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Store-operated Ca^{2+} entry in rhabdomyosarcoma cells

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ABSTRACT

Rhabdomyosarcoma (RMS), the most common pediatric soft tissue sarcoma, has an intrinsic or early-acquisition of resistance to chemo- and radiation therapy. Molecular determinants pivotal for RMS migration, metastatic invasion, cell proliferation, and survival are incompletely identified. Migration and cell proliferation were shown to correlate with cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). Store-operated Ca^{2+}-entry (SOCE) that increases intracellular $[Ca^{2+}]$ is accomplished by Orai1, a pore-forming ion channel unit, the expression of which is stimulated by the transcription factor NFκB. The present study explored the expression of Orai1 and its regulators STIM1 and NFκB in human rhabdomyosarcoma cell lines and analyzed their impact on cell proliferation and migration.

For the study human rhabdomyosarcoma cell lines RD (embryonal) and RH30 (alveolar) were analyzed for Orai1, STIM1, and NFκB transcription by RT-PCR and their corresponding proteins in Western blot. $[Ca^{2+}]_i$ was detected via Fura-2 fluorescence and SOCE – resulting from $[Ca^{2+}]_i$ increase following store depletion with extracellular Ca^{2+} removal and inhibition of the sarcoendoplasmatic reticular Ca^{2+} ATPase – detected with thapsigargin. Cell migration was analyzed in transwell and mitotic cell death with the clonogenic assay.

In summary, Orai1, STIM1, and NFκB are expressed in embryonal (RD) and alveolar (RH30) rhabdomyosarcoma. SOCE inhibitor BTP2, Orai1 inhibitor 2-APB, or NFκB inhibitor wogonin virtually abrogated (BTP2, 2-APB) or significantly reduced (wogonin) SOCE. Moreover, SOCE inhibitors 2-APB and BTP2 and wogonin significantly inhibited migration and proliferation of both, RD and RH30 cells.

These results suggest that Orai1 signaling is involved in SOCE into rhabdomyosarcoma cells thus contributing to migration, invasion and proliferation.

1. Introduction

Rhabdomyosarcoma is the most common pediatric soft tissue sarcoma (STS) and the third most common extra-cranial solid tumor of childhood. It presents 5% of all pediatric cancers and 7–8% of all solid tumors in children [1]. The two main histological subtypes of this malignancy are embryonal (RME) and alveolar (RMA) RMS [2]. In RMA, specific genetic alterations such as the translocation t(2; 13)(q35; q14) occurring in 55% of all cases and t(1; 13)(p36; q14) in 22% are found. In contrast, no specific genetic alterations are present in RME [3,4]. The optimal treatment of RMS depends on the histology, localization and age of the patients and normally consists of systemic chemotherapy and local control consisting of radiotherapy and/or surgery [5,6]. The prognosis of advanced stage RMS remains poor depending on localization of the primary lesion, histological subtype, stage of disease, and the age at diagnosis [5,7]. Cellular mechanisms accounting for survival or death of RMS cells and underlying cell migration with subsequent metastatic invasion remained incompletely understood. Therefore it is crucial to improve the knowledge of basic pathways that contribute to the aggressiveness of RMS.

Mechanisms participating in the orchestration of tumor cell

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migration, proliferation and death include timely alterations of cytosolic Ca^{2+} activity [8–10]. Regulation of cytosolic Ca^{2+} concentration involves Ca^{2+} release from intracellular stores with subsequent activation of store-operated Ca^{2+} entry (SOCE) or Ca^{2+} release-activated Ca^{2+} channel I_{CRAC}, which are accomplished by the pore-forming Ca^{2+} channel subunits Orai1, Orai2 and/or Orai3 [11–15] as well as their regulators STIM1 and/or STIM2 [16–20]. Ca^{2+} could be extruded in exchange for Na^{+} [21–23] by K^{+}-dependent (NCKX) and K^{+}-independent (NCX) Na^{+}/Ca^{2+} exchangers [24–27]. Up-regulation of Ca^{2+} signaling may confer survival and therapy resistance of tumor cells [28]. Expression of Orai1 and STIM1 is up-regulated by the transcription factor NFκB [29].

The present study explores for the first time, whether Orai1 and STIM1 are expressed in rhabdomyosarcoma cells and contribute to the regulation of migration and proliferation of those cells.

2. Materials and methods

2.1. Cell lines and culture conditions

The RME cell line RD (ATCC, Manassas, VA, USA) and the RMA cell line RH30 (DSMZ, Braunschweig, Germany) were routinely cultured in DMEM medium (Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum (Biochrom), 1% penicillin/streptomycin (Biochrom) and 1% L-glutamine (Biochrom) in a humidified atmosphere containing 5% CO_2 at 37 °C. Cell identity was proven by SLR analysis of the DNA profile using PowerPlex 16 (Promega, Mannheim, Germany). All cells were tested to be mycoplasma negative.

2.2. Real time PCR

Determination of Orai1, STIM1 and NFκB transcript levels was performed by RT-PCR [30]. Total RNA was extracted from RD and RH30 cells using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Reverse transcription of total RNA was performed using High capacity cDNA Reverse Transcription Kit (Applied Biosystems). Polymerase chain reaction (PCR) amplification of the respective genes were set up in a total volume of 20 μl using 40 ng of cDNA, 500 nM forward and reverse primer and 2x GoTaq® qPCR Master Mix (Promega) according to the manufacturer's protocol. Cycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 58 °C for 30 s and 72 °C for 20 s. For the amplification the following primers were used (5′-3′orientation):

Orai1, fw TGATGAGCCTCAACGAGCACTCCATG;
Orai1 rev TGCTGATCATGAGCGCAAACAGGTG;
STIM1, fw CCTGTGGAAGGCATGGAAGT
STIM1, rev CTGAGGCAGCTCCACATATGT;
NFκB, fw CGAGACAGTGACAGTGTCTGC,
NFκB, rev GCTCTCTGAGCACCTTTGGATG;
TBP, fw GCC CGA AAC GCC GAA TAT,
TBP, rev CCG TGG TTC GTG GCT CTC.

Specificity of PCR product was confirmed by analysis of a melting curve. Real-time PCR amplifications were performed on a CFX96 Real-Time System (Bio-Rad). All experiments were done in duplicate. Amplification of the house-keeping gene *tbp* (TATA binding protein) was performed to standardize the amount of sample RNA. Relative quantification of gene expression was achieved using the Δct method as described earlier [30].

2.3. Western blotting

The protein expression levels were analyzed by Western blotting. In brief, RD and RH30 cells were washed with ice cold phosphate-buffered saline (PBS) and cells were lysed with cell lysis buffer (Cell Signaling Technology). The extracts were centrifuged at 13,000 rpm for 20 min at 4 °C and the protein concentration of the supernatant was determined. Total protein (30 μg) was subjected to 10% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (VWR) and the membranes were then blocked for 1 h at room temperature with 10% non-fat dried milk in Tris-buffered saline (TBS) containing 0.1% Tween-20. For immunoblotting the membranes were incubated overnight at 4 °C with antibodies directed against Orai1 (1:500, Proteintech), STIM1 (1:800, Cell Signaling Technology) or NFkappaB (1:200 Santa Cruz). A GAPDH antibody (1:1000, Cell Signaling Technology) was used for a loading control. Specific protein bands were visualized after subsequent incubation with a 1:3000 dilution of anti-rabbit IgG conjugated to horseradish peroxidase and a Western Sure Premium Chemiluminescent Substrate (LI-COR). Specific bands were quantified by LI-COR Image Studio software (LI-COR) [31].

2.4. Ca^{2+} measurements

Fura-2 fluorescence was utilized to determine intracellular Ca^{2+} measurements [32]. Cells were loaded with Fura-2/AM (2 μM, Invitrogen) for 20 min at 37 °C. Cells were excited alternatively at 340 nm and 380 nm through an objective (Fluor 40 × /1.30 oil) built in an inverted phase-contrast microscope (Axiovert 100, Zeiss). Emitted fluorescence intensity was recorded at 505 nm. Data were acquired using specialized computer software (Metafluor, Universal Imaging, Downingtown, USA). Cytosolic Ca^{2+} activity was estimated from the 340 nm/380 nm ratio. SOCE was determined by extracellular Ca^{2+} removal and subsequent Ca^{2+} re-addition in the presence of thapsigargin (1 μM, Invitrogen) [33]. For quantification of Ca^{2+} entry, the slope (delta ratio/s) and peak (delta ratio) were calculated following re-addition of Ca^{2+} [32,34].

Experiments were performed with Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 2 $CaCl_2$, 2 Na_2HPO_4, 32 HEPES, 5 glucose, pH 7.4. To reach nominally Ca^{2+}-free conditions, experiments were performed using Ca^{2+}-free Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 2 Na_2HPO_4, 32 HEPES, 0.5 EGTA, 5 glucose, pH 7.4. For calibration purposes ionomycin (10 μM, Sigma-Aldrich) was applied at the end of each experiment. In the experiments RMS cells were treated with 2-APB (10 μM), or Wogonin (50 μM) or BTP-2 (2 μM) for 24 h before the experiment. The Ringer solution contained (in mM): 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 32.2 Hepes, 2 Na_2HPO_4, 2 $CaCl_2$, and 5 glucose at pH 7.4 (NaOH). Ca^{2+}-free solution contained (in mM): 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 2 Na_2HPO_4, 32.2 Hepes, 0.5 EGTA, and 5 glucose at pH 7.4 (NaOH).

2.5. In vitro migration assay

For transwell migration assays, 2.5×10^4 to 5×10^4 cells were plated in the top chamber with a membrane (24-well insert; pore size, 8 μm; BD Biosciences) [35]. Cells were plated in DMEM medium without serum. DMEM medium supplemented with serum was used as a chemoattractant in the lower chamber. The cells were incubated with and without 2-APB or Wogonin or BTP_2 for 24 h (RH30) or 48 h (RDs) in a humidified atmosphere of 37 °C and 5% CO_2. The transwells were incubated with 4% paraformaldehyde (PFA) for 15 min at room temperature, cells that did not migrate through the pores were removed and afterwards stained with Giemsa. The migrated cells bound on the lower surface to the membrane were then counted at three different regions using Axio