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Differentiation impairs low pH-induced Ca^{2+} signaling and ERK phosphorylation in granule precursor tumour cells

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ABSTRACT

Extracellular acidification is a hallmark of a number of debilitating pathologies including cancer, ischemia and inflammation. We have recently shown that in human granule precursor tumour cells a fall in extracellular pH triggers increases in intracellular Ca^{2+} concentration through activation of G-protein coupled proton-sensing receptors coupling to phospholipase C. This pH-dependent rise in cytosolic Ca^{2+} led to activation of the extracellular signal-regulated kinase ERK, providing a mechanistic explanation of how extracellular acidification can promote tumour growth. We now find that differentiation of granule precursor tumour cells profoundly affects their ability to respond to extracellular acidification with gene transcription. Differentiating cells have a lower Ca^{2+} release probability from intracellular Ca^{2+} stores upon acidification and cells that respond have a significantly smaller and slower Ca^{2+} signal than proliferating cells. Importantly, Ca^{2+} release in differentiating cells fails to evoke ERK phosphorylation. This altered responsiveness of differentiating cells is not due to reduced proton-sensing receptor expression or diminished Ca^{2+} store content. Rather, our results suggest that in differentiating cells, the proton-sensing receptor couples less effectively to phospholipase C activation and IP_3 formation. Hence, the ability of human granule cells to respond to extracellular acidification by generating Ca^{2+} signals and ERK activation is state-dependent, being lost upon differentiation.

1. Introduction

A fall in extracellular pH is characteristic of a number of debilitating pathologies including cancer, ischemia and inflammation. In tumours, the external pH can be as low as 5.8 [1]. This extracellular acidity is, at least in part, due to the altered glucose metabolism in tumour cells, resulting in increased build-up of lactic acid that is then excreted from the cells thereby decreasing extracellular pH. Further acidification is due to the extracellular hydration of cell-derived CO_2 into H^+ and HCO_3^-, a reaction that is catalysed by extracellular-facing membrane-tethered carbonic anhydrase isoforms (e.g. CA9, CA12) expressed extensively in tumours [2–6]. Despite this hostile environment, tumour cells thrive and the acidosis, in fact, promotes tumour aggressiveness and metastasis [1,4,5]. Whilst it is well understood how hypoxia, which also occurs in tumour tissue, contributes to tumour cell growth and survival [2,3], little is known about how external acidosis can promote transformed cell survival. Amongst the proteins known to be modulated by external pH are the acid-sensing ion channels [7] and the vanilloid family of Transient Receptor Potential channels, TRPV channels [8,9]. Importantly, a novel family of G-protein coupled receptors activated by a fall in external pH was recently identified [10,11]. To date, 3 distinct pH-sensing G-protein coupled receptors have been identified: ovarian cancer G-protein coupled receptor 1 (OGR1), T-cell death-associated gene 8 (TDAG8) and G-protein coupled receptor 4 (GCR4) [11]. We have recently found that human granule precursor cancer cells respond to extracellular acidification with complex intracellular Ca^{2+} signaling and activation of the extracellular signal-regulated kinase (ERK) pathway [12]. Tumour cells are by definition proliferating cells, and differentiating cells often lose the ability to respond to specific growth factors, but it is not known if acid-sensing is affected. Here, we show that marked changes in the response to pH depend on the state of the cell, and this involves loss of functional linkage of G-protein coupled receptors to phospholipase C.

2. Materials and methods

2.1. Cell culture

Human desmoplastic medulloblastoma cell line (DAOY) was obtained from American Type Culture Collection. Cells were routinely maintained at 37 °C, 5% CO_2, in DMEM supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (standard

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culturing medium). They were plated in 6 cm culture dishes and were subcultured approximately every 3 days when cells were 70% confluent. To induce differentiation, cells were kept in medium in which serum was reduced to 1% and 50 ng/ml basic Fibroblast Growth Factor (bFGF) was added (R&D systems, Minneapolis, USA) for 7 days (medium change on 3rd day).

2.2. Cell counting

Proliferating DAOY cells were grown on a 6 cm dish until 70% confluent. They were treated with trypsin and floating cells were resuspended in 6 ml of standard culturing medium (see above). One drop of this cell suspension was then added per 6 cm culture dish (total of 6 dishes) containing 15 coverslips and 10 ml of standard culturing medium each. The following day, cell culture medium was changed in 3 dishes (standard culturing medium) and in the remaining 3 dishes it was replaced by differentiation medium. The next day (Proliferating Day 1: P1; Differentiating Day 1: D1), 1 coverslip was taken from each dish and incubated in Fura-2 AM as described for imaging experiments (see below). Following recovery, coverslips were placed under the imaging system and cells were counted in the field of view. Four independent fields of view were chosen randomly per coverslip. Cell numbers counted per coverslip were then averaged for the 3 coverslips for each condition. This was repeated at P3/D3, P5/D5 and P7/D7. At P3/D3, cell culture medium (standard and differentiating medium) was changed.

2.3. Total RNA isolation

Total RNA was isolated from DAOY cells by using the RNeasy Mini-Kit (Qiagen,Valencia, CA) and treated with DNase I (Sigma, Dorset, UK). RNA concentrations were determined by measuring absorbance at 260 nm.

2.4. RT-PCR and quantitative PCR

First-strand cDNA was prepared from 1 μg of total RNA using the Superscript III Kit (Invitrogen, Carlsbad, CA) in the presence of 1 μg of Oligo(dT). PCR was performed with primers specific for the pH-sensing receptor OGR1 (forward primer: 5′-GATGGGGAACATCACTGCAGA-3′, reverse primer: 5′-AACTGGTGGAAGCGGAAGG-3′) and using the following protocol: 35 cycles with 30 s at 95 °C, 30 s at 50 °C and 30 s at 72 °C. The PCR products were electrophoresed through a 1.8% agarose gel and visualized by ethidium bromide staining. The identity of the amplified PCR products was verified by an ABI PRISM DNA sequencing system (PerkinElmer). Human brain was obtained from AMS Biotechnology (Europe) Ltd. (UK). The detection of OGR1 mRNA levels was also performed by quantitative reverse transcription (qRT)-PCR analysis with an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) and EXPRESS SYBR® GreenER™ qPCR Supermix with Premixed ROX (Invitrogen, Paisley, UK). The following primers were used for qPCR: OGR1 forward: 5′-GATGGGGAACATCACTGCAGA-3′, reverse: 5′-TCAGGTTGCACAGGTACACG-3′. GAPDH was used as internal control and results were normalised to GAPDH expression in proliferating and differentiating cells, respectively. There was no difference in expression level of GAPDH between proliferating and differentiating DAOY cells.

2.5. ERK phosphorylation

DAOY cells were cultured in 6 cm dishes. To assess background ERK phosphorylation, protein was harvested as described below from some dishes only exposed to medium. In the experiment, medium was replaced by either 2 ml Ca^{2+}-free standard external solution (pH 7.35; composition see below) or 2 ml pH 6 Ca^{2+}-free standard external solution (composition see below) for 3 min.

2.6. Western Blotting

Dishes were washed twice with ice-cold PBS, 30 μl of lysis buffer (150 mM NaCl, 10 mM Tris–Cl (pH 7.5)), supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO). After harvesting the cell lysate, it was centrifuged at 13,000 rpm for 10 min and then frozen (−20 °C) until further use.

Samples of equal amounts of protein (~80 μg) were analyzed by SDS-PAGE on 10% polyacrylamide gels. Bands were transferred onto a nitrocellulose membrane, which was incubated in TBST (150 mM NaCl, 20 mM Tris, 0.02% Tween, pH 7.4) containing 5% non-fat milk for 1 h at room temperature. Membranes were washed with TBST three times and incubated with mouse anti-pERK1/2 antibody (New England Bio Lab, Hartfordshire, UK) or rabbit total ERK2 antibody (Santa Cruz Biotechnology; dilution 1:2000) for 1 h at room temperature. After another wash with TBST, membranes were incubated with peroxidase-linked anti-mouse or anti-rabbit IgG (Amersham Biosciences Europe GmbH, Freiburg, Germany; 1:2000 dilution) for 1 h at room temperature. Bands were detected by an ECL-plus Western blotting detection system (Amersham Biosciences) following a final wash. Western blots were repeated 3 times using different cell lysates.

2.7. IP_3 measurements

HitHunter FP IP_3 assay kit (DiscoveRx, Fremont, USA) was used for measuring changes in IP_3 production following changes in intra- and extracellular pH.

Black 384-well non-binding plates (Greiner Bio-one, UK) were used for the fluorescence polarisation assay. Experiments were performed according to the manufacturer's instructions. A PHERAstar (BMG LABTECH GmbH, Germany) plate reader was used for analysis.

2.8. Immunocytochemistry

Proliferating cells were cultured on 13 mm cover slips in normal DMEM medium with 10% FBS two days before treatment. For differentiation, DAOY cells were kept for 7 days in medium with 1% serum and 50 ng/ml bFGF. Cells were then fixed in 4% paraformaldehyde for 20 min at room temperature, and incubated with anti-rabbit anti-OGR1 antibody (1:1000, Caltag Medsystems, UK) in PBS containing 2% bovine serum albumin (BSA) for 1 h. Cells were washed three times with PBS and incubated with secondary antibodies: Alexa Fluor 488 coupled anti-rabbit IgG antibody (Molecular Probes, Eugene, OR) in a dilution of 1:1000 for 1 h at room temperature. After washing with PBS twice for 15 min, the cells were counterstained with distinct nuclear stains; proliferating cells were stained with DAPI whereas differentiating cells were stained with propidium iodide. Cells were then mounted with Vectashield (Vector Laboratories). A conventional fluorescent microscope (LeicaDMRB) was used for taking images; fluorescence was measured at 488 nm (OGR1), 350 nm (DAPI) and 535 nm (PI).

2.9. Fluorescent Ca^{2+} imaging experiments

DAOY cells were plated on coverslips (1.3 cm) and kept in dishes (3.5 cm; 4 coverslips per dish). For Fura 2-AM (Molecular Probes) incubations, medium was replaced with 1 ml standard Ca^{2+} external solution (composition see below) supplemented with 4 μM Fura-2-AM. Coverslips were left to incubate for 45 min at room temperature, then washed 3 times and left to recover for at least 15 min in standard external solution. Coverslips were then taken