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Acidic pH*e* regulates cytoskeletal dynamics through conformational integrin β1 activation and promotes membrane protrusion

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ABSTRACT

An acidic extracellular pH (pH*e*) in the tumor microenvironment has been suggested to facilitate tumor growth and metastasis. However, the molecular mechanisms by which tumor cells sense acidic signal to induce a transition to an aggressive phenotype remain elusive. Here, we showed that an acidic pH*e* (pH 6.5) stimulation resulted in protrusion and epithelial-mesenchymal transition (EMT) of cancer cells, which promoted migration and matrix degeneration. Using computational molecular dynamics simulations, we reported acidic pH*e*-induced opening of the Integrin dimers (α5β1) headpiece which indicated the activation of integrin. Moreover, acidic pH*e* promoted maturation of focal adhesions, temporal activation of Rho GTPases and microfilament reorganization through integrin β1-activated FAK signaling. Furthermore, mechanical balance of cytoskeleton (actin, tubulin and vimentin) contributed to acidic pH*e*-triggered protrusion and morphology change. Taken together, these findings revealed that integrin β1 could be a novel pH-regulated sensitive molecule which confers protrusion and malignant phenotype of cancer cells.

1. Introduction

Acidic pH*e* (from pH 5.8–7.4) [1] is an important feature of the tumor microenvironment [2], along with stiffness of the extracellular matrix (ECM), various cellular constituents, and growth factors. Based on measurements of lactic acid in tumors and normal tissues, Warburg proposed that, even in the presence of sufficient oxygen, tumors rely on anaerobic glycolysis rather than oxidative phosphorylation as a metabolic energy source, which results in lactate accumulation. The "Warburg effect" is now a widely accepted answer as to why tumors have an acidic pH*e*, and other chemicals, such as CO_2 [3], also contribute to the formation of an acidic microenvironment. Acidic pH*e* in the cancer microenvironment can promote tumor growth and metastasis *via* increased VEGF [4] and matrix metalloproproteinase-9 [5] production, regulation of apoptosis [6], autophagy [7] and immune [8] and increased expression of responsive genes, such as SREEP2 [9].

A number of steps are involved in tumor metastasis, including tumor cell detachment and migration, degradation of the ECM, intravasation and transportation in the circulatory system, and finally growth at a distant site. Epithelial-mesenchymal transition (EMT) is known to be a powerful promoter and regulator of most of the abovementioned steps. During EMT, dynamic reorganization of cytoskeleton and protrusion are determinants for the morphology, migration and invasive abilities of carcinoma cells. Additionally, recent studies reported the crucial role of low pH in the EMT process [10–12], but the role of acidic pH*e* on protrusion and cytoskeletal dynamics in EMT progression remains unknown.

Activation of many types of proteins depends on environmental pH with pH-dependent structural plasticity, such as heat shock protein sip1 [13] and bacterial sensor kinase PhoQ [14]. The integrin protein family governs various cellular behaviors critical to cancer initiation, progression and metastasis. Integrins promote actin rearrangement *via* the recruitment of molecules, including the Rho family of GTPases, that stimulate cytoskeleton rebuilding [15]. Integrin α5β1 is an essential factor of fibronectin mediated adhesion with enhanced RhoA GTPase activation and localization of focal contacts in cellular protrusions [16]. In addition, the interactions of integrin αIIbβ3 with kinases, such as Src, have been implicated in initiation of integrin outside-in signaling to the actin cytoskeleton [17]. Therefore, whether acidic pH*e* itself could directly regulate integrin activity is still an open question. Due to the particular spatial structure of integrin family, we hypothesize that integrins as transmembrane proteins that respond to both intra- and

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extracellular cues, most probably act as pH sensors to facilitate the accurate regulation of cellular protrusion, architecture, spreading and migration in cancer progression.

Accordingly, in this study, we aimed to elucidate whether integrin β1 have the potential to regulate acidic pH*e* induced cytoskeletal dynamics, protrusion and EMT. We found that an acidic pH*e* could induce mesenchymal-like morphological changes in melanoma, cervical carcinoma and osteosarcoma cells in 2D and 3D culture. We further explored the molecular mechanisms for acidic pH*e* induced EMT by evaluating its effects on *in vitro* adhesion, migration and invasion and signal transduction. Moreover, we analyzed dynamic reconstructions of the cytoskeleton proteins (actin, tubulin and vimentin) and protrusion divined by acidic pH*e*. Molecular dynamics (MD) simulations were applied to understand of how acidic extracellular pH affects integrin conformation. Integrin β1 plays an important role in the regulation of actin filaments and downstream EMT related proteins under acidic pH*e*. Our data demonstrated that acidic pH*e* could trigger cell protrusion and cytoskeletal dynamics *via* integrin β1-activiation of the FAK-Src signaling.

2. Materials and methods

2.1. Cell culture and treatment

A375, HeLa and U2-OS cells, obtained from the American Type Culture Collection (Manassas), were maintained in DMEM or DMEM/F12 cell culture medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% antibiotic-antimycotic (Gibco) at 37 °C in a 5% CO_2 atmosphere. HCl (3 mM) was added to the medium to mimic an acidic pH*e* (approximately pH 6.5). The pH of acidic medium could last for 48 h under cell culture condition. Cell lines were authenticated using Short Tandem Repeat analysis as described in 2012 in ANSI Standard (ASN-0002) by the ATCC Standards Development Organization (SDO) (Genetic Testing Biotechnology, China).

2.2. Phalloidin staining and cell form factor (FF) analysis

Phalloidin staining of cells was performed as published before [18]. Cells were fixed with 4% formalin for 15 min at room temperature, and stained with a TRITC-phalloidin solution (5 μg/ml) (Sigma Aldrich, CA, USA) in PBS for 40 min at 37 °C. Images were acquired under confocal microscope (Nikon A1, Nikon Corporation, Tokyo, Japan) or research microscope (Axio Imager 2, Zeiss, Oberkochen, Germany). The elliptical factor (EF, EF = length/breadth) was calculated to quantify the morphological changes of cells. EF of cells and fractions of cells with protrusion > 5 μm, strong stress fibers and cortical actin were statistically analyzed using Image J software (NIH, MD, USA). Cell shape measurements are elevated as the form factor (FF), $4\pi(\text{area})/(\text{perimeter})^2$, which gives a value of 1 for a perfectly circular perimeter and decreasingly smaller positive values for less circular perimeters. Cell FFs was analyzed using Image J. In each experiment, > 100 cells from each group were measured.

2.3. Cell proliferation, adhesion and wound healing assays

After cells were seeded into 96-well plates at 5000 cells/well for 12 h, cell proliferation was determined using the Cell Counting Kit-8 assay kit (CCK-8) (Dojindo). The absorbance at 450 nm was measured by spectrophotometry (Spectra Max190, Molecular Devices). The adhesion and wound healing assays were performed as published before [19]. After culturing in acidic pH*e* for 24 h, cells were trypsinized, washed and resuspended in culture medium. Cells (1×10^4) were added into each well and incubated for 6 h. Non-adherent cells were removed by washing with PBS and images were acquired using an inverted fluorescence microscope (Nikon TE-2000, Tokyo, Japan). After A375 cells had grown to confluence, a wound was created by scratching using a sterile pipette tip. The medium was aspirated and displaced cells were washed with PBS. Images were acquired immediately and at 24 h.

2.4. Time-lapse tracking

Cells were (1×10^5) seeded in 3.5 mm dishes the day before experiments. Medium was then changed into acidic pH*e* medium before taking images. Each image was taken by Nikon TE-2000 U. The cells in each group were taken for 15 min interval and recorded for 2 h duration. Cell migration routes, distance, and velocity were analyzed and by Image J. Cells measured: control group (n = 13) and acid group (n = 8).

2.5. Gene silencing

siRNA targeting human vimentin (5′-CUGGCACGUCUUGACCUU GAAdTdT-3′), FAK (5′-ACACCAAAUUCGAGUACUAdTdT-3′) and scrambled control siRNA were designed and synthesized by RiboBio (RiboBio, Guangzhou, China). A375 cells were transfected with 50 nM annealed siRNA using Lipofectamine3000 (Invitrogen, CA, USA). The cells were continued to incubate for a total of 24 h for the downstream experiments.

2.6. Real time-PCR

Real time-PCR was performed as published before [19]. Total RNA was extracted using the RNAiso Plus kit (Takara, Shiga, Japan) and reverse transcribed using the PrimeScriptTM RT reagent kit (Takara). Real-Time was performed on a CFX96 Touch™ Detection System (Bio-Rad, CA, USA) with the SYBR® Premix Ex Taq™ II kit (Takara). Gene expression was normalized to that of β-actin using the 2-ΔΔCt method. Data were presented as fold-changes. The primers used are listed in Supplementary table 1.

2.7. Protein extraction and western blotting analysis

Western blotting and immunofluorescence analysis were performed as published before [19]. Each lysate was resolved by non-reducing SDS-PAGE for active integrin β1 [20] or reducing SDS-PAGE for other proteins, and samples were blotted for indicated antibodies. E-cadherin, vimentin, Snail, Twist1, β-actin (Santa Cruz Biotech, CA, USA), integrin β1 (BD, NJ, USA), Src (Tyr-416) (CST, MA, USA), FAK (BD), p-FAK (Tyr-397) (CST) and active integrin β1 (clone HUTS-4, Millipore, MA, USA).

2.8. Immunofluorescence analysis

Cells grown on coverslips were fixed with 4% formaldehyde for 15 min at room temperature, permeabilized with 0.4% Triton X-100 for 15 min, and blocked with 1% bovine serum albumin (BSA) for 1 h. The cells were incubated with primary antibodies at 1:50 dilution at 4 °C overnight, washed with PBS, and incubated with a secondary antibody at 4 °C overnight. Slides were counter-stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Gibco, NY, USA). Fluorescent images were acquired with an Axio Imager 2 microscope.

2.9. Rho GTPase activity analysis

GTPases were enriched using the Pierce® GTPase Enrichment Kits and ActivX® GTP Probes assay kit (Thermo). After the enrichment, the protein levels of RhoA, Rac1, and Cdc42 were detected by Western blotting analysis.

2.10. PA soft substrates preparation

PA gels were prepared as described previously [21]. Briefly, glass