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Enhanced aerobic glycolysis of nasopharyngeal carcinoma cells by Epstein-Barr virus latent membrane protein 1

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ABSTRACT

Latent membrane protein 1 (LMP1) is a principal viral oncoprotein in Epstein-Barr virus (EBV)-associated malignancies, including nasopharyngeal carcinoma (NPC), which acts through regulating tumorigenesis and metabolic reprogramming of cancers. In the presence of oxygen, we demonstrated that glucose consumption, lactate production and lactate dehydrogenase (LDH) activity were significantly increased upon LMP1 expression in NPC cells and in a LMP1 variant derived from NPC patients-transformed BALB/c-3T3 cells. The amounts of the α subunit of hypoxia-inducible factor-1 (HIF-1α), a key regulator of aerobic glycolysis, and its targets, pyruvate dehydrogenase kinase 1 (PDK1) and the pyruvate kinase M2 (PKM2) isoform, were also consistently elevated by LMP1. Moreover, in parallel with reductions in the oxygen consumption rate and mitochondrial membrane potential in cells, an augmented extracellular lactate concentration was observed due to LMP1 induction. In conclusion, our results proved facilitation of the Warburg effect by LMP1 through alteration of mitochondrial function in NPC cells.

1. Introduction

Accumulating evidence has revealed an intimate relationship between virus infection and viral carcinogenesis. A common human virus, the Epstein-Barr virus (EBV), has been identified as an etiological factor in several human malignancies, including Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC), Hodgkin lymphoma (HL), post-transplantation lymphoproliferative disease and gastric cancer [1–4]. During EBV infection, a limited set of latent viral products is expressed in tumor cells, and notably, evidence has revealed that latent membrane protein 1 (LMP1) plays an important role, giving rise to oncogenesis. LMP1 is a 63-kDa integral membrane protein that consists of a 23-amino-acid N-terminal cytoplasmic tail, six transmembrane domains and 200-amino-acid C-terminal activating regions (CTARs) [5]. Of interest, via oligomerization and lipid-raft partitioning, LMP1 activity is similar to that of tumor necrosis factor receptor (TNFR) CD40, in a ligand-independent manner. Through CTARs engagement with adaptor proteins (tumor necrosis factor receptor-associated factor (TRAF) and TNFR-associated death domain protein (TRADD)), LMP1 recruits a multitude of cellular signaling transduction pathways, such as phosphatidylinositol 3-kinase (PI3K)/Akt, nuclear factor-κB (NF-κB), mitogen-activated protein kinases (MAPKs)/ATF2, Janus-activated kinase 3 (JAK3)/signal transducers and activators of transcription (STATs), to stimulate cell proliferation, anti-apoptosis, angiogenesis, and invasion, as well as metastasis [6,7].

In contrast to normal cells, cancer cells rewire anabolic and catabolic pathways to maintain infinite proliferation and nutrient acquisition. Intriguingly, an increased glucose uptake and facilitation of glycolysis from glucose to lactate are observed in tumor cells in spite of functional mitochondrial oxidative phosphorylation (OXPHOS),

Abbreviations: EBV, Epstein–Barr virus; HPV, human papilloma virus; HCV, hepatitis C virus; LMP1, latent membrane protein 1; NPC, nasopharyngeal carcinoma; BL, Burkitt's lymphoma; HL, Hodgkin lymphoma; HIF-1α, hypoxia-inducible factor-1α; TNFR, tumor necrosis factor receptor; TRAF, TNFR-associated factors; TRADD, TRAF-associated death domain protein; PI3K, phosphatidylinositol 3-kinase; NF-κB, nuclear factor-κB; MAPKs, mitogen-activated protein kinases; JAK3, Janus-activated kinase 3; STATs, signal transducers and activators of transcription; OXPHOS, oxidative phosphorylation; LDH, lactate dehydrogenase; PDK1, pyruvate dehydrogenase kinase 1; PKM2, pyruvate kinase M2 isoform; PEP, phosphoenolpyruvate; GLUT1, glucose transporter; PDH, pyruvate dehydrogenase; HKs, hexokinases; PFKFB2, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2; F2, 6BP, fructose-2,6-bisphosphate; PFK1, phosphofructokinase 1; OGDH, oxoglutarate dehydrogenase; TCA cycle, tricarboxylic acid cycle; FGF2, fibroblast growth factor 2; FGFR1, FGF receptor 1; Hox, homeobox genes; COX4, cytochrome oxidase 4 isoform

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even under normoxic conditions. This phenomenon is known as the Warburg effect [8]. In addition, tumor cells also metabolize glutamine to synthesize nucleotides and fatty acids in response to nutrient stress [9–11]. Remarkably, multiple glycolytic enzymes are involved in aerobic glycolysis (the Warburg effect) and tumor development. For instance, the pyruvate kinase M2 (PKM2) isoform, a rate-limiting enzyme, converts phosphoenolpyruvate (PEP) to pyruvate by transferring a phosphate group from PEP to ADP, manifesting as an elevated expression and an enhanced Warburg effect in tumor cells [12,13]. It is worthy of note that PKM2 is a downstream target of hypoxia inducible factor-1 (HIF-1), and interacts with HIF-1 to reprogram glucose metabolism via transactivation of the expressions of other glycolytic genes, such as glucose transporter (GLUT1), which enhances glucose uptake and increases lactate dehydrogenase (LDH), leading to lactate production from pyruvate conversion. In addition, pyruvate dehydrogenase kinase 1 (PDK1) prevents oxidative decarboxylation of pyruvate by repression of pyruvate dehydrogenase (PDH) activity [12–14]. Consequently, amplification of HIF-1-mediated aerobic glycolysis in cancer cells has a profound effect in terms of reprogramming metabolism-related requirements for tumor growth and proliferation.

NPC is a prevalent EBV-associated malignancy in Southern China, Hong Kong and Taiwan [1,3]. As mentioned above, LMP1 is a principal EBV oncoprotein, and therefore, in this study, we assessed the influence of LMP1 on the Warburg effect in NPC cells.

2. Materials and methods

2.1. Specimens, cell lines and plasmids

Specimens from 3 NPC patients were collected, and the study protocol was approved by the Research Ethics Committee at Buddhist Tzu Chi General Hospital, Hualien, Taiwan. EBV-negative human NPC cell line NPC-TW01, E2-plus cells, a cell line established from N-LMP1 (clone 1510), a LMP1 variant derived from Taiwanese NPC patients-transformed BALB/c-3T3 stable clone (3T3/N-LMP1) E2-induced tumor cells, and 3T3/Neo (Neo) cells containing a vector control, as well as established Tet-on-expressing normal human foreskin keratinocytes (RHEK-1)-derived cell lines RHEK/Tet-LMP1 and RHEK/Tet-On, were gifts from Dr. Ching-Hwa Tsai (National Taiwan University, Taiwan), Dr. Kai-Ping N Chow (Chang Gung University, Taiwan) [15] and Dr. Won-Bo Wang (National Taiwan University, Taiwan), and were cultured as described previously [16]. Plasmid pSG5Flag-LMP1 was kindly provided by Dr. Kenneth Izumi (National Institute of Health, USA). asHIF-1α/pcDNA3.1(−) plasmid was generated for antisense RNA expression. Briefly, an isolated HIF-1α fragment in an antisense orientation from asHIF-1α/yTA plasmid, which was obtained by PCR subcloning with forward primer 5′-CTCCATCTCCTACCCACATAC-3′ and reverse primer 5′-ATCCATTGATTGCCCCAGC-3′ using pHA-HIF-1α plasmid (a gift from Dr. Kou-Juey Wu, China Medical University, Taiwan) as a template and T & A cloning vector (Yeastern Biotech, Taipei, Taiwan), was inserted into *Kpn*I/*Bam*HI restriction sites of pcDNA3.1(−) vector (Invitrogen, Carlsbad, CA, USA).

2.2. Transient transfection, $CoCl_2$ treatment and Western blotting analysis

Fifty to sixty percent confluent cells were transfected with plasmid mixture using jetPrime reagent (Polyplus-transfection, Illkirch, France) according to the manufacturer's instructions. After transfection, cells were harvested and subjected to protein extraction. For $CoCl_2$ treatment, 80–90% confluent cells were treated with 800 μM $CoCl_2$, a hypoxia-mimetic agent, for the indicated time period, and total protein was then collected. For Western blot analysis, mouse anti-LMP1 antibody was purchased from Dako (Glostrup, Denmark). Rabbit anti-HIF-1α antibody, anti-PDK1 antibody and anti-PKM2 antibody were purchased from Novus Biologicals (Littleton, CO, USA), Enzo Life Sciences (Plymouth meeting, PA, USA) and Epitomics, Inc. (Burlingame, CA, USA), respectively. The densities of the bands were measured using ImageJ software, and values were normalized to the densitometric values of the internal control in each sample. The relative fold changes of protein amounts were then calculated for the experimental set as compared with the mock or vector control.

2.3. Biochemical analysis

Cells were incubated with culture medium supplemented with (CM) or without fetal bovine serum (FBS) (SFM). When cells were grown to 70–80% confluence, conditional medium was collected and subjected to measurement of glucose and lactate levels, as well as LDH activity, using a Siemens Dimension RXL Max Integrated Chemistry System at the Department of Laboratory Medicine, Tzu Chi Medical Center (Hualien, Taiwan).

2.4. Oxygen consumption rate and lactate production assay

Cells were seeded into 96-well plates and then grown to 70–80% confluence. Measurements of oxygen consumption rate and lactate production were carried out using an Oxygen Consumption/Glycolysis Dual Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions.

2.5. Measurement of mitochondrial membrane potential ($\Delta\Psi m$)

Seventy to eighty percent confluent cells were stained with cationic lipophilic fluorescent dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazolylcarbocyanine iodide (JC-1), using a JC-1 Mitochondria Membrane Potential Assay Kit (Caymen Chemical) according to the manufacturer's instructions. Cells were then harvested and subjected to flow cytometry. Healthy cells with functional mitochondria (high ΔΨm) containing red JC-1 aggregates were detected in the FL2 channel; however, unhealthy cells with collapsed mitochondria (low ΔΨm) containing JC-1 green monomers were detected in the FL1 channel.

2.6. DNA microarray analysis

Total RNA was extracted from cells with or without LMP1 expression plasmid transfection. The RNA samples were then labeled with Cy3- or Cy5-CTP using a Low Input Quick-Amp Labeling Kit (Agilent Technologies, Palo Alto, CA, USA). Subsequently, fluorescent-labeled cRNA probes were mixed and subjected to competition hybridization using an Agilent SurePrint G3 Human GE 8×60 K Microarray. Images were scanned using an Agilent Microarray Scanner and analyzed using Agilent Feature Extraction 10.5.1.1 software. After quantification and normalization by the rank-consistency-filtering LOWESS method, the expression fold change was calculated as the intensity of the test (LMP1 transfectant) in comparison with the intensity of the mock control.

2.7. Animal and microPET/CT imaging analysis

Congenic/NOD.CB17-Prkdc-scid/JTcu mice of 5 weeks of age were purchased from the Laboratory Animal Center, Tzu Chi University (Hualien, Taiwan), and underwent subcutaneous injection of 10^7 3T3/Neo cells or E2-plus cells in 100 μL of PBS into the flank region of the right or left hind limb. The animal experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee, Tzu Chi University (Hualien, Taiwan). Subsequently, tumor-bearing mice were intravenously injected with ^{18}F-fluorodeoxyglucose (^{18}F-FDG) under isoflurane anesthesia. High-resolution positron emission tomography (micro-PET) and X-ray computed tomography (micro-CT) images were obtained using a FLEX Triumph PET/SPECT/CT preclinical imaging system (Gamma Medica-Ideas, Northridge, CA, USA)

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