Biochimica et Biophysica Acta 1832 (2013) 1105–1116

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

V-ATPase is a candidate therapeutic target for Ewing sarcoma

Sofia Avnet [a,*], Gemma Di Pompo [a], Silvia Lemma [a], Manuela Salerno [a], Francesca Perut [a], Gloria Bonuccelli [a], Donatella Granchi [a], Nicoletta Zini [b], Nicola Baldini [a,c]

[a] Laboratory for Orthopaedic Pathophysiology and Regenerative Medicine, Istituto Ortopedico Rizzoli, via di Barbiano 1/10, 40136, Bologna, Italy
[b] IGM-CNR, Unit of Bologna, c/o Istituto Ortopedico Rizzoli, via di Barbiano 1/10, 40136 Bologna, Italy
[c] Department of Biomedical and Neuromotor Sciences, University of Bologna, via di Barbiano 1/10, 40136, Bologna, Italy

ARTICLE INFO

Article history:
Received 19 October 2012
Received in revised form 29 March 2013
Accepted 2 April 2013
Available online 8 April 2013

Keywords:
Ewing sarcoma
Glycolysis
Extracellular acidification
V-ATPase

ABSTRACT

Suppression of oxidative phosphorylation combined with enhanced aerobic glycolysis and the resulting increased generation of protons are common features of several types of cancer. An efficient mechanism to escape cell death resulting from intracellular acidification is proton pump activation. In Ewing sarcoma (ES), although the tumor-associated chimeric gene EWS-FLI1 is known to induce the accumulation of hypoxia-induced transcription factor HIF-1α, derangements in metabolic pathways have been neglected so far as candidate pathogenetic mechanisms. In this paper, we observed that ES cells simultaneously activate mitochondrial respiration and high levels of glycolysis. Moreover, although the most effective detoxification mechanism of proton intracellular storage is lysosomal compartmentalization, ES cells show a poorly represented lysosomal compartment, but a high sensitivity to the anti-lysosomal agent bafilomycin A1, targeting the V-ATPase proton pump. We therefore investigated the role of V-ATPase in the acidification activity of ES cells. ES cells with the highest GAPDH and V-ATPase expression also showed the highest acidification rate. Moreover, the localization of V-ATPase was both on the vacuolar and the plasma membrane of all ES cell lines. The acidic extracellular pH that we reproduced in vitro promoted high invasion ability and clonogenic efficiency. Finally, targeting V-ATPase with siRNA and omeprazole treatments, we obtained a significant selective reduction of tumor cell number. In summary, glycolytic activity and activation of V-ATPase are crucial mechanisms of survival of ES cells and can be considered as promising selective targets for the treatment of this tumor.

1. Introduction

Cancer cells survive and proliferate in competition with somatic cells and according to the physical and biological properties of their microenvironment [1]. During cancer progression, as the tumor mass increases in size, neoplastic cells outgrow their blood supply and lack of adequate access to oxygen and nutrients. It is well documented that tumors induce a program of adaptive responses to thrive under hypoxic conditions by switching their metabolism to upregulated glycolysis and by releasing pro-angiogenic factors [2]. However, according to Otto Warburg's findings [3], tumor cells may continue to metabolize carbon by the glycolytic pathway even under adequate oxygen conditions. Therefore, regardless of hypoxia [4], aerobic glycolysis is a constant feature in cancer development, as suggested by the high levels of glucose consumption detected by positron emission tomography in most malignancies [5]. The high, constant level of glycolytic activity of tumor cells leads to an increased production of lactic acid that decreases the pH of the extracellular microenvironment. Previous authors have demonstrated that the extracellular pH (pHe) of different tumors is in the range of 5.7–7.3 [6–8], whereas the pHe level of normal tissues is significantly more alkaline (7.2–7.5). The existence of an acidic intracellular pH (pHi) is also indicated in vivo by magnetic resonance spectroscopy [9]. To maintain pH homeostasis and escape apoptosis induced by an increase in proton concentration in the cytosol, cancer cells increase the activity and/or expression of several pH regulators, resulting in the alkalinization of pHi and acidification of pHe [10]. Indeed, an increased expression and activity of the transmembrane vacuolar (H^+)-ATPase (V-ATPase) is a constant feature of several tumor types, and its inhibition has been suggested as a promising therapeutic target [11–14]. V-ATPase is an ATP-driven proton pump that acidifies the intracellular compartment and transports protons across the plasma membranes, both in physiological processes and in human diseases [15]. V-ATPase is a large multisubunit complex composed of a peripheral domain (V_1), responsible for hydrolysis of ATP, and an integral domain (V_0) that carries out proton transport [15]. In sarcoma cells, the survival mechanism under acidic conditions and the activity of V-ATPase are still entirely unexplored.

Abbreviations: pHe, extracellular pH; pHi, intracellular pH; V-ATPase, vacuolar (H^+)-ATPase; HIFs, hypoxia-inducible factors; HSP60, heat shock protein 60; GAPDHm, glyceraldehyde-3-phosphate dehydrogenase; OHPHOS, oxidative phosphorylation; BEC, bioenergetic; PAS, periodic acid-Schiff reaction; BF-1, bafilomycin A1; OME, omeprazole

* Corresponding author at: Laboratory for Orthopaedic Pathophysiology and Regenerative Medicine, Istituto Ortopedico Rizzoli, via di Barbiano 1/10, 40136 Bologna, Italy. Tel./fax: +39 0516366897.
E-mail address: sofia.avnet@ior.it (S. Avnet).

0925-4439/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbadis.2013.04.003

In this paper, we focused on Ewing sarcoma (ES), a rapidly growing, highly malignant bone tumor developing metastases in the vast majority of patients unless multiagent chemotherapy is applied [16]. ES is the second most frequent bone tumor in childhood and adolescence, and is characterized by the presence of EWS–ETS gene rearrangements [17]. Recently, some authors have reported that, in ES, hypoxia enhances the malignant phenotype through an upregulation of the EWS–FLI1 fusion gene by hypoxia-inducible factor (HIF)-1α [18]. HIF-1α and HIF-1β are the main mediators of the hypoxic response that favors cell proliferation through the control of the expression of numerous genes that regulate glucose uptake, metabolic reprogramming from oxidative phosphorylation (OXPHOS) to glycolysis, and lactic acid production [19,20]. Interestingly, glycogen granules accumulate in normal or cancer cells in a HIF-1-dependent manner [21], and the positive glycogen reaction to the cytochemical periodic acid-Schiff (PAS) stain is an additional feature that is currently used for the diagnosis of ES in parallel to the molecular detection of the EWS-FLI1 fusion gene [22].

In this study, we endorsed V-ATPase as a survival mechanism of ES cells under acidic conditions, and as a promising selective therapeutic target to be considered for future trials.

2. Material and methods

2.1. Cell lines

A-673, SK-N-MC, RD-ES, SK-ES-1 ES cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and cultured in RPMI (Sigma, Manassas, VA) buffered at 7.4 pH plus 20 units/mL penicillin, 100 μg/mL streptomycin (Gibco, Life Technologies, Paisley, UK), 10% fetal bovine serum FCS (FCS, Euroclone, Milan, Italy) (complete medium). Normal human fibroblasts (hFG) were isolated from the gingiva of healthy donors. Tissue explants were cultured in complete medium [23]. All the cell cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere. In the different assays complete medium was also used at different pH (5.8 or 6.5). The specific pH in the culture medium was maintained by using different concentrations of sodium bicarbonate needed for the preset pH in 5% CO_2 atmosphere, according to the Henderson–Hasselbach equation. At the end-point of each experiment, the final pH in the supernatant was always measured to ascertain the maintenance of the pH value along the incubation time. For all assays but the bone invasion assay, cells were cultured on human fibronectin (Gibco, Life Technologies).

2.2. Western blotting

Western blotting was carried out to detect ATP synthase subunit α, Complex II subunit 30 kDa, Complex III subunit Core 2, heat shock protein 60 (HSP60), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), V_0c and V_1B2 subunits of the V-ATPase protein, and actin in total lysates. Cells were maintained in complete medium and lysated at semi-confluence with RIPA buffer [Tris pH 7.6 50 mM, NaCl 150 mM, Triton-X 100 5%, sodium deoxycholate 0.25%, EGTA pH 8 1 mM, NaF 1 mM] (Sigma) supplemented with protease inhibitors (Roche, Milan, Italy). Equal amounts of protein lysates were subjected to reducing SDS-PAGE on a polyacrylamide gel, transferred to nitrocellulose membranes, and subjected to immunoblot analysis. Blots were probed with MitoProfile Total OXPHOS human antibody Cocktail 1:300 (Abcam, Cambridge, UK), or with an anti-GAPDH (1:2000, Santa Cruz Biotechnology, Dallas, TX), anti-HSP60 (1:2000, Sigma), anti-V_1B2 V-ATPase (1:200, Sigma), anti-V_0c V-ATPase (1:50, Abcam), anti-actin (1:1000, Cell Signaling, Danvers, MA) polyclonal antibodies. Incubation with horseradish peroxidase-conjugated anti-rabbit, anti-mouse (GE Healthcare, Buckinghamshire, UK), or anti-goat antibodies (Santa Cruz Biotechnology, Dallas, TX) followed. To detect different antigens within the same blot, nitrocellulose membranes were stripped with Restore Western Blot Stripping buffer (Thermo Fisher Scientific, Rockford, IL), and then reprobed. The reaction was revealed by a chemiluminescence substrate (ECL Western Blotting Detection Reagents, GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Immunoblot assays were repeated five times for the BEC index assay and three times for all the other assays. Signal from each band was quantified by dedicated software for densitometric evaluation (Quantity One, Biorad Laboratories Headquarters, Hercules, CA).

2.3. Mitochondrial activity

To stain mitochondria, rhodamine-based MitoTracker Orange CMTMRos (Molecular Probes, Life Technologies) was used. MitoTracker stains live mitochondria with an intact membrane potential. Briefly, cells were incubated with MitoTracker diluted in serum free RPMI to a final concentration of 250 nM for 10–15 min at 37 °C. Then, cells were fixed with 3% paraformaldehyde and 300 mM sucrose (Sigma) in PBS. Analysis of labeled cells was performed using a confocal microscope (Nikon D-Eclipse C-1, Tokyo, Japan).

2.4. Immunofluorescence

For the immunofluorescence staining with an antibody against the surface of intact mitochondria, cells were washed with PBS, fixed in 3% paraformaldehyde in PBS containing 300 mM sucrose for 20 min at 22 °C. After washing in PBS, permeabilization was performed with 0.1% Triton X-100 for 5 min. Then, cells were incubated with the MAB1273 antibody 1:100 (Millipore, Billerica, MA), and subsequently with anti-mouse Alexa green 488 nm (Molecular Probes, Life Technologies), and observed by confocal microscopy. For the co-localization analysis of LC3B or V-ATPase V_0a1 with lysosomes, before washing and fixation, living cells were incubated with Lysotraker (0.25 μM, Molecular Probes, Life Technologies) in complete medium for 30 min at 37 °C. After the incubation with the primary antibody (anti-V-ATPase V_0a1 antibodies 1:30, Sigma, and anti-LC3B antibody 1:50, Cell Signaling), and secondary anti-mouse antibody Alexa green 488 nm (1:1000, Molecular Probes, Life Technologies), nuclei were stained with Hoechst 33258 (1.25 μg/ml Sigma). Cells were then analyzed by a confocal microscope (Nikon TI-E). For the co-localization analysis of V-ATPase V0a1 with plasma membrane, cells were washed and fixed as described above, but not permeabilized. After the immunofluorescence stain for the ATPase V_0a1, cells were also stained with a Fluorescent Cell Linker Dye (PKH26, Sigma) with long aliphatic tails that bind lipid regions of the cell membrane.

2.5. Lysosome staining

Living adherent cells at low confluence and maintained in complete medium were treated with 0.37% neutral red (Sigma) for 2 h at 37 °C and 5% CO_2, washed with PBS, and observed. Alternatively, for fluorescence experiments, cells were incubated with 1 μg/ml acridine orange dye (Sigma) for 10 min at room temperature in complete medium in the dark, and rinsed with PBS prior to fluorescence microscopy analysis.

2.6. Electron microscopy

For ultrastructural evaluation, pellets of ES cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (Sigma), pH 7.4, for 1 h at RT, postfixed with 1% osmium tetroxide (Sigma), dehydrated in a graded series of ethanol, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope (Zeiss, Milan, Italy).