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Switch from αvβ5 to αvβ6 integrin is required for CD9-regulated keratinocyte migration and MMP-9 activation

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ABSTRACT

Our previous research found that tetraspanin CD9 is downregulated in migrating epidermis during wound healing, and CD9 downregulation contributes to keratinocyte migration via matrix metalloproteinase-9 (MMP-9) activation. However, little is known about the mechanisms involved in CD9-regulated keratinocyte migration and MMP-9 activation. In this study, we revealed that the expressions of integrin subunits β5 and β6 were regulated by CD9. Furthermore, CD9 silencing triggered the switch from αvβ5 to αvβ6 integrin in HaCaT keratinocytes and CD9 overexpression reversed the switch. Importantly, integrin αvβ6 functional blocking antibody 10D5 significantly inhibited CD9 silencing-induced keratinocyte migration and MMP-9 activation, suggesting that the switch from αvβ5 to αvβ6 integrin plays a key role in CD9-regulated cell migration and MMP-9 activation in keratinocytes.

1. Introduction

Wound healing is a dynamic and well-ordered biological process requiring the collaborative efforts of many different tissues and cell lineages [1,2]. Keratinocyte migration is an early event in the re-epithelialization process, an essential step for wound healing [3]. The behavior of keratinocyte migration, matrix synthesis, and signaling pathways present at a wound site are roughly understood. Our previous results revealed that CD9, a member of the tetraspanin superfamily, is critical for cutaneous wound healing. CD9 is downregulated in migrating epidermis and re-elevated to the basal level when re-epithelialization is complete [4]. CD9 is an integral transmembrane protein comprised of four transmembrane domains, two extracellular loops, and short intracellular ends [5,6] and has been implicated in various cellular and physiological processes like cell motility, migration, and adhesion [7]. Moreover, we also found that CD9 downregulation enhances keratinocyte migration by upregulating matrix metalloproteinase-9 (MMP-9) [8]. MMP-9, a gelatinase subclass, was revealed to contribute to keratinocyte migration during wound repair [9–11]. However, little is known about how CD9 regulates keratinocyte migration and MMP-9.

Integrins are heterodimeric transmembrane receptors that comprise the α- and β-subunit for extracellular matrix (ECM), which play important roles in embryonic development, wound healing, inflammation, and tumorigenesis [12]. The activity of integrins must be regulated to execute the function, either by outside-in signals from ECM or inside-out signals from multiple intracellular pathways [13,14]. Integrins known to be expressed in the epithelium include integrins α3β1, α6β4, αvβ5, and αvβ6 [15]. Interestingly, αvβ6 is not expressed constitutively in healthy epithelia but is upregulated during tissue remodeling, including wound healing and carcinogenesis [16,17]. Aslo, freshly isolated epidermal keratinocytes do not stain for αvβ6 integrin but begin to express after subculturing [18]. Successful epithelial wound healing is associated with specific integrin changes in the cell-matrix adhesion process and extracellular matrix degradation [19]. αv forms a heterodimer exclusively with β5 in normal epidermis [20]. Both αvβ5 and αvβ6 are expressed in hyperproliferative stratified squamous epithelia [18]. Reepithelialization is associated with a switch from αvβ5 to αvβ6 during cutaneous wound healing [21]. The down-regulation of αvβ5 in association with αvβ6 expression probably reflects a hierarchy in the preference of αv

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to heterodimerize with different subunits [22]. CD9 also shows well-ordered change during wound repair [4] and is implicated in many integrin regulations [23,24]. Moreover, αvβ6 integrin is revealed to promote corneal wound repair [25], upregulate MMP-9, and promote cell migration in normal oral keratinocytes [26]. Increasing research found that αvβ6 integrin is involved in promoting MMP-9 expression and cell migration [27–29]. Hence, we hypothesized that CD9 regulates the switch from αvβ5 to αvβ6 integrin, which participates in keratinocyte migration and MMP-9 expression.

In the present study, we found that CD9 downregulation triggered the switch from αvβ5 to αvβ6 integrin in HaCaT keratinocytes and CD9 overexpression reversed the switch. Furthermore, CD9 silencing-induced keratinocyte migration and MMP-9 activation and expression were significantly inhibited by αvβ6 integrin functional blocking, while αvβ5 integrin had no effect in the processes. Thus, we report here, for the first time, that the switch from αvβ5 to αvβ6 integrin plays a key role in CD9-regulated cell migration and MMP-9 activation in keratinocytes.

2. Materials and methods

2.1. Cell culture

HaCaT keratinocytes were obtained from Cell Bank of the Chinese Academy of Sciences in Beijing, China. Cells were cultured in RPMI 1640 medium (Hyclone, USA) supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% fetal bovine serum (Hyclone, USA). The cells were incubated at 37 °C, 5% CO_2, and 95% humidity.

2.2. Recombinant adenovirus vectors for CD9 expression silencing or overexpressing

The recombinant adenovirus vectors for silencing CD9 expression (siCD9) or overexpressing CD9 (Ad-CD9) and the negative vector containing non-specific shRNA (siCtr) and the negative control adenovirus vector (Vector) were purchased from Shanghai GeneChem, Co. Ltd (Shanghai, China). All vectors contained the gene for GFP, which served as a marker. HaCaT cells were infected with these vectors at a multiplicity of infection of 10 for 48 h for these experiments.

2.3. Western blot

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and collected in 1× loading buffer on ice, and homogenized. Protein concentration was determined by RCDC protein assay kit (Sigma, USA). Cell lysates were resolved by SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes according to standard procedures. The membranes were blocked with 5% skim milk and probed with primary antibodies for β5, β6, β1, αv, and α3 (1:1000; Santa Cruz Biotechnology, USA) and MMP-9 (1:1000; Abcam, USA) anti-GAPDH at 1:5000 dilution. Horseradish peroxidase-conjugated IgG was used as a secondary antibody and GAPDH was used as loading control. The results were analyzed with ChemiDoc imaging system (Bio-Rad, USA).

2.4. Immunoprecipitation

To analyze the integrin subunit αvβ5 and αvβ6 complex formation at the plasma membrane, equal amounts of membrane protein (500 μg) extracted with the Plasma Membrane Protein Extraction Kit (Biovision, USA) were incubated with 2 μg of anti-αv antibody for 1 h at room temperature and then the complexes were precipitated with protein A/G-Sepharose (Santa Cruz) overnight at 4 °C. The lysate-antibody-agarose bead mixture was washed four times with PBS and then probed with anti-β5, anti-β6, and anti-αv (1:1000 dilution) (Santa Cruz Biotechnology, USA) using Western blot.

2.5. Immunofluorescence microscopy

Cells cultured on fibronectin-coated glass coverslips were fixed in 4% paraformaldehyde for 20 min. The fixed cells were incubated with mouse primary antibody αvβ5-heterodimer-specific antibody P1F6 (1:100 dilution; Millipore, USA) or αvβ6-heterodimer-specific antibody E7P6 (1:100 dilution; Millipore, USA) at 4 °C overnight, washed with PBS and followed by incubation with goat anti-mouse secondary antibody conjugated to cyanine 3 (Cy3; 1:100 dilution; Beyotime, Shanghai, China) at 37 °C for 1 h. Nuclei were stained with DAPI (Hyclone, USA). The integrin αvβ5 or αvβ6 expression was observed under Leica Confocal Microscope (Leica Microsystems, Wetzlar, Germany). The fluorescence intensity of individual cells was measured and analyzed with the NIH ImageJ image software (http://rsb.info.nih.gov/ij/).

2.6. Flow cytometry

Subconfluent HaCaT keratinocytes were washed with PBS, trypsinized and resuspended in FACS buffer (PBS containing 1 mM $MgCl_2$ and 0.1% BSA). Cells were incubated with antibody P1F6 or E7P6 at 4 °C for 40 min and washed twice with PBS. Alexa Fluor® 488 Goat anti-mouse secondary antibody (1:100 dilution; Invitrogen, USA) was applied to the cells at 4 °C for 30 min. Cells were washed twice with PBS and resuspended in 0.5 mL PBS with 10% fetal bovine serum. Labelled cells were subsequently analysed on a FACScans flow cytometer using Cellquest software (Beckton Dickinson).

2.7. Cell scratch wounding assay and time-lapse videomicroscopy

Scratch wounding assay was performed as we described previously [8]. HaCaT Cells infected with recombinant adenoviruses were grown to confluence in the 24-well plates in serum conditioned RPMI 1640. Scratch wounds were created in confluent monolayers using a sterile 20 μL pipette tip and different fields were filmed for 20 h using a Zeiss videomicroscope then analysed using the NIH ImageJ image software (http://rsb.info.nih.gov/ij/). For inhibition experiments, αvβ6 function blocking antibody 10D5 (10 μg/mL) or αvβ5 function blocking antibody P1F6 (10 μg/mL) was added into RPMI 1640 medium at 37 °C for 30 min before scratching.

2.8. In vitro cell migration assays

Cell-migration assays were performed using polycarbonate filters (8 mm pore size, Transwell; BectonDickinson, USA). For inhibition experiments, function blocking antibody 10D5 or P1F6 (10 μg/mL) was added into upper and lower chambers. The lower chamber was filled with 600 μL of RPMI 1640 medium supplemented with 10% FBS, and the cells were plated at a density of 3×10^5 in 100 mL of migration buffer in the upper chamber of triplicate wells followed the incubation at 37 °C for 20 h. Then the transwell inserts were fixed with 10% formalin, stained with 0.5% crystal violet in 10% ethanol for 10 min and washed with PBS for three times. Cells in the upper compartment were removed using a cotton wool swab, and the filter was mounted onto glass slides. Cells from five random fields were counted under 100× magnification. Mean cell numbers for each sample were from triplicate inserts.