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O-GlcNAcylation affects β-catenin and E-cadherin expression, cell motility and tumorigenicity of colorectal cancer

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ABSTRACT

O-GlcNAcylation, the addition of β-N-acetylglucosamine (*O*-GlcNAc) moiety to Ser/Thr residues, is a sensor of the cell metabolic state. Cancer diseases such as colon, lung and breast cancer, possess deregulated O-GlcNAcylation. Studies during the last decade revealed that O-GlcNAcylation is implicated in cancer tumorigenesis and proliferation. The Wnt/β-catenin signaling pathway and cadherin-mediated adhesion are also implicated in epithelial-mesenchymal transition (EMT), a key cellular process in invasion and cancer metastasis. Often, deregulation of the Wnt pathway is caused by altered phosphorylation of its components. Specifically, phosphorylation of Ser or Thr residues of β-catenin affects its location and interaction with E-cadherin, thus facilitating cell-cell adhesion.

Consistent with previous studies, the current study indicates that β-catenin is *O*-GlcNAcylated. To test the effect of *O*-GlcNAcylation on cell motility and how *O*-GlcNAcylation might affect β-catenin and E-cadherin functions, the enzyme machinery of *O*-GlcNAcylation was modulated either with chemical inhibitors or by gene silencing. When *O*-GlcNAcase (OGA) was inhibited, a global elevation of protein *O*-GlcNAcylation and increase in the expression of E-cadherin and β-catenin were noted. Concomitantly with enhanced *O*-GlcNAcylation, β-catenin transcriptional activity were elevated. Additionally, fibroblast cell motility was enhanced. Stable silenced cell lines with adenoviral OGA or adenoviral *O*-GlcNAc transferase (OGT) were established. Consistent with the results obtained by OGA chemical inhibition by TMG, OGT-silencing led to a significant reduction in β-catenin level. *In vivo*, murine orthotropic colorectal cancer model indicates that elevated *O*-GlcNAcylation leads to increased mortality rate, tumor and metastasis development. However, reduction in *O*-GlcNAcylation promoted survival that could be attributed to attenuated tumor and metastasis development. The results described herein provide circumstantial clues that *O*-GlcNAcylation deregulates β-catenin and E-cadherin expression and activity in fibroblast cell lines and this might influence EMT and cell motility, which may further influence tumor development and metastasis.

1. Introduction

O-GlcNAcylation regulates practically every cellular process, and seems to play a major role in the etiology of diseases [1]. *O*-GlcNAcylation is a reversible post-translation modification consisting of a single *N*-acetylglucosamine (*O*-GlcNAc) moiety attached via a β-*O*-glycosidic linkage to serine or threonine residues of the protein [2]. In many ways, *O*-GlcNAcylation is similar to protein phosphorylation. Since both modifications can occur on the same sites (i.e. serine/threonine residues) and thus, might entail a direct competition between the two. In addition, these two modifications can coexist at adjacent or even proximal sites that may impose a steric hindrance on each other [3,4]. However, unlike phosphorylation, which is regulated by hundreds of kinases and phosphatases, cycling of *O*-GlcNAc residues on intracellular proteins is known to be controlled by two highly conserved enzymes; *O*-linked *N*-acetylglucosamine transferase (OGT), which transfers *N*-acetylglucosamine from uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) to protein substrates, and *N*-acetyl-β-glucosaminidase (OGA, *O*-GlcNAcase), which removes the *O*-GlcNAc modification. Together, these enzymes dynamically alter the post-translational state, translocation and function of proteins in response to cellular signals [2,5,3].

It seems that *O*-GlcNAcylation is a hallmark of cancers and may be involved in tumor invasion and metastasis [6,7]. Increasing *O*-GlcNAcylation enhances the migration/invasion of breast and liver cancer

Abbreviations: CTD, carboxyl terminal domain; GlcNAc, N-acetylglucosamine; OGA, O-GlcNAcase; OGT, O-GlcNAc transferase; Ser, serine; WGA, wheat germ agglutinin; Thr, threonine; TMG, Thiamet G

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cells, lowering *O*-GlcNAcylation by knockdown of OGT inhibits tumor invasion and metastasis *in vivo* and *in vitro* in breast and prostate cancer cells [8,9,10]. Recent study has proposed a link between O-GlcNAcylation, SIRT1/ERK/FOXM1 signaling axis and breast cancer metastasis [11].

The most well-established example of changes in cancer-cell pattern and function is termed the epithelial–mesenchymal transition (EMT). Although EMT is a critical normal process during development and wound healing, recently properties of EMT have been implicated in cancer metastasis [12]. It is believed that the migratory characteristics acquired by EMT enable the invasive capabilities of the cancer cell [13]. During EMT, the cells lose their cell–cell junctions via different mechanisms, while retaining expression of migration-promoting molecules. These increase the motility of individual cells and enable the development of an invasive phenotype [14].

β-Catenin is a versatile protein playing fundamental roles in cells from control of intercellular junction integrity, to regulation of transcriptional processes as a co-transcription factor mediating the canonical Wnt signaling pathway [15]. β-catenin plays a role in intracellular adhesion. It links cadherin adhesion receptors to the actin cytoskeleton through the actin binding protein α-catenin. In the absence of a Wnt stimulus, the majority of β-catenin is located at the cytoplasmic side of the membrane as a component of cadherin-based cell–cell interaction. Deregulated Wnt signaling results in, increased levels of β-catenin in the nucleus and constitutive transcription of Wnt target genes, some of which are involved in cell proliferation, invasion and metastasis. Thus, increased transcriptional activity of β-catenin has been associated with the development and progression of many cancers.

In this study, we investigated how *O*-GlcNAcylation might affect β-catenin and E-cadherin functions, and further, how that might affect cell motility. Our results indicate that elevation of *O*-GlcNAcylation enhances β-catenin and E-cadherin expression levels, and enhances β-catenin transcriptional activity. This elevation is concomitant with enhancement of cell migration. Further, the in vivo test depicts that dysregulated *O*-GlcNAcylation affects tumor development, metastasis and mortality. The results obtained herein provide substantial evidences that *O*-GlcNAcylation may enhance cell migration through the regulation of β-catenin and E-cadherin levels.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were obtained from GIBCO Invitrogen (Carlsbad, CA, USA). Penicillin/streptomycin, trypsin-EDTA and enhanced chemiluminescence (ECL) kits were purchased from Biological Industries (Beit-Haemek, Israel). All antibodies and agarose beads were purchased from Santa-Cruz Biotechnology (Santa Cruz, CA, USA), except the following: anti-*O*-GlcNAc antibody (CTD110.6) (Gerald Hart laboratory, Johns Hopkins School of Medicine, Baltimore, MD, USA), wheat germ agglutinin (WGA) was purchased from Vector (Burlingame, CA, USA). Protein assay reagent was obtained from Bio-Rad (Hercules, CA USA). Protease inhibitor cocktail was purchased from Calbiochem (Gibbstown, NJ, USA). Thiamet-G (TMG) was kindly donated by Prof. Gerald W.Hart (Johns Hopkins School of Medicine, Baltimore, MD, USA)

2.2. Cell culture

CT26 murine colon carcinoma cells and NIH-3T3 murine fibroblasts were initially obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were grown at 37 °C, under a humidified atmosphere with 5% CO_2, in complete DMEM supplemented with 10% fetal bovine serum, and 1% penicillin-streptomycin.

2.3. Protein extraction

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested by scraping into chilled PBS containing 1 mM phenylmethylsulphonyl fluoride (PMSF) and centrifuged at 800 × *g* for 5 min. For whole-cell lysate, cells were suspended with RIPA buffer (10 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, and protease inhibitor cocktail 1:200), and were lysed by vibra cell sonicator (model VCX 130) (30% amplitude, 3 cycles of 3 s each, with 3 s interval), (Sonics and Materials Inc., Danbury, Conn., USA). The samples were centrifuged at 12,000 × *g* for 15 min at 4 °C, and supernatants were stored at − 70 °C until analysis.

2.4. β-Catenin Immunoprecipitation

Protein concentration was determined by Bradford assay (Bio-Rad) and 0.5 mg of cell lysate protein was used for immunoprecipitation. Anti-β-catenin antibody was linked to prewashed Sepharose A + G beads mix (1:1). Ninety µl of bead mixture were incubated (by gentle shaking) with anti-β-catenin antibody at 4 °C overnight, after which the samples were centrifuged for 1 min at 16,000 × *g*, and the supernatant was discarded. Prior to immunoprecipitation, the lysates were precleared by incubation (shaking) with 90 µl of Sepharose-protein A + G mixture for 20 min at 4 °C. Then the samples were centrifuged for 1 min at 16,000 × *g*, and the supernatants were added to the sepharose bead-bound β-catenin antibodies. The immunoprecipitation reaction was performed by gentle shaking at 4 °C for 3 h. The immunoprecipitated complex was pelleted by centrifugation at 2,800 × *g* at 4 °C for 2 min, washed three times with RIPA buffer, and eluted with Laemmli sample buffer (5% (w/v) SDS, 25% (v/v) glycerol, and 156 mM Tris-HCl pH 6.8, and bromophenol blue). The samples were boiled at 95 °C for 5 min and centrifuged, and the supernatant (bound fraction) was resolved by SDS-PAGE.

2.5. Affinity purification of β-catenin with Wheat Germ Agglutinin (WGA)

Samples were incubated with 400 µl of wheat germ agglutinin (WGA) in WGA buffer (300mMNaCl, 5 mM CaCl2, 1 mM MgCl2, 25 mM Tris-HCl pH 7.8), in the presence of different concentrations of *O*-GlcNac (1, 0.5, 0.25 and 0 M). After 2 h of rotation at 25 °C, unbound proteins were removed by three washes with WGA buffer and bound *O*-GlcNAcylated proteins were eluted with sample buffer. Alternatively, samples were treated with OGA enzyme at 37 °C for 2 h, and then purified by WGA.

2.6. Western blotting

Protein concentration was determined by Bradford assay (Bio-Rad) and equal amounts of whole-cell lysates (25 µg protein/lane) were separated on 10% SDS-PAGE and electroblotted onto nitrocellulose membranes. The membranes were blocked for 1 h with TBS containing 0.1% (v/v) Tween-20 (designated TBST) supplemented with 4% bovine serum albumin (BSA) or with 5% non-fat dry milk. The membranes were then probed overnight at 4 °C with the primary antibodies anti-*O*-GlcNAc CTD (1:5000), β-catenin (1:1000), E-cadherin (1:700) and Actin (1:1000). Next, the membranes were incubated for 1 h with the appropriate HRP-conjugated secondary antibody, and protein cross-reactive bands were detected using an ECL kit. Each immunoblot was repeated at least three times (using different protein extracts), and relative protein levels and immuno-reactive intensities (normalized according to the actin level) were quantified using Quantity One (Bio-Rad) software and presented as the mean ± SEM of adjusted volume intensity (adj. Vol. Int. × mm^2). Statistical analysis was performed using the T-test (at least $p \leq .05$) with the SPSS software.