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Keratinocytes negatively regulate the N-cadherin levels of melanoma cells via contact-mediated calcium regulation

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ABSTRACT

In human skin, melanocytes and their neighboring keratinocytes have a close functional interrelationship. Keratinocytes, which represent the prevalent cell type of human skin, regulate melanocytes through various mechanisms. Here, we use a keratinocyte and melanoma co-culture system to show for the first time that keratinocytes regulate the cell surface expression of N-cadherin through cell-cell contact. Compared to mono-cultured human melanoma A375 cells, which expressed high levels of N-cadherin, those co-cultured with the HaCaT human keratinocyte cell line showed reduced levels of N-cadherin. This reduction was most evident in areas of A375 cells that underwent cell-cell contact with the HaCaT cells, whereas HaCaT cell-derived extracellular matrix and conditioned medium both failed to reduce N-cadherin levels. The intracellular level of calcium in co-cultured A375 cells was lower than that in mono-cultured A375 cells, and treatment with a cell-permeant calcium chelator (BAPTA) reduced the N-cadherin level of mono-cultured A375 cells. Furthermore, co-culture with HaCaT cells reduced the expression levels of transient receptor potential cation channel (TRPC) 1, −3 and −6 in A375 cells, and siRNA-mediated multi-depletion of TRPC1, -3 and -6 reduced the N-cadherin level in these cells. Taken together, these data suggest that keratinocytes negatively regulate the N-cadherin levels of melanoma cells via cell-to-cell contact-mediated calcium regulation.

1. Introduction

In the epidermis, melanocytes are surrounded by neighboring keratinocytes, and the two cell types together maintain epidermal homeostasis. Keratinocytes control melanocytes via soluble factors (e.g., growth factors, hormones, cytokines) [1], the extracellular matrix [2] and direct cell-cell interaction [3]. Among the molecules that are commonly involved in this intercellular communication, the cadherins mediate cell-cell adhesion in a calcium-dependent manner. The classical cadherins include E−, N- and P-cadherin, which are expressed with cell- and tissue-specificity [4]. In normal epidermis, E-cadherin is expressed on the cell surfaces of both melanocytes and keratinocytes, where it functions as a major cell adhesion molecule [5]. Disruption of E-cadherin may allow melanocytes to escape from the tight control of keratinocytes, and thus may be an initial step in the transition from a melanocyte to a melanoma cell, in which E-cadherin expression is decreased but N-cadherin is increased. This cadherin switching enables melanoma cells to invade into the dermis by interacting with N-cadherin-expressing cells, such as fibroblasts and endothelial cells [6]. Indeed, N-cadherin promotes the heterotypic adhesion of melanoma cells to dermal fibroblast cells and their migration over fibroblasts [7], and re-expression of E-cadherin in melanoma cells induces apoptosis and suppresses their invasion through gap junctional communication with keratinocytes [8].

The above-described intercellular communication induces the exchange of small molecules, including ions (e.g., Ca^{2+}, H^{+}), secondary messengers (e.g., phosphatidyl inositides, cAMP) and

Abbreviations: TRPC, transient receptor potential cation channel; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ECM, extracellular matrix.

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metabolic products (amino acids) [9]. Calcium influxes mediated by the cell-cell interaction induce bone matrix protein production [10] and neurite outgrowth [11]. In the epidermis, calcium plays critical roles in cellular functions, including the regulation of keratinocyte and melanocyte differentiation and proliferation through surface-expressed calcium-sensing receptors [12]. Reduction of calcium in melanoma cells was shown to strongly suppress their cell migration in vitro and colony formation in vivo [13]. Oda et al. recently found that the calcium channel, TRPC (transient receptor potential canonical) 3, is widely expressed in human melanoma and that calcium treatment induces the growth and migration of melanoma cells [14]. Furthermore, in a B16 melanoma cell-A431 keratinocyte co-culture system, cell-cell interaction was shown to trigger the release of calcium from intracellular stores in keratinocytes, leading to melanin transfer [3]. Together, the previous findings suggest that calcium signaling plays important roles in the cell-cell communication between keratinocytes and melanoma cells. However, no previous report has examined the potential ability of the keratinocyte-melanoma interaction to alter the level of N-cadherin in melanoma cells. Here, we use a co-culture system to show that keratinocytes downregulate the calcium levels of melanoma cells through cell-cell contact, and thereby negatively regulate N-cadherin expression in melanoma cells.

2. Materials and methods

2.1. Reagents and antibodies

Polyclonal antibodies against GAPDH, E-cadherin and N-cadherin and the monoclonal antibody against cytokeratin-18 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal antibodies against TRPC 1 and TRPC 3–7 were purchased from Alomone Labs (Jerusalem, Israel). BAPTA-AM was purchased from TCI America (Portland, OR, USA).

2.2. Cell culture

The A375 human melanoma cell line, B16F10 mouse melanoma cell line and HaCaT human keratinocyte cell line were maintained in Dulbecco's-modified Eagle's medium (DMEM; WelGene, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and gentamicin (50 μg/ml, Sigma, St. Louis, MO, USA). Cells were cultured at 37 °C in 5% CO_2 in a humidified atmosphere. For the co-culture system, A375 cells and HaCaT cells were mixed 1:2 and plated to six-well plates with a total of 3.6×10^5 cells/well. After 48 h, the A375 cells were separated from the HaCaT cells using 1 mM EDTA (in phosphate buffered saline; PBS), and further processed as described below.

2.3. Immunoblotting

The cells were washed twice with PBS and lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 2 mM Na_3VO_4) containing protease inhibitors (1 μg/ml aprotinin, 1 μg/ml antipain, 5 μg/ml leupeptin, 1 μg/ml pepstatin A, 20 μg/ml phenylmethylsulfonyl fluoride). The lysates were clarified by centrifugation at 13,000×*g* for 15 min at 4 °C, denatured with SDS sample buffer, boiled, and analyzed by SDS- polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were transferred to nitrocellulose membranes (NC; GE Healthcare, Germany) and probed with the appropriate antibodies. The signals were detected by an Odyssey imaging system (Li-COR Biosciences, Lincoln, NE, USA).

2.4. Cell fractionation

Hypoosmotic solution (20 mM Tris/HCl, pH 7.5, 2 mM 2-mercaptoethanol, 5 mM EGTA, 2 mM EDTA) containing protease inhibitors was added to the culture plates. The cells were scraped off the plates and sonicated, and the obtained cell lysate was centrifuged at 13,000×*g* for 15 min at 4 °C. The membrane fraction was collected by solubilizing the pellet in RIPA buffer containing protease inhibitors and centrifuging the sample at 13,000×*g* for 15 min at 4 °C. Equal amounts of lysates were resolved by SDS-PAGE, transferred to NC membranes, and probed with the indicated antibodies. The signals were detected by an Odyssey imaging system.

2.5. Small interfering RNAs (siRNAs)

siRNAs against human TRPC 1 (No. 1156730), TRPC 3 (No. 1156740) and TRPC 6 (No. 1156770) were purchased from Bioneer (Daejeon, Korea). Transient transfections were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the provided instructions.

2.6. Flow cytometry

Cells were cultured in 60-mm tissue culture dishes, washed with PBS, released with 0.2% EDTA followed by 5% FBS in PBS, pelleted, resuspended in PBS and counted. The cells were then incubated overnight at 4 °C with anti-N-cadherin antibody in 10% FBS in PBS, washed three times with PBS containing 0.05% Tween-20, and incubated with APC-conjugated anti-rabbit antibody in 10% FBS in PBS for 1 h. The cell surface expression of N-cadherin was analyzed by fluorescence-activated cell sorting (FACSCalibur; BD Biosciences, San Jose, CA, USA).

2.7. Immunofluorescence analysis

Cells were grown in 12-well plates containing coverslips and fixed with 3.5% paraformaldehyde for 10 min. The cells were washed with PBS, blocked with 0.5% BSA and incubated overnight with primary antibodies at 4 °C. The cells were washed again with PBS and incubated with Texas Red-conjugated goat anti-rabbit antibody (Invitrogen) for 1 h at 25 °C. For staining of intercellular proteins, the cells were fixed with 3.5% paraformaldehyde and permeabilized with 0.5% Triton X-100. The cells were blocked with 0.5% BSA and incubated with antibody overnight at 4 °C. The coverslips were mounted on glass slides with mounting solution containing 4′,6-diamidino-2-phenylindole (DAPI) and observed by fluorescence microscopy.

2.8. In vitro calcium imaging

HaCaT cells were seeded into μ-slides (Ibidi, Munich, Germany) and stained with 20 nM of MitoTracker, red-fluorescent dye (Invitrogen). A375 cells were transfected with the calcium-sensing FRET probe, Twitch-1, in the pcDNA3 vector [15] and co-cultured with HaCaT cells. After 24 h, the culture medium was replaced by Leibovitz's L-15 medium without phenol red (Life Technologies, Carlsbad, CA, USA) supplemented with 10% FCS and intracellular calcium levels in A375 cells were measured by FRET analysis. Briefly, Twitch-1-expressing A375 cells were imaged for donor and acceptor emissions using a DeltaVision platform (GE Healthcare, Chicago, IL, USA) that comprised a motorized, inverted microscope (model IX71 [Olympus, Tokyo, Japan]; Universal Apochromat/340 40 × /1.35 oil and Plan ApochromatN 60 × /1.42 oil objectives; optical filters 438/24 for CFP and 513/17 for YFP excitation, 470/24 for

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