained in 30 μg/ml bis-benzimide trihydrochloride (Hoechst #33258; Sigma Aldrich, Milwaukee WI, USA). Apoptosis was counted on an Olympus BH2 fluorescence microscope using nuclear compaction and fragmentation as criteria for apoptosis according Lazebnik et al. [28].

3. Results

3.1. ASMase activity increases with cell density

These studies utilized BAEC, a cultured endothelial cell system shown by us to closely mimic endothelial responses observed in vivo in murine allografts and human xenografts [29,30]. Initial studies examined the impact of cell cycle kinetics on ASMase activity. As in vivo, endothelial cells undergo contact inhibition in cell culture [31]. Fig. S1A shows that BAEC growth slows as confluence is approached, and that the cells fully growth arrest 3 days after reaching confluence under the conditions of our assay. Confluence (day 0) for the purpose of these studies is defined as the day the monolayer occupies the entire surface of the culture plate. After confluence is reached, BAEC density continues to increase from 2.5 (day 0) to 4.0 × 10^5 cells/cm^2 (Fig. S1A), as individual cells continue to pack more tightly. While 50% of sub-confluent BAEC are in the G2/S phase of the cell cycle as detected by BrdU staining, growth-arrested BAEC display ≤5% BrdU staining (Fig. S1B). In the current study, we assessed cellular ASMase activity in relation to these growth states. Fig. 1 shows that baseline ASMase activity increases with growth of BAEC. Even after confluence (day 0) and growth arrest (day 4), cellular ASMase activity continues increasing (Fig. 1A), up to 3.5-fold of the initial activity