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Original Articles

Short-chain ceramides depress integrin cell surface expression and function in colorectal cancer cells

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ABSTRACT

Colorectal cancer (CRC) is highly metastatic, significantly so to liver, a characteristic that embodies one of the most challenging aspects of treatment. The integrin family of cell–cell and cell–matrix adhesion receptors plays a central role in migration and invasion, functions that underlie metastatic potential. In the present work we sought to determine the impact of ceramide, which plays a key modulatory role in cancer suppression, on integrin cell surface expression and function in CRC cells in order to reveal possible ceramide-centric effects on tumor cell motility. Human CRC cells LoVo, HT-29, and HCT-116 were employed, which represent lines established from primary and metastatic sites. A cell-permeable, short-chain analog, C6-ceramide, was used as ceramide mimic. Exposure of cells to C6-ceramide (24 h) promoted a dose-dependent (2.5–10 μM) decrease in the expression of cell surface β1 and β4 integrin subunits in all cell lines; at 10 μM C6-ceramide, the decreases ranged from 30 to 50% of the control. Expression of cell surface αVβ6 integrin, which is associated with advanced invasion in CRC, was also suppressed by C6-ceramide. Decreases in integrin expression translated to diminished cellular adhesion, 50% of the control at 5 μM C6-ceramide, and markedly reduced cellular migration, approximately 30–40% of the control in all cell lines. Physicochemical examination revealed potent efficacy of nano-formulated C6-ceramide, but inferior activity of dihydro-C6-ceramide and L-C6-ceramide, compared to the unsaturated counterpart and the natural D-enantiomer, respectively. These studies demonstrate novel actions of ceramides that may have application in suppression of tumor metastasis, in addition to their known tumor suppressor effects.

Introduction

Colorectal cancer (CRC), which disproportionally impacts African-Americans [1], is the third most common cancer in both men and women [2]. In 2015, an estimated 52,000 deaths are predicted. Although resection may be curative for CRCs that have not spread, cure is rarely possible for most patients with metastatic disease. When CRC is detected early, the 5-year survival is around 90%. However, only approximately 40% of patients are diagnosed with localized disease. Current regimens are designed to limit the growth potential of tumors by attenuating the rate of cell division or through nutrient restriction by hindering angiogenesis. However, modalities that decrease the most lethal aspect of colon cancer, metastatic potential, have not been largely reported. Poor prognosis in patients with later stage disease necessitates the search for new treatment strategies, and in this instance, strategies that are directed to target the biology underlying metastasis might prove beneficial.

CRC is highly metastatic to the liver, and this characteristic embodies one of the most challenging aspects of treatment [3,4]. The integrin superfamily of cell–cell and cell–matrix adhesion receptors plays a central role in migration and invasion during normal and pathological states. In addition, integrin heterodimers are also cell signaling molecules that propagate outside-in and inside-out signaling cascades to culminate in a variety of cellular responses

Abbreviations: CRC, colorectal cancer; C6-ceramide, *N*-hexanoyl-D-*erythro*-sphingosine; DH-C6-ceramide, dihydro-C6-ceramide; ATCC, American Type Culture Collection; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; HBSS, Hanks' balanced salt solution; PBS, phosphate buffered saline; BSA, bovine serum albumin; PI, propidium iodide; SDS, sodium dodecyl sulfate; 4-HPR, N-(4-hydroxyphenyl) retinamide.

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including cell proliferation. The duality of integrin receptors in facilitating cell migration as well as governing growth potential places integrins at the interface of normal cell function and cancer biology. Integrins contribute to the initial establishment, expansion, and spread of solid tumors [5]; for this latter role, integrin receptors have been pursued as both anti-cancer targets and diagnostic markers. Augmented expression and function of integrins have been shown to correlate with invasive potential and poor outcomes in a spectrum of cancers [6], particularly α6β4 and αVβ6 expressions have emerged as unique markers in CRC and overexpression has been linked to enhanced adhesion that potentiates CRC invasive properties [7–12].

Although the sphingolipid ceramide has of late taken center stage in its role as a tumor suppressor [13–15], little information is available on the impact of ceramide and/or ceramide-generating agents on integrin expression and function. To this end, we have employed a short-chain ceramide analog, C6-ceramide, to investigate whether this sphingolipid, which orchestrates a mosaic of signaling responses [15], imparts influence on integrin cell surface expression with functional outcomes in human CRC cells. Here we show for the first time that C6-ceramide exposure decreases the cell surface expression of the β1 and β4 integrin subunits and cell surface expression of the αVβ6 integrin subtype, actions that functionally translated to a reduction in cellular adhesion and migration. We thus propose that short-chain ceramides could augment CRC therapy by reducing tumor growth and limiting metastatic potential and disease progression.

Materials and methods

Cell lines and reagents

The human CRC cell lines LoVo and HT-29 were obtained from the American Type Culture Collection (ATCC), Manassas, VA. HCT 116 was a gift from Carol Cardarelli, James Madigan, and Michael Gottesman, National Cancer Institute, Bethesda, MD. Cells were propagated in RPMI-1640 medium (Life Technologies, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA), 100 units/mL penicillin and 100 μg/mL streptomycin. The cell lines were not tested or authenticated over and above documentation provided by the ATCC, which includes antigen expression, DNA profile, and cytogenic analysis. Cells were grown in humidified conditions in a tissue culture incubator with 95% air and 5% CO_2 at 37 °C. Confluent cells were subcultured using 0.05% trypsin/0.53 mM EDTA solution (Invitrogen Corp, Carlsbad, CA). C6-ceramide (*N*-hexanoyl-D-*erythro*-sphingosine) and dihydro-C6-ceramide (DH-C6-ceramide) were obtained from Avanti Polar Lipids, Alabaster, AL, and the L-enantiomer of C6-ceramide was obtained from Matreya, Pleasant Gap, PA. C6-ceramides were dissolved in DMSO and stored as a 10 mM stock at –20 °C. Monooleoylglycerol (monoolein), dissolved in DMSO and stored at –20 °C, was from Nu-Chek Prep, Elysian, MN. Hanks' balanced salt solution (HBSS) was from Life Technologies, Carlsbad, CA, and propidium iodide (PI) was obtained from Sigma, St. Louis, MO. alamarBlue was a product of Thermo Fisher. Human plasma fibronectin (catalog # 354008) was purchased from BD Biosciences. Integrin β1 antibody, which reacts with the human β-subunit (catalog # MAB2253), integrin β4 antibody, which reacts with human β4 integrin (catalog # MAB1964), and integrin αVβ6 antibody specific for αVβ6 (catalog # MAB2077Z) were from Millipore (Billerica, MA). Alexa Fluor 488 goat anti-mouse IgG antibody was obtained from Life Technologies. Millicell hanging cell culture inserts for 24-well plates, 8 μm polyethylene terephthalate (PET), were purchased from Millipore. Conditioned medium for cell migration assays was prepared by culturing respective cell lines at 2×10^6 cells/T-75 flask in RPMI-1640 medium containing 10% FBS for 96 h. The serum-containing medium was then discarded and replaced with 10 mL of serum-free medium for 48 h. This medium was collected, centrifuged at 16,000 × *g* for 10 min to pellet debris, decanted, and adjusted to contain 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, and 2.0 mM $CaCl_2$. Tissue culture plates and dishes were from Corning.

Integrin cell surface expression

Integrin expression was determined by flow cytometry. Briefly, 1×10^6 cells plated in 10-cm dishes were grown for 24 h in RPMI-1640 medium containing 10% FBS, after which cells were exposed for 24 h to vehicle control (DMSO) or C6-ceramide, in medium containing 2.5% FBS. Following treatment, monolayers were washed with phosphate buffered saline (PBS) and disaggregated by addition of citric saline (135 mM KCl, 15 mM sodium citrate, pH 7.4). Cells were isolated by centrifugation (500 × *g*) for 5 min and washed twice with isotonic Tyrode's solution (Sigma Chemical Company, St. Louis, MO) containing 0.1% sodium azide. For flow cytometry, 1×10^6 cells were resuspended in 1.0 mL of HEPES-buffered Tyrode's solution containing 10 mM HEPES, pH 7.4, 1 mM $MgCl_2$, 0.1% bovine serum albumin (BSA), and incubated at room temperature with primary antibody (1:100 dilution) for 30 min, after which 1.0 mL of 3.7% paraformaldehyde was added for 20 min. Cells were then washed twice with HEPES–Tyrode's solution, resuspended in 0.2 mL of the same and incubated with secondary antibody Alexa Fluor 488 goat anti-mouse IgG (1:200 dilution) for 15 min. The preparation was then washed twice with HEPES–buffered Tyrode's solution and resuspended in the same for analysis by flow cytometry. The cells were stained with PI in order to exclude dead cells in the analyses. The percentage of positive cells as well as the mean fluorescence intensities were obtained; the latter was used as measure of relative antigen density.

Cell adhesion

Assays were conducted in 96-well plates coated with 50 μL of fibronectin (25 μg/mL); plates were incubated overnight at 4 °C. The 96-well plates were then washed with PBS and incubated at 37 °C in a 5% CO_2 atmosphere for 30 min with blocking buffer (HBSS containing 1% BSA) prior to seeding cells. Cells, seeded in 10-cm plates in medium containing 10% FBS, were grown to 60% confluence, switched to medium containing 2.5% FBS, and C6-ceramide was added for the designated times. Cells were collected using citric saline, and viability was determined using trypan blue exclusion. Cells were resuspended at 1.0×10^6 cells/mL in adhesion buffer (10 mM HEPES, pH 7.3, 135 mM NaCl, 2.8 mM KCl, 0.4 mM $NaHCO_3$, 1.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, 1.0 mM $MnCl_2$, 0.5% BSA, 5.5 mM glucose), seeded at 5×10^4 viable cells per well in adhesion buffer and placed in the culture incubator for 1–2 h. The 96-well plates were then gently washed 3 times with PBS, and cells were fixed with 3.7% paraformaldehyde for 10 min followed by 100% methanol for 10 min. Cells were stained with 0.05% crystal violet in 20% methanol for 10 min, washed 3 times with PBS to remove excess dye and allowed to dry overnight. Crystal violet-containing cells were solubilized in 1% SDS in 50% ethanol, and absorbance was measured at 590 nm.

Cell migration

Trans-well cups (Millipore-24 Well Millicell) were coated by addition of 50 μL of fibronectin (25 μg/mL) and allowed to incubate overnight at 4 °C and then rinsed with PBS. Wells in a 24-well plate were then filled with 0.6 mL of conditioned media and incubated at 37 °C with 5% CO_2 prior to cell seeding. For the experiment, LoVo, HT-29, and HCT-116 cells (1×10^6) were seeded in 6-cm dishes and treated with C6-ceramide the following day in medium 2.5% FBS for 24 h. Cells were collected by using 1% citric saline, resuspended at 1×10^6/mL in migration medium and seeded at 1×10^5 viable cells per trans-well cup in migration medium (RPMI-1640 medium, 10 mM HEPES, pH 7.3, 1.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, 1.0 mM $MnCl_2$, 0.1% BSA). After 24 h of incubation at 37 °C, 5% CO_2, the cups were washed 3 times with PBS, fixed with 3.7% paraformaldehyde for 10 min followed by 100% methanol for 10 min, and stained with 0.005% crystal violet for 10 min. The trans-well cups rinsed with PBS to remove excess dye and allowed to dry overnight. Migrated cells were photographed on an inverted microscope (20× magnification) and quantified using the automatic counting feature in Photoshop.

Cell viability

Cell viability was assessed using the alamarBlue protocol according to the manufacturer's instructions. Cells (10,000/0.1 mL of 10% FBS-containing medium) were seeded in 96-well plates, cultured overnight and treated for 24 h by addition of C6-ceramide in medium containing 1% FBS (final well volume 0.2 mL).

Preparation of C6-ceramide nanoliposomes

Pegylated C6-ceramide nanoliposomes were prepared from specific lipids at particular molar ratios as previously described [16,17]. Briefly, 1,2-distearoyl-*sn*-glycero-3-phosphocholine, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000](PEG2000)-DSPE, and C6-ceramide were dissolved in chloroform, dried to a film under a stream of nitrogen, and then hydrated by addition of 0.9% NaCl. Solutions were sealed, heated at 55 °C for 60 min, vortex mixed and sonicated for 5 min until light no longer diffracted through the suspension. The solution was quickly extruded at 55 °C by passage through 100 nm polycarbonate filters in an Avanti Mini-Extruder (Avanti Polar Lipids). Ghost nanoliposomes were prepared in the same manner excluding C6-ceramide.

Statistical analysis

Results are expressed as mean ± SE (standard error) and were analyzed by ANOVA. Differences among treatment groups were assessed by Tukey's test. Differences were considered significant at $p \leq 0.05$. An asterisk (*) used in specific figures denotes significance; figure legends also provide comments on statistical significance.