International Journal of Biological Macromolecules xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Protein disulfide isomerases: Impact of thapsigargin treatment on their expression in melanoma cell lines

Zélia Silva [a], Teresa Veríssimo [a], Paula A. Videira [a], Carlos Novo [a,b,*]

[a] CEDOC, Chronic Diseases Research Center, NOVA Medical School/Faculdade Ciências Médicas da Universidade Nova de Lisboa, Campo dos Mártires da Pátria, 130, 1169-056 Lisboa, Portugal
[b] UEI Parasitologia Médica, Instituto de Higiene e Medicina Tropical da Universidade NOVA de Lisboa, Rua da Junqueira 100, 1349-008 Lisboa, Portugal

ARTICLE INFO

Article history:
Received 10 November 2014
Received in revised form 15 April 2015
Accepted 16 April 2015
Available online xxx

Keywords:
Melanoma
Thapsigargin
Protein disulfide isomerase

ABSTRACT

Anti-cancer treatments usually elevate the content of unfolded or misfolded proteins in the endoplasmic reticulum (ER). Here we aimed to get insights into the relation between sensitivity of melanoma cell lines to the ER stress inducer thapsigargin (THG) and the genetic expression of protein disulfide isomerase family members (PDIs). The expression of PDIs was analysed by flow cytometry and real-time PCR. The results showed that SK-MEL-30, the less THG sensitive cell line, displays higher basal PDIs' expression levels and the sensitivity is increased by the PDIs inhibitor bacitracin. While SK-MEL-30 PDIs' expression is not THG dose-dependent, an increase in glucose related protein 78 (GRP78), PDIA5, PDIA6, and thioredoxin-related-transmembrane proteins' (TMX3 and TMX4) expression, in response to higher drug concentrations, was observed in MNT-1. The differences in PDIs' gene expression in MNT-1 suggest a different response to ER stress compared to the other cell lines and highlight the importance of understanding the diversity among cancer cells.

1. Introduction

In tumour cells, the ER tends to accumulate unfolded or misfolded proteins. This high content of protein molecules activates the "unfolded protein response" (UPR) through the three ER sensors: pancreatic ER kinase PKR-like ER kinase (PERK), activating transcription factor-6 (ATF6) and inositol-requiring enzyme 1 (IRE1) after their dissociation from the glucose related protein 78/immunoglobulin binding protein (GRP78/Bip) chaperone whose presence has been reported in melanoma [1,2]. UPR activation also leads to the expression of members of the protein disulfide isomerase family members (PDIs) in the ER. Essential for the acquisition of a correct folding for proteins, PDIs are part of the quality-control system and UPR activation [3]. In the melanoma cell lines A375 and 526 [4], M14 [5], WM-115 and WM-266-4 [6], it was reported their presence. The few reported studies relative to the relationship between ER stress and PDIs expression on melanoma cells prompted us to perform this study with the main objectives of (a) identifying the PDIs expressed and (b) analysing the effect of induced ER stress on the genetic expression of the PDIs. If the cells are unable to control the calcium levels, certain mechanisms within the cell are activated, which eventually causes it to commit suicide. This is the body's natural defence against damaged cells which could potentially develop into cancer. As ER stress inducer, we chose thapsigargin (THG) because it dramatically affects cell calcium level and is a strong cell stress inducer, which is currently being tested on humans. In this study we used three phenotypically different melanoma cell lines M8, SK-MEL-30 and MNT-1.

2. Materials and methods

2.1. Melanoma cell lines and in vitro culture conditions

Cell line SK-MEL-30, a human malignant melanoma cell line derived from tumour tissue (subcutis metastasis) of a 67-year-old Caucasian man was obtained from the Leibniz-Institut DSMZ (Germany); MNT-1, a highly pigmented human melanotic malignant melanoma cell line, enriched with mature stages III and IV melanosomes, but containing melanosomes in all stages was kindly

Abbreviations: ER, endoplasmic reticulum; THG, thapsigargin; PDI, protein disulfide isomerase; TMX, thioredoxin-related-transmembrane proteins; UPR, unfolded protein response; PERK, pancreatic ER kinase PKR-like ER kinase; ATF6, activating transcription factor-6; IRE1, inositol-requiring enzyme 1; GRP78/Bip, glucose related protein 78/immunoglobulin binding protein chaperone; CRT/CNX, calnexin/calreticulin; Bac, bacitracin; 7-AAD, 7-aminoactinomycin D.

* Corresponding author at: UEI Parasitologia Médica, Instituto de Higiene e Medicina Tropical da Universidade NOVA de Lisboa, Rua da Junqueira 100, 1349-008 Lisboa, Portugal. Tel.: +351 213 652 647.
E-mail address: cnovo@ihmt.unl.pt (C. Novo).

http://dx.doi.org/10.1016/j.ijbiomac.2015.04.029
0141-8130/

offered by Dr. José Ramalho (Faculdade de Ciências Médicas, NOVA University of Lisbon, Portugal) and M8, an HLA-A, -B, -C and -E-positive (HLA-A1, -A2, -B12 and -B40/male) but HLA-G-negative melanoma cell line, kindly offered by Dr. Edgardo D. Carosella (Institut Universitaire d'Hématologie, Paris, France [7]. SK-MEL-30 and M8 were cultured in medium RPMI-1640 (Sigma–Aldrich, USA) and MNT-1 was cultured in DMEM (Sigma–Aldrich, USA). Both media were supplemented with 10% (v/v) foetal bovine serum (Sigma–Aldrich, USA), 2 mM L-glutamine (Sigma–Aldrich, USA), 1% (p/v) sodium pyruvate (Gibco) and 100 μg/mL penicillin/streptomycin (Gibco). RPMI-1640 was further supplemented with 1% (p/v) non-essential amino acids (Sigma–Aldrich, USA). All cells were maintained at 37 °C, 95% humidity and 5% CO_2. The absence of mycoplasma contamination was confirmed by fluorescence microscopy of nuclei stained with DAPI.

2.2. Cell viability assays under thapsigargin effect

To examine the effect of drugs on cell viability we used thapsigargin (THG) (Sigma–Aldrich, USA). SKMEL-30, M8 and MNT-1 cells were grown to approximately 80% confluence and were treated with THG at several concentrations for 48 h; the final concentration of the organic solvent DMSO used in the assays did not exceed 0.2% (v/v). To inhibit PDI activity, bacitracin zinc salt (Bac) (Sigma–Aldrich, USA) was added concomitantly to THG to SKMEL-30 cells. Cultures supplemented with medium without THG or Bac but containing 0.1% and 0.2% DMSO were used as controls. After THG treatment, cell viability was tested by staining with APC conjugated Annexin V (BD Biosciences, USA) and 7-aminoactinomycin D (7-AAD) (Sigma–Aldrich, USA), and analysed by flow cytometry in an "Attune Acoustic Focusing Cytometer" (Applied Biosystems).

2.3. Expression of PDIs and chaperones

The identification of the PDIs expressed in melanoma cell lines was performed by flow cytometry. A total of 1.0×10^5 cells from each cell line were stained with commercially available antibodies: Anti-TMX1 (HPA003085), Anti-TMX3 (HPA014157), Anti-TMX4 (Ab1) (HPA015752), Anti-TMX4 (Ab2) (HPA000399), Anti-PDIA5 (WH0010954M1), Anti-PDIA6 (SAB1402501), Anti-ERp57 (E5031), Anti-GRP78/BiP (G8918), Anti-CNX (C7617), Anti-CRT (C7492) (all from Sigma–Aldrich, USA) and Anti-PDI (610946), Anti-ERp72 (610970) (from BD Biosciences, USA). Alexa Fluor®488 conjugated Goat Anti-Mouse IgG (H + L) (Molecular Probes) or FITC conjugated Anti-Rabbit IgG (whole molecule), *F(ab')*2 fragment (Sigma–Aldrich, USA) were used as secondary antibodies. To detect proteins expressed intracellularly, cells were previously fixed and permeabilized using Cytofix-CytoPerm (BD Biosciences, USA) as recommended by the manufacturer. Cell staining was performed for 30 min at 4 °C, in the dark.

2.4. Quantitative real-time PCR (RT-qPCR)

The effect of THG treatment on mRNA expression was evaluated for SK-MEL-30 and MNT-1. Cells were treated as described above for the cell viability assays for 18 h. Total RNA was isolated from melanoma cells using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma–Aldrich, USA). The total RNAs from each sample were reverse transcribed to generate the corresponding cDNAs using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative mRNA measurements were performed in each sample using TaqMan Universal Master PCR mix and TaqMan® Gene Expression Assays (Applied Biosystems). For each primer/probe set, the assays ID were the following: ERp57 Hs00607126_m1, ERp72 Hs01115905_m1, ERdj5 Hs00943467_m1, GRP78 Hs99999174_m1, CNX Hs00233492_m1, CRT Hs00189032_m1, PDI Hs00168586_m1, PDIA5 Hs00895698_m1, PDIA6 Hs00194922_m1, TMX1 Hs00997973_g1, TMX3 Hs00287225_m1, TMX4 Hs01062739_m1 and ACTB Hs99999903_m1. The relative expression for each gene was calculated according to the ΔΔCt method [8]. To normalize the variations in mRNA expression levels between different samples' β-actin (Applied Biosystems) expression was used as an endogenous control. The relative mRNA levels were calculated by using the formula $2^{-\Delta Ct}*1000$, which infers the number of a certain gene per 1000 molecules of β-actin. ΔCt stands for the difference between the cycle threshold of the target gene and that of β-actin. mRNA isolated from samples without THG treatment was used as calibrator. RT-qPCR analyses were conducted using an ABI7500 Fast real-time PCR system (Applied Biosystems).

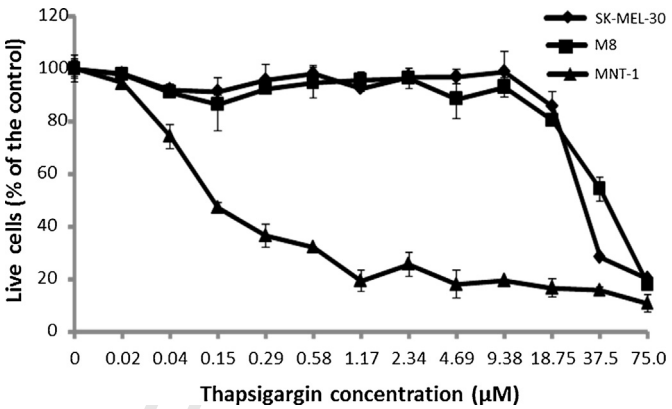

Fig. 1. Effect of THG on cell viability. Cells were incubated with THG at several concentrations for 48 h. THG maximum final concentration was 75 μM and the final concentration of DMSO used did not exceed 0.2%. Plotted values represent the percentage of live cells relative to the condition where cells were cultured in the culture medium with solvent alone (control). Results are the mean of the percentage of cell viability of at least three replicates and respective error bars for each THG concentration used.

2.5. Statistical analysis

Student's *t*-test was performed. Statistical data are shown as the means ± standard errors for triplicate samples and are representative of at least three different experiments.

3. Results

The results obtained for the effect of THG on cell viability, determined by flow cytometry (Fig. 1) showed that MNT-1 is more sensitive than SK-MEL-30 or M8 both with similar sensitivities to THG. Also, the data obtained for the PDIs expressed in these melanoma cell lines through staining with commercial anti-human antibodies, revealed that PDI, Erp57, PDIA4, PDIA5, PDIA6, TMX1, TMX3, TMX4, as well the chaperones Calnexin/Calreticulin (CRT/CNX) and GRP78 are expressed in the three melanoma cell lines. The results led us to select SK-MEL-30 and MNT-1 as cell lines with low and high sensitivity to THG respectively, for evaluation of the PDIs mRNA expression by RT-qPCR. It is known that even in the absence of ER stress inducers there is a constitutive activation of the UPR in melanoma cells lines [9]. In SK-MEL-30 cells the basal mRNA expression level was always higher than the correspondent expression in MNT-1 cells cultured for 18 h without THG obtained by RT-qPCR (Table 1). This could be one of the reasons for the higher cell viability observed in SK-MEL-30 versus MNT-1 cells in response to THG treatment. After THG induction, in SK-MEL-30 cells the RQ level is in general independent of the concentration of drug, but the MNT-1 cells showed a pronounced raise of mRNA