FEBS Letters 580 (2006) 3746–3752

Silencing of the human microsomal glucose-6-phosphate translocase induces glioma cell death: Potential new anticancer target for curcumin

Anissa Belkaid[a], Ian B. Copland[b], Duna Massillon[c], Borhane Annabi[a,*]

[a] *Laboratoire d'Oncologie Moléculaire, Département de Chimie, Centre BIOMED, Université du Québec à Montréal, C.P. 8888, Succ. Centre-ville, Montréal, Que., Canada H3C 3P8*
[b] *Department of Medicine, Lady Davis Institute for Medical Research, Montreal, Que., Canada*
[c] *Department of Nutrition, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA*

Received 19 April 2006; revised 19 May 2006; accepted 31 May 2006

Available online 9 June 2006

Edited by Veli-Pekka Lehto

Abstract **G6P translocase (G6PT) is thought to play a crucial role in transducing intracellular signaling events in brain tumor-derived cancer cells. In this report, we investigated the contribution of G6PT to the control of U-87 brain tumor-derived glioma cell survival using small interfering RNA (siRNA)-mediated suppression of G6PT. Three siRNA constructs were generated and found to suppress up to 91% G6PT gene expression. Flow cytometry analysis of propidium iodide/Annexin-V-stained cells indicated that silencing the G6PT gene induced necrosis and late apoptosis. The anticancer agent curcumin, also inhibited G6PT gene expression by more than 90% and triggered U-87 glioma cells death. Overexpression of recombinant G6PT rescued the cells from curcumin-induced cell death. Targeting G6PT expression may provide a new mechanistic rationale for the action of chemopreventive drugs and lead to the development of new anti-cancer strategies.**

Keywords: Glioma; Glucose-6-phosphate translocase; Curcumin; Cell death

1. Introduction

The incidence of malignant brain tumors is increasing in both children and adults, and this type of cancer is often unmanageable due to its diffuse infiltrating nature [1,2]. Although the prognosis is very grim, the standard therapies for malignant gliomas, i.e., surgical resection and radiation only retard glioma growth for a short period and, paradoxically, can facilitate recurrence in the long run [3]. Hence, new approaches are needed to target the very infiltrating nature of this cancer and prevent recurrence. Lately, many dietary polyphenols have been shown to have anti-cancer properties due to their chemopreventive and anti-tumor activities [4,5]. Among these, we have recently demonstrated that both the green tea polyphenol epigallocatechin-3-gallate (EGCg) [6,7], as well as chlorogenic acid (CHA) [8], efficiently inhibited several glioblastoma cell invasive processes. Interestingly, glucose-lowering properties have also been attributed to EGCg and CHA, which also make them valuable anti-diabetic agents [9,10]. Both the modulation of blood glucose levels and the chemopreventive properties of EGCg and CHA could be potentially linked to a common intracellular target, the microsomal glucose-6-phosphatase (G6Pase) system [11,12].

The G6Pase system catalyses the hydrolysis of glucose-6-phosphate (G6P) to glucose and phosphate as a final step in both glucose-producing pathways in the liver: gluconeogenesis and glycogenolysis [13]. G6Pase is a multicomponent endoplasmic reticulum (ER) enzyme which rate-limiting step in G6P hydrolysis is thought to be catalyzed by a G6P translocase (G6PT). Whereas only a low number of tissues do express the G6Pase catalytic subunit and are gluconeogenic, G6PT's ubiquitous expression and functionality in non-gluconeogenic tissues such as brain remains poorly characterized [14]. Recent evidence, however, suggest that CHA, the most potent functional inhibitor of G6PT, triggers a host of cellular events including apoptosis in neutrophils and differentiated promyelocytic HL-60 cells [15], and inhibition of matrix metalloproteinase (MMP) secretion in the human Hep3B hepatocellular carcinoma cell line [16]. CHA also inhibits glioma cell migration, response to chemotactic growth factors, and secretion of MMP [8], all prerequisite processes needed for tumor growth. Whether G6PT is involved in the survival of brain tumor-derived cancer cells is currently unknown.

Aside from regulating the rate limiting step of G6P transport through the ER membrane, alternate G6PT roles include adenosine triphosphate (ATP)-mediated calcium sequestration in the ER lumen [17], and function as a G6P receptor/sensor [18]. Such underestimated G6PT-mediated ER functions may collectively be responsible for crucial survival processes such as cell proliferation, cell cycle division, extracellular matrix (ECM) degradation, and response to growth factors during brain tumor development [19]. Moreover, enhanced glucose utilization in vitro, as well as in vivo, is correlated with the degree of malignancy, but also with poor prognosis for patients with glioma tumors [20,21]. Selective interference with G6PT functions may thus be an attractive therapeutic approach to metabolic control of glioma cell growth. Interestingly, glioma cell proliferation and survival have recently been shown to

*Corresponding author. Fax: +1 514 987 0246.
E-mail address: annabi.borhane@uqam.ca (B. Annabi).

Abbreviations: ATP, adenosine triphosphate; CHA, chlorogenic acid; 2-DG, 2-deoxy-D-glucose; ECM, extracellular matrix; ER, endoplasmic reticulum; G6P, glucose-6-phosphate; G6Pase, glucose-6-phosphatase; G6PT, G6P translocase; GSD, glycogen storage disease; MMP, matrix metalloproteinase; PI, propidium iodide; siRNA, small interfering ribonucleic acid

0014-5793/$32.00
doi:10.1016/j.febslet.2006.05.071

be affected by curcumin (diferuloyl-methane), the yellow pigment found in the spice turmeric [22,23]. Because curcumin regulates key enzymes involved in carbohydrate metabolism [24,25] and shows chemopreventive properties [26,27], we investigated the effects of curcumin on G6PT gene expression and U-87 glioma cells survival.

2. Materials and methods

2.1. Cell culture and transfection method

The U-87 glioma cell line was purchased from American Type Culture Collection and cultured in Eagle's minimum essential medium (MEM) containing 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin, at 37 °C under a humidified atmosphere containing 5% CO_2. The rabbit polyclonal antiserum against human G6PT (p46) was a kind gift from Dr. Gerald van de Werve (Centre de Recherche du CHUM, University of Montreal, Que.) [28]. The G6PT plasmid was generously provided by Dr. Christopher Newgard (University of Texas Southwestern Medical Center, Dallas, TX) and recombinant protein expression validated [28]. U-87 glioma cells were transiently transfected with the cDNA construct or with 20 nM small interfering ribonucleic acid (siRNA) (see below) using Lipofectamine 2000 (Invitrogen, Burlington, Ont.). The occurrence of G6PT specific gene knockdown as well as G6PT overexpression was also evaluated by semi-quantitative RT-PCR. All experiments involving these cells were performed 36 h following transfection. Mock transfections of U-87 cultures with pcDNA (3.1+) expression vector alone were used as controls.

2.2. RNA interference

RNA interference experiments were performed using Lipofectamine 2000. Three siRNA oligonucleotides for human G6PT (gene ID: NM_001467) and mismatch siRNA were synthesized by EZBiolab Inc. (Westfield, IN), and annealed to form duplexes. The sequences of the three siRNA used in this study are as follows: siG6PT #1: 5′-GCACUACAGUUGGAGCACAdTdT-3′ (sense) and 5′-UGUGCUCCAACUGUAGUGCdTdT-3′ (antisense), siG6PT #2: 5′-CUGUGAUCUUCUCAGCCAUdTdT-3′ (sense) and 5′-AUGGCUGAGAAGAUCACAGdTdT-3′ (antisense); siG6PT #3: 5′-CGAAACAUCCGCACCAAGAdTdT-3′ (sense) and 5′-UCUUGGUGCGGAUGUUUCGdTdT-3′ (antisense).

2.3. Semi-quantitative and quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from cultured monolayers of U-87 cells using TRIzol reagent (Life Technologies, Gaithersburg, MD). One microgram of total RNA was used for first strand cDNA synthesis followed by specific gene product amplification with the One-Step RT-PCR kit (Invitrogen) for semi-quantitative PCR products abundance analysis. Primers for G6Pase-α (forward: 5′-TTCAGCCACATCCACAGCATC-3′, reverse: 5′-GGGGTTTCAAGGAGTCAAAGACG-3′), for G6Pase-β (forward: 5′-ACTCTTCCTGACTTCTTGTGTGCC-3′, reverse: 5′-TTGCCTTTGCTCTTTGGGGG-3′) and for G6PT (forward: 5′-CAGGGCTATGGCTATTATCGCAC-3′, reverse: 5′-ATGGCTCAAACCACTTCCGCAG-3′) were all derived from human sequences. β-actin cDNA amplification was used as an internal house-keeping gene control. PCR conditions were optimized so that the gene products were examined at the exponential phase of their amplification [8] and the products were resolved on 1.8% agarose gels containing 1 μg/ml ethidium bromide. For quantitative RT-PCR, cDNA synthesis was performed by using 2 μg of total RNA, random hexamers and MULV reverse transcriptase reagents (ROCHE) as instructed by the manufacturer. Real-time PCR was performed with the SybrGreen Universal Master Mix (Invitrogen) according to the manufacturer's protocol, in which 50 ng of cDNA was amplified for G6PT gene and 5 ng amplified for 18S ribosomal RNA using specific primers at a final concentration of 200 nM in 2× SybrGreen Master Mix in a total volume of 50 μl. The thermocycler parameters for the real-time PCR consisted of two initial steps (50 °C for 2 min, followed by 95 °C for 10 min), 40 cycles of DNA amplification (95 °C for 15 s, 58 °C for 15 s, 72 °C for 20 s). At the end of the PCR a melting curve (disassociation curve) was run to ensure that only a single specific product was amplified. Relative transcript quantities were calculated as ΔCT values, as recommended by the manufacturer with 18S ribosomal RNA as the endogenous reference amplified from the samples.

2.4. Analysis of cell death by flow cytometry

Cell death was assessed by flow cytometry in cells treated with curcumin (Sigma, Oakville, ON), as well as in untransfected (mock) cells or cells transfected with the G6PT cDNA or with siG6PT #3 oligonucleotides. Adherent and floating cells were harvested by trypsin digestion and gathered to produce a single cell suspension. The cells were pelleted by centrifugation and washed with phosphate-buffered saline (PBS). Then, 2×10^5 cells were pelleted and suspended in 200 μL of buffer solution and stained with annexin-V–fluorescein isothiocyanate and propidium iodide (PI) according to the manufacturer's protocol (BD Biosciences, Mississauga, Ont.). The cells were diluted by adding 300 μL of buffer solution and processed for data acquisition and analysis on a Becton–Dickinson FACS Calibur flow cytometer using CellQuest Pro software. The *X*- and *Y*-axes indicate the fluorescence of annexin-V and PI, respectively. It was possible to detect and quantitatively compare the percentages of gated populations in all of the four regions delineated. In the early stages of apoptosis, phosphatidylserine is well known to translocate to the outer surface of the plasma membrane, which still remains physically intact. As annexin-V binds to phosphatidylserine but not to PI, and the dye is incapable of passing the plasma membrane, it is excluded in early apoptosis (annexin-V^+/PI^-). Cells in late apoptosis are stained with annexin-V and PI (annexin-V^+/PI^+). Necrotic cells have lost the integrity of their plasma membrane and are predominantly stained with PI (annexin-V^-/PI^+).

2.5. Statistical data analysis

Data are representative of three or more independent experiments. Statistical significance was assessed using non-parametric one-way ANOVA with GraphPad Prism Version 4.0. Probability values of less than 0.05 were considered significant, and an asterisk (∗) identifies such significance in each figure.

3. Results

3.1. Specific G6PT gene silencing in U-87 glioma cells

We first assessed microsomal G6PT gene expression, as well as the expression of glucose-6-phosphatase (G6Pase) isoforms α and β isoforms. Total RNA was extracted from HepG2 hepatoma and U-87 glioma cells, and then gene expression levels were analyzed by RT-PCR. As it would be expected for a cell line derived from a gluconeogenic tissue, HepG2 cells expressed all three components of the G6Pase system, with a higher expression of G6Pase-β (Fig. 1A). In contrast to HepG2, only G6PT and G6Pase-β transcripts were significantly expressed in U-87 glioma cells, with very low to undetectable levels of G6Pase-α (Fig. 1A). This is in agreement with previous reports demonstrating a lack of G6Pase-α expression in brain-derived cells [14]. Because previous evidence demonstrated that functional inhibition of the microsomal G6PT with CHA abrogates the cell migration and chemotactic response of U-87 cells to growth factors [8], we have generated siRNA constructs designed to specifically downregulate G6PT gene expression in U-87 glioma cells. Three siRNA constructs were designed and cell transfection performed as described in Section 2. Semi-quantitative RT-PCR analysis showed that G6PT transcription was specifically downregulated by all three constructs, while G6Pase-β gene expression remained unaffected by any of the constructs (Fig. 1B). Relative PCR product abundance was quantified