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Disialoganglioside GD3-synthase over expression inhibits survival and angiogenesis of pancreatic cancer cells through cell cycle arrest at S-phase and disruption of integrin-β1-mediated anchorage

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ABSTRACT

Gangliosides play important roles in the development, differentiation and proliferation of mammalian cells. They bind to other cell membrane components through their terminal sialic acids. Different gangliosides influence cellular functions based on the positions and linkages of sialic acids. Expression of gangliosides mainly depends on the status of sialic acid-modulatory enzymes, such as different types of sialyltransferases and sialidases. One such sialyltransferase, disialoganglioside GD3 synthase, is specifically responsible for the production of GD3. Pancreatic ductal adenocarcinoma, making up more than 90% of pancreatic cancers, is a fatal malignancy with poor prognosis. Despite higher sialylation status, the disialoganglioside GD3 level is very low in this cancer. However, the exact status and function of this disialoganglioside is still unknown. Here, we intended to study the intracellular mechanism of disialoganglioside GD3-induced apoptosis and its correlation with the adhesion and angiogenic pathways in pancreatic cancer. We demonstrated that disialoganglioside GD3 synthase-transfected cells showed enhanced apoptosis and it caused the arrest of these cells in the S-phase of the cell cycle. Integrins, a family of transmembrane proteins play important role in cell–cell recognition, invasion, adhesion and migration. disialoganglioside GD3 co-localised with integrin-β1 and thereby inhibited it's downstream signalling in transfected cells. Transfected cells exhibited inhibition of cell adhesion with extracellular matrix proteins. Enhanced GD3 expression down regulated angiogenesis-regulatory proteins and inhibited epidermal growth factor/vascular endothelial growth factor-driven angiogenic cell growth in these cells. Taken together, our study provides support for the GD3-induced cell cycle arrest, disruption of integrin-β1-mediated anchorage, inhibition of angiogenesis and thereby induced apoptosis in pancreatic cancer cells.

Abbreviations: B-MAA, biotinylated-*Maackia amurensis* lectin; b-FGF, basic fibroblast growth factor; B-SNA, biotinylated-*Sambucus nigra* lectin; DAPI, 4′,6-diamidino-2-phenylindole; ECM, extra-cellular matrix; eIF4E, eukaryotic translation initiation factor 4E; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FACS, fluorescence-activated cell sorter; FGFR, fibroblast growth factor receptor; FITC, fluorescein isothiocyanate; GD1a, disialo-ganglioside GD1a; GD3: disialo-ganglioside GD3, GM3, monosialo-ganglioside GM3; HMEC, human microvascular endothelial cell; HUVAC, human umbilical vein endothelial cells; HIF1α, hypoxia-inducible factor 1-alpha; IGFR, insulin-like growth factor receptor; MAA, *Maackia amurensis* lectin; MFI, mean fluorescence intensity; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; MU, 4-methylumbelliferone; MUAc, 4-methylumbelliferyl acetate; Neu5Ac, 5-*N*-acetylneuraminic acid; PDAC, pancreatic ductal adenocarcinoma; SIAE, sialic acid acetylesterase; SNA, *Sambucus nigra* lectin; ST, sialyltransferase; VEGF, vascular endothelial growth factor, 4-MU-NeuAc, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid.

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1. Introduction

5-*N*-Acetylneuraminic acid (Neu5Ac), commonly known as sialic acid, constitutes a family of *N*- and *O*-substituted 9-carbon monosaccharides (Varki and Schauer, 2009). There are 52 different derivatives of sialic acids exist in nature. Sialic acids with a negative charge due to its carboxyl group along with the vast diversity in its structure, perform and modulate various cellular functions like camouflaging the antigenicity of receptors, causing repulsive actions between cells and manipulating the structural conformation of glycoproteins and glycolipids on cell membranes, especially in the lipid rafts (Sinha et al., 1999). One of the main attributes of cancer cells is abnormal glycosylation mainly through sialylation and fucosylation. Malignancies with metastatic properties are directly linked with altered sialylation in many cancers (Miyagi et al., 2012). The capacity of the host defense system decreases dramatically with increased sialic acid expression in malignancy thereby unable to induce apoptosis of the cancer cells (Sinha et al., 1999; Miyagi et al., 2012).

Gangliosides are structurally diverse acidic glycosphingolipids that are present in the outer leaflet of cell membranes. These are amphipathic constituents, composed of a sialic acid-containing hydrophilic carbohydrate moiety attached to hydrophobic ceramides (Sorice et al., 1997). They bind to other cell membrane components through their terminal sialic acids (Kohla et al., 2002). Different gangliosides influence cellular functions based on the positions and linkages of sialic acids. They are involved in a variety of functions, including acting as antigens or as receptors for bacterial toxins, mediating cell adhesion and modulating signal transduction (Hakomori, 2003). Gangliosides also play important roles in development (Fenderson et al., 1990), differentiation (Schengrund, 1990) and proliferation (Hakomori, 2002). Approximately more than 200 gangliosides have been identified to date, as classified for their carbohydrate components (Kamerling and Boons, 2007).

Expression of gangliosides mainly depends on the status of sialic acids modulatory enzymes like sialyltransferases, *O*-acetyl transferases, sialidases and esterases (Schauer, 2009). The balance between these enzymes gives the signature of the ganglioside profile of a cellular system. Some sialyltransferases are specifically responsible for particular ganglioside production, such as ST8 Sia1, ST3Gal V and ST6GalNAc IV are known as GD3-synthase, GM3-synthase and GD1a-synthase, respectively. Membrane bound sialidase (Neu3) specifically cleaves the terminal sialic acid of gangliosides (Monti et al., 2010).

GD3 is often depicted as a tumour-related ganglioside in epithelial originated meningioma and glioma (Fredman, 1994). It portrays its association with colorectal carcinoma, sarcoma and leukaemia. Normally an increase of GD3 induces apoptosis through the mitochondrial pore formation or activating CD95/FAS (Kristal and Brown, 1999; Malisan and Testi, 2002).

9-*O*-acetylated disialoganglioside GD3 is a minor subset of gangliosides, found in the majority of normal tissues, except thymus and placenta (Dyatlovitskaya and Bergelson, 1987) and also on the surface of a small population of normal human peripheral blood T cells (Merritt et al., 1996). 9-*O*- and 7-*O*-acetylated GD3 are found to be differentially regulated during differentiation and maturation of human T and B lymphocytes (Wipfler et al., 2011; Erdmann et al., 2006). They are found on lymphoblasts in childhood acute lymphoblastic leukaemia and gliomas and promote their survival (Mukherjee et al., 2008; Birks et al., 2011). Therefore, *O*-acetylated GD3 inhibits its pro-apoptotic activity (Chen et al., 2006).

Pancreatic ductal adenocarcinoma (PDAC), comprising of more than 90% of pancreatic cancers, is a fatal malignancy with poor prognosis. It is the fourth most common reason for cancer-related deaths in USA and the frequency of occurrence is almost similar to the frequency of mortality (Jemal et al., 2005). PDAC starts with a cluster of genetic alterations in proliferative pancreatic ductal epithelial cells (Hruban et al., 2000). Among the genetic alterations, the activation of the oncogenes like K-Ras and epidermal growth factor receptor (EGFR), and the inactivation or deletion of the tumour suppressor genes like p16, p53, and DPC4, are predominant (Bardeesy and DePinho, 2002; Jaffee et al., 2002). Other oncogenes that harbour mutations in pancreatic cancer include c-Myc and Akt (Feldmann and Maitra, 2008). However, the exact status and function of GD3 in pancreatic cancer is still unknown.

Metastasis is a multifaceted and multistep procedure involving unsynchronised cell proliferation, alterations in cell–cell and cell–matrix interactions, aberration in cell adhesion and abnormalities in angiogenesis (Nguyen and Massague, 2007). The characteristics of cancer cells to adhere to the basal cells or extra cellular matrix (ECM) and to metastasise to distant organs are some of the most crucial features of cancer. Adhesion molecules, for example the integrins, a family of transmembrane glycosylated proteins having eighteen α subunits and eight β subunits, can associate to form twenty-four unique heterodimers and provide cell–matrix adhesion and cell–cell recognition (Hynes, 1992). They are also critical for cancer cell motility (Holly et al., 2000). In pancreatic cancer, expressions of integrins α6β1 (Weinel et al., 1995; Vogelmann et al., 1999; Sawai et al., 2003) and αvβ3 (Hosotani et al., 2002) play a role in cell invasion. However, the highly metastatic PDAC cells, MIAPaCa2, do not express the α2β1 collagen-binding integrin (Grzesiak and Bouvet, 2006). Diverge combination and expressions of integrins relating to pancreatic tumour types makes it complicated to depict any common conclusion on the function of integrins.

In the management of PDAC, we have earlier demonstrated that mahanine, a herbal compound from an Indian dietary plant *Murraya koenigii*, demonstrated ROS-mediated apoptosis in pancreatic adenocarcinoma (Sarkar et al., 2013). Recently, we also showed that another herbal steroidal lactone showed apoptotic effects via G2/M cell cycle arrest in pancreatic cancer (Sarkar et al., 2014). To further to deal with PDAC control, we intended to study the intracellular mechanism of GD3-induced apoptosis in PDAC cells, and consequently tried to correlate this with the adhesion and angiogenic pathways.

Here we demonstrated GD3-induced apoptosis in MIAPaCa2 by arresting the cells in S-phase of the cell cycle. GD3 also co-localised with integrin-β1, which further inhibited its downstream signalling in MIAPaCa2 cells. It also inhibited cell adhesion with ECM proteins, down regulated the expression of angiogenesis regulatory proteins and inhibited EGF/vascular endothelial growth factor (VEGF)-driven angiogenic cell growth in PDAC cells.

2. Materials and methods

Most of the methodologies like real time polymer chain reaction, flow cytometric and Western blot analysis, gene microarray using Illumina human Sentrix 6V2, confocal microscopy, immunohistochemistry, cell viability and scratch wound assay together with reagents were furnished in the supplemental files.

2.1. Reagents

Primary antibodies against Bax, Bcl-2, Caspase-3, PARP, Chk1, Chk2, Cdc25A, Cyclin E, Cyclin A, Cdk2, Cdc2, *p*-Cdc2 (Tyr^{15}), *p*-c-Myc (Thr^{58}/Ser^{62}), c-Myc, p21, p27, Integrin-β1, FAK, Src, Grb2, *p*-Akt (Thr^{308}), *p*-Akt (Ser^{473}), Akt, eIF4E, HIF1α, p70S6K, FGFR1, IGFR1, bFGF, VEGFc and β-actin were from Cell Signalling Technology (Danvers, MA). VEGF, EGF, collagen, fibronectin, laminin, 2′-(4-methylumbelliferyl)-α-d-*N*-acetylneuraminic acid (4-MU-NeuAc),