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Short communication

Defective myogenic differentiation of human rhabdomyosarcoma cells is characterized by sialidase Neu2 loss of expression

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Abstract

Sialidase Neu2 is a glycohydrolytic enzyme whose tissue distribution has been detected principally in differentiated skeletal muscle. In this study we show that Neu2 expression is absent in different embryonal and alveolar human tumor rhabdomyosarcoma (RMS) cells, which are genetically committed myoblasts characterized by delayed differentiation. Forced myogenic differentiation of an embryonal RMS cell line, as obtained via pharmacological and genetic p38 activation or via follistatin overexpression, was characterized by Neu2 loss of expression despite the significant rise of different muscle-specific markers, suggesting therefore that the defective myogenic program of RMS cells is accompanied by Neu2 suppression.

Keywords: Neu2; Sialidases; Rhabdomyosarcoma; p38; Follistatin

1. Introduction

Cytosolic sialidase Neu2 belongs to a family of glycohydrolytic enzymes (EC 3.2.1.18) whose activity catalyzes the removal of terminal sialic acids from glycoconjugates (Miyagi et al., 1993; Monti et al., 2002; Tringali et al., 2004). The tissue distribution of Neu2 has been principally restricted to skeletal muscle (Miyagi et al., 1993; Monti et al., 1999), and several in vitro studies suggest that Neu2 expression and enzymatic activity normally increase during myogenic differentiation (Fanzani et al., 2003, 2006, 2008; Sato and Miyagi, 1996). Human rhabdomyosarcoma (RMS) is the most common pediatric soft tissue sarcoma that arises from mesenchymal precursors with the potential to differentiate into skeletal muscle cells (Tonin et al., 1991), but fail to undergo proper differentiation because of the presence of chromosomal aberrations (Galili et al., 1993; Loh et al., 1992). We have investigated Neu2 expression in different alveolar and embryonal RMS cell subtypes by means of RT-PCR analysis, gene reporter and enzymatic assays; additionally, Neu2 expression has been investigated in embryonal RD cells pharmacologically and genetically manipulated to restore the p38 pathway (Puri et al., 2000) or blunt the myostatin signaling (Langley et al., 2004; Ricaud et al., 2003), in order to force significantly the myogenic program.

2. Materials and methods

All reagents were from Sigma–Aldrich, if not otherwise indicated.

2.1. Cell cultures and treatments

Human embryonal RD cells were purchased from European collection of cell cultures (ECACC). Human RMS and murine C2C12 cells were cultured under standard conditions at 37 °C and 5% CO_2 in humidified air incubator in growth medium (GM)

Abbreviations: bp, base pairs; Cav-3, Caveolin-3; DMEM, Dulbecco's modified Eagle's Medium; FBS, fetal bovine serum; HS, horse serum; MKK6, MAP kinase kinase 6; MyHC, Myosin heavy chain; PBS, phosphate buffered solution; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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consisting of DMEM high glucose supplemented with 10% FBS and 100 μg/ml penicillin–streptomycin. To induce differentiation, confluent cells were cultured every day with differentiation medium (DM) consisting of DMEM high glucose supplemented with 2% HS. Rat L6MLC/IGF1 myoblasts were cultured essentially as described elsewhere (Musarò and Rosenthal, 1999). TPA treatment was used at the indicated doses when the cells reached 80% confluence in GM, without replacing the medium over the entire time-course.

2.2. Sialidase assay

Sialidase was assayed by fluorometry, as described by Fanzani et al. (2008). A pH curve was set up for each experiment, usually between 5 and 6.5 points, to determine the optimum of the sialidase activity assayed in the cytosolic fraction.

2.3. RT-PCR analysis

Total RNA was obtained by Tri-reagent extraction, digested with DNase (DNA-free, Ambion), and reverse-transcribed (2 μg) in the presence of 400 units of MMLV-RT (Promega). The following primers (250 nM) were used: mouse Neu2 forward 5′-CGAGCCAGCAAGACGGATGAG-3′ and reverse 5′-GGCTCTACAAGCTTACTCACTACCCGG-3′ amplify a 679 bp long fragment (33 cycles); rat Neu2 forward 5'-CCG TCCAGGACCTCACAGAG-3′ and reverse 5′-TCACTGAGCA CCATGTACTG-3′ amplify a 727 bp long fragment (33 cycles); human Neu2 forward 5′-CCTGCAGAAGGAGAGCGTGTT-3′ and reverse 5′-GGTGAAGTTTCCGGTAGGCGTA-3′ amplify a 550 bp long fragment (45 cycles); human Cav-3, forward 5′-ACCCCAAGAACATTAACGAG-3' and reverse: 5′-TGCA GAAGGTGCGGATGCAG-3′ amplify a 310 bp long fragment (38 cycles); human MyHC forward 5′-GGCAGAGAAGAC AGGTG AGCCTCAG-3′ and reverse: 5′-CCTCATCT GGCTTTAGCACCGTAGC-3′ amplify a 571 bp long fragment (38 cycles); human follistatin forward 5′-CTCTTCAAGTGG ATGATTTTC-3′ and reverse: 5′-ACAGTAGGCATTATTG GTCTG-3′ amplify a 344 bp long fragment (27 cycles). Gene expression levels were normalized to tubulin mRNA expression.

2.4. Plasmid transfection

Transfections were carried out with Lipofectamine 2000 reagent (Invitrogen), according to manufacturer's instructions. RD cells stably transfected with the pBabe vector harbouring the short human form of follistatin were obtained after 20 days selection in presence of 2 μg/ml puromycin.

2.5. Luciferase assay

Luciferase activity was measured using the Promega Dual luciferase assay system, after transfection of the cells with a mix consisting of pGL2-Basic-Neu2 promoter (1410 bp long) vector and pRL-TK-Renilla luciferase control vector. Data were corrected for transfection efficiency by measuring the Renilla luciferase activity, according to manufacturer's instructions.

2.6. Immunoblotting analysis

Protein concentration was obtained by bicinchoninic acid assay (Pierce). Samples were separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. To detect Cav-3, immunoblots were made with a diluted 1:1000 mouse monoclonal antibody against N-terminal portion of Cav-3 (clone 26, BD Transduction Laboratories), using Triton-insoluble membrane fractions. To detect MyHC, immunoblots were done with a diluted 1:1000 mouse monoclonal antibody (Hybridoma Bank, University of Iowa), using total cell lysates from harvested cells at 4 °C in RIPA lysis buffer (1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS in 50 mM NaCl, 20 mM Tris–HCl pH 7.6) containing a mix of protease inhibitors. Blots were incubated with secondary antibodies conjugated with horseradish peroxidase (Chemicon) and revealed by enhanced chemiluminescence (Chemicon). To normalize protein expression, an anti-tubulin antibody was used.

2.7. Immunofluorescence microscopy

Myoblasts cultured on laminin (Roche) coated glass coverslips were fixed with ice-cold methanol, incubated in a humid atmosphere with a diluted 1:100 mouse monoclonal MyHC antibody (Hybridoma Bank, University of Iowa), followed by a diluted 1:500 anti-mouse Cy3 conjugated secondary antibody (Jackson Immunoresearch), washed and mounted with PBS/Glicerol (1:9 v/v). Fluorescent staining was observed under an Axiovert S100 microscope (Zeiss). Pictures were taken with a digital camera (SensiCam) using the Image-Pro Plus software version 6.2.

3. Results

3.1. Analysis of the sialidase Neu2 expression levels in different rhabdomyosarcoma cell lines

The expression of sialidase Neu2 in human tumor rhabdomyosarcoma (RMS) cells committed to myoblast lineage that fail to differentiate properly was investigated. To this purpose, we used a commercially available embryonal RD cell line, as well as 2 clones termed embryonal RD/18 and alveolar RD/12 (Lollini et al., 1991) to compare with the murine C2C12 and L6MLC/IGF1 myoblasts, which express sialidase Neu2 during differentiation (Fanzani et al., 2003; Musarò and Rosenthal, 1999). After treatment with differentiating medium (DM) for up to day 5, C2C12 and L6MLC/IGF1 myoblasts underwent formation of numerous myotubes, whereas RMS cells had a delayed ability to fuse (Fig. 1A, phase contrast pictures), as represented in the case of embryonal RD cells (comparable behavior was also observed for RD/18 and RD/12 cells, not shown). Under the same conditions, immunofluorescence images clearly showed the presence of multinucleated myotubes positive for MyHC ($MyHC^+$) in C2C12 and L6MLC/IGF1