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Autocrine galectin-1 promotes collective cell migration of squamous cell carcinoma cells through up-regulation of distinct integrins

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ABSTRACT

We found that high galectin-1 (Gal-1) mRNA levels were associated with invasive squamous cell carcinoma (SCC) cells that expressed Snail, an epithelial-to-mesenchymal transition (EMT) regulator. Both Gal-1 overexpression and soluble Gal-1 treatment accelerated invasion and collective cell migration, along with activation of cdc42 and Rac. Soluble Gal-1 activated c-Jun N-terminal kinase to increase expression levels of integrins α2 and β5, which were essential for Gal-1 dependent collective cell migration and invasiveness. Soluble Gal-1 also increased the incidence of EMT in Snail-expressing SCC cells; these were a minor population with an EMT phenotype under growing conditions. Our findings indicate that soluble Gal-1 promotes invasiveness through enhancing collective cell migration and increasing the incidence of EMT.

1. Introduction

Epithelial-to-mesenchymal transition (EMT) is a process by which a cancer cell becomes invasive. The process is typically characterized by loss of the cell–cell adhesion molecule E-cadherin, and acquisition of the mesenchymal protein vimentin [1,2]. Snail is a zinc finger transcription factor, and a master regulator of EMT that triggers the process through direct repression of E-cadherin expression [1,2]. Previous microarray analyses using oral squamous cell carcinoma (SCC) cells indicate that 63 genes, including Gal-1, are involved in Snail-induced EMT [3].

Gal-1 is a member of the β-galactoside-binding lectin family of proteins. These are expressed at various levels in many tissues under normal and pathological conditions. It has been reported that increased Gal-1 expression in SCC tissues is associated with prognosis. Gal-1 localizes to the nucleus and cytoplasm, and is present in the extracellular matrix. Regardless of whether the signal peptide is absent from its primary polypeptide sequence, Gal-1 exists as a non-covalent homodimer in its secreted form. This binds to the galactoside group in a glycosylated protein *via* a carbohydrate recognition domain [4–6].

During this study, we elucidated a novel mechanism through which autocrine Gal-1 induces cell migration and invasion by up-regulating the expression of integrins α2 and β5 through c-Jun N-terminal kinase (JNK) signaling pathways.

2. Materials and methods

2.1. Cell culture and reagents

The human vulval SCC cell line A431, and the human oral SCC cell lines OM-1, HOC719-PE, HOC719-NE, and HOC 313 were maintained as described previously [3,7]. HEK293FT cells were purchased from Invitrogen (CA, USA). All cell lines were cultured in Dulbecco's modified Eagle's medium (Sigma–Aldrich, MO, USA), supplemented with 10% fetal bovine serum (Biowest, Tokyo, Japan) and 1% penicillin streptomycin (Sigma–Aldrich) at 37 °C/5% CO_2. The JNK inhibitor SP600125 and the nuclear factor-kappa B (NF-kB) inhibitor ammonium pyrrolidinedithiocarbamate (APDC) were purchased from Sigma–Aldrich. Human recombinant galectin-1 (#450-39) was obtained from Peprotech (NJ, USA). Primary antibodies included those directed against Galectin-1 (sc-28248; Santa Cruz Biotech, TE, USA), V5 (Invitrogen), E-cadherin (#3195; Cell Signaling Technology, MA, USA), integrin α2 (CD49b) (#611016; BD Biosciences, CA, USA), integrin β5 (#4708; Cell Signaling Technology), phospho-JNK (#4668; Cell Signaling Technology), phospho-c-Jun (#9261; Cell Signaling Technology) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; MAB374; Millipore, MA, USA). We purchased horseradish peroxidase (HrP)-conjugated anti-rabbit IgG and HrP-conjugated anti-mouse IgG from GE Healthcare Bio-Sciences. Neutralizing antibodies against integrin

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α2 (MAB1950), integrin β1 (MAB1987) and integrin αV (MAB1980) were purchased from Millipore.

2.2. Reverse-transcription polymerase chain reaction (PCR) assays

Total RNA was isolated using an RNeasy kit (Qiagen, Venlo, Netherlands). First strand cDNA synthesis was conducted with cDNA synthesis kits (Toyobo, Osaka, Japan). Aliquots of the first strand cDNA library were subjected to PCR using Go tag Green Master Mix (Promega, WI, USA) when amplicons were to be subjected to agarose gel electrophoresis. For real-time detection, we used Thunderbird™ SYBR® qPCR Mix (Toyobo) in quantitative PCR (qPCR) assays. Gene-specific primer pairs are presented in Supplementary Table 1. The qPCR data were analyzed using a CFX connect Real-Time PCR Detection System (Bio-Rad, CA, USA). Relative comparison of samples with a calibrator was determined according to the Thunderbird™ SYBR® qPCR Mix (Toyobo) user manual.

2.3. Plasmids

The Galectin-1 (NM_002305.3) open reading frame was amplified from an OM-1 cDNA library using LA Taq with a GC buffer kit (Takara, Shiga, Japan) and cloned into a pCR8 Gateway entry plasmid (Invitrogen) using a pCR8/GW/TOPO TA cloning kit (Invitrogen). The entry vector was converted into pLenti 6.2/V5-DEST (Invitrogen) using an LR recombinase II kit (Invitrogen). This resulted in a V5-tagged Gal-1-expressing pLenti plasmid. The V5-tagged Snail-expressing pLenti plasmid has been previously described [8].

2.4. Recombinant virus production and infection

The ViraPower™ Letiviral Packaging Mix was purchased from Invitrogen. Host HEK293FT cells were co-transfected with pLenti plasmid and ViraPower™ using FuGENE6 (Promega), according to the manufacturer's protocol. After 48 h, the virus-containing supernatant supplemented with 8 μg/ml polybrene (Sigma) was used to infect target cells. Uninfected cells were removed by the addition of 10 μg/ml blasticidin (Invitrogen) during culture.

2.5. Immunoblotting

Cells harvested in phosphate-buffered saline (PBS) were precipitated and suspended in lysis buffer from a Mammalian Cell Lysis Kit (Sigma–Aldrich). For secreted proteins, growth medium was replaced with serum-free medium, and cells were incubated for a further 3 h. The conditioned medium was incubated with 4 volumes of acetone at −20 °C for 1 h. After centrifugation (15,000g, 10 min, 4 °C) the protein precipitate was dissolved in lysis buffer from the Mammalian Cell Lysis Kit. Total protein concentration was determined using a BCA protein assay kit (Pierce, IL, USA). Protein samples (20 μg) were denatured and reduced in sample buffer (ATTO) separated using 4–20% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to Immobilon-P (Millipore) membranes. The membranes were incubated (12 h, 4 °C) with the appropriate primary antibody diluted in Tris-buffered saline supplemented with Triton-X100 and 5% (w/v) bovine serum albumin (BSA). The immunoblot was labeled with the appropriate HrP-conjugated secondary antibody and signals visualized with an ECL Advance Western Blotting Detection Kit (GE Healthcare Bio-Sciences). Acquired images were analyzed using a LAS 4000 mini (GE Healthcare Bio-Sciences).

2.6. Pull-down assays

Cells were harvested using an Active GTPase Pull-Down and Detection kit (Pierce) according to the manufacturer's instruction. Briefly, a cell extract was applied to each affinity column for the GTP-bound form of Rho, Rac or cdc42. The bound fraction was eluted and recovered in sample buffer. Cell extracts and bound fractions were subjected to immunoblotting with anti-Rho, anti-Rac and anti-cdc42 antibodies, which were supplied in the kit.

2.7. Wound healing assays

Confluent monolayers of cells were prepared in culture plates. Wounds were prepared by scraping each plate with a sterile 200-μl pipette tip. Initial wound size and closing distance was evaluated using phase-contrast microscope images at various time points using a BZ-9000 microscope (Keyence, Osaka, Japan).

2.8. In vitro three-dimensional culture

Immortalized fibroblast GT1 cells were suspended in type I collagen (Koken, Tokyo, Japan) containing growth medium, then poured into 12-well culture plates [9]. Subsequently, 1×10^6 SCC cells in 1 ml of growth medium were seeded onto the collagen gel. After 1 week in culture, a small shrunken gel disc was placed onto a reversed nylon mesh strainer well (BD Biosciences) to maintain the air–liquid interface culture for a further week. Each gel was fixed with Mildform (Wako, Osaka, Japan) and embedded in paraffin. Thinly sliced sections were stained with hematoxylin and eosin (HE; Wako) and analyzed.

2.9. Invasion assays

Invasiveness was assessed using Biocoat-Matrigel invasion chambers (BD Biosciences) according to the manufacturer's instructions. Briefly, 2.5×10^5 cells were seeded into each upper chamber and cultured for 24 h. The upper chamber was fixed by soaking in 4% paraformaldehyde in PBS. After removing cells from the topside of the Matrigel-coated membrane, cells invading the other side of the membrane were stained with HE and counted with the aid of a microscope.

2.10. RNA interference (RNAi)

Small interfering RNAs (siRNAs) for integrin β5 and control siRNAs were obtained from Sigma Aldrich. Target-specific siRNA duplexes were 5′-CUA UGU CUG CGG CCU GUG UTT-3′ (sense) and 5′-ACA CAG GCC GCA GAC AUA GTT-3′ (antisense). We transfected 2.5×10^5 cells were with 100 pmol of double-stranded (ds)RNA using RNAi Max (Invitrogen) according to the manufacturer's guidelines. The knock-down efficiency was determined 48 h later by immunoblotting with an anti-integrin β5 antibody (data not shown). Wound healing assays were started at 24 h post-transfection once cells were confluent.

2.11. Fluorescent cell imaging

Cells were seeded in 24-well EZ view glass-bottom plates (Iwaki, Shizuoka, Japan), fixed with 4% paraformaldehyde in PBS for 15 min, and permeabilized with 0.2% Triton X-100 in PBS for 30 min. The permeabilization step was omitted for cell surface staining analysis. Cells were incubated for 8 h with a primary antibody incubated in PBS containing 5% (w/v) BSA, and subsequently incubated for 1 h with Alexa Fluor® 568- or Alexa Fluor® 488-conjugated secondary antibodies (Invitrogen). To visualize F-actin, cells were incubated with Alexa Fluor® 488 Phalloidin (Invitrogen) diluted in PBS for