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Original Article

High NRF2 expression controls endoplasmic reticulum stress induced apoptosis in multiple myeloma

Yu Sun [a], Amina Abdul Aziz [a], Kristian Bowles [a, b], Stuart Rushworth [a, *]

[a] Norwich Medical School, The University of East Anglia, Norwich Research Park, NR4 7TJ, United Kingdom
[b] Department of Haematology, Norfolk and Norwich University Hospitals NHS Trust, Colney Lane, Norwich, NR4 7UY, United Kingdom

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ABSTRACT

Multiple myeloma (MM) is an incurable disease characterized by clonal plasma cell proliferation. The stress response transcription factor Nuclear factor erythroid 2 [NF-E2]-related factor 2 (NRF2) is known to be activated in MM in response to proteasome inhibitors (PI). Here, we hypothesize that the transcription factor NRF2 whose physiological role is to protect cells from reactive oxygen species via the regulation of drug metabolism and antioxidant gene plays an important role in MM cells survival and proliferation. We report for the first time that NRF2 is constitutively activated in circa 50% of MM primary samples and all MM cell lines. Moreover, genetic inhibition of constitutively expressed NRF2 reduced MM cell viability. We confirm that PI induced further expression of NRF2 in MM cell lines and primary MM. Furthermore, genetic inhibition of NRF2 of PI treated MM cells increased ER-stress through the regulation of CCAAT-enhancer-binding protein homologous protein (CHOP). Finally, inhibition of NRF2 in combination with PI treatment significantly increased apoptosis in MM cells. Here we identify NRF2 as a key regulator of MM survival in treatment naive and PI treated cells.

Introduction

Multiple myeloma (MM) is an incurable disease characterized by clonal plasma cell proliferation [1–3]. Genetic studies demonstrate that MM is a highly complex and heterogenous disease that undergoes clonal evolution towards a multi-drug resistant disease over time [4–7]. Thus, treatment relapse from the development of drug resistance clones is inevitable and presently MM remains incurable [8]. Therefore, better patient outcomes are expected to come from an improved understanding of the mechanisms of drug resistance which results in the development of novel treatment strategies that 're-sensitise' MM cells to chemotherapy.

MM cells are dependent on the unfolded protein response to alleviate the endoplasmic reticulum (ER) stress caused by the excessive amounts of paraprotein being produced [9]. The proteasome inhibitors bortezomib and carfilzomib increase the accumulation of proteins, which elevate ER-stress and increase intracellular oxidative stress. This, in part accounts for proteasome inhibitor induced apoptosis in MM cells [10]. The transcription factor (nuclear factor erythroid 2 [NF-E2]-related factor 2 (NRF2)) is a key mediator of oxidative stress through the direct regulation of over 200 genes, as well as through mechanisms of post transcriptional modification [11–13]. These genes are involved in various cellular processes including the regulation of glutathione (GSH) synthesis, detoxification and the regulation of inflammatory processes [14–17]. The transcription factor NRF2 has been shown to contribute to the malignant phenotypes of several cancers through effects on proliferation and drug sensitivity [18]. Moreover, in MM we identified the pro-tumoural function of heme oxygenase-1 (HO-1), an NRF2 regulated gene, through chemotherapy resistance [19].

NRF2 is regulated by Kelch-like ECH-associated protein 1 (KEAP1), which facilitates the ubiquitination and subsequent degradation of NRF2 by the proteosome [18]. Therefore, because proteosome inhibitors prevent the degradation of NRF2 by KEAP1, an increased transcriptional activity is induced in most cell types including malignant plasma cells [15,20]. Recently, NRF2 has also been shown to be involved in regulating ER-stress through the negative regulation of CCAAT-enhancer-binding protein homologous protein (CHOP) [21]. CHOP is induced by the transcription factor, Activating Transcription Factor 4 (ATF4), as part of the ER-stress response which then mediates apoptosis. Studies have shown that high NRF2 levels inhibit the expression of CHOP and

* Corresponding author. Department of Molecular Haematology, Norwich Medical School, University of East Anglia, Norwich, NR4 7TJ, United Kingdom.
E-mail address: s.rushworth@uea.ac.uk (S. Rushworth).

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therefore prevent ER-stress induced apoptosis [22]. Others have shown that modulating redox homeostasis in MM could increase sensitivity of MM to bortezomib [20]. Finally, a recent study has shown that elevated glutathione levels can block bortezomib induced stress responses [23]. Therefore, since NRF2 activation positively regulates glutathione levels and negatively regulates CHOP we wanted to determine in MM if NRF2 is highly expressed and if silencing the expression of NRF2 reduced cell viability. In addition, we aimed to determine the relationship between NRF2 activation, increased glutathione levels and CHOP deregulation in response to proteasome inhibitors.

Materials and methods

Materials

Anti- β-actin (R&D Systems, Abingdon, UK #MAB1536), anti-NRF2 (Abcam, Cambridge, UK #62352), anti-GAPDH (Cell Signaling Technology, Cambridge, MA, USA #D16H11), anti-Sam68 (Santa Cruz Biotechnology, Santa Cruz, USA), anti-CHOP (Cell Signaling Technology #1649). All other reagents were obtained from Sigma-Aldrich (St Louis, MO, USA), unless indicated.

Cell lines and primary cell isolation

DNA-fingerprinting authenticated MM derived cell lines were obtained from the European Collection of Cell Cultures. MM cell lines were maintained in medium RPMI 1640 supplemented with 10% (v/v) foetal bovine serum, 1% penicillin-streptomycin. Primary MM cells were obtained from MM patients' heparinized BM aspirates with informed consent in accordance with the Declaration of Helsinki and under approval from the United Kingdom National Research Ethics Service (07/H0310/146).

Histopaque 1077 density-gradient centrifugation method was used to isolate primary cells from MM patients' heparinized BM aspirates. The cells were then cultured in DMEM supplemented with 20% (v/v) foetal bovine serum and 1% penicillin-streptomycin. Primary MM cells were purified from other haematopoietic cells using magnetic-activated positive selection cell sorting with CD138+ MicroBeads (Miltenyi Biotec, Auburn, CA). All cells were incubated at 37 °C with 5% CO_2 and 95% relative humidity.

Viability and apoptosis assay

Cell viability was determined by measuring levels of intracellular ATP using Cell Titer-GLO (Promega, Southampton, UK) according to manufactures instructions. Plates were measured on FLUOstar optima Microplate Reader (BMG LABTECH, Germany). CyFlow Cube 6 flow cytomter (Sysmex, Milton Keynes, UK) was used to detect cell apoptosis. Cells were counter stained with Annexin-V and Propidium Iodide (PI), then analysed by flow cytometry.

Quantitative RT-PCR

ReliaPrep RNA cell miniprep Kit (Promega) was used to extract total RNA, according to the manufacturer's instructions. Reverse transcription (RT) was performed using the qPCRBIO cDNA synthesis kit (PCR Biosystems, London, UK). Relative quantitative real-time PCR using qPCRBIO SyGreen Mix (PCR Biosystems) was performed on cDNA generated from the reverse transcription of purified RNA. After pre-amplification (95 °C for 2 min), the PCRs were amplified for 45 cycles (95 °C for 15 s and 60 °C for 10 s and 72 °C for 10 s) on a 384-well LightCycler 480 (Roche, Burgess Hill, UK). Each mRNA expression was normalised against glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Sequences of real-time PCR primers (Sigma) used in this study are listed in Table 1.

Table 1
Oligonucleotide sequences for real-time PCR Sequences (5' to 3').

GAPDH	F GCACCACCAACTGCTTAGC
	R GGCATGGACTGTGGTCATA
NRF2	F CGTTTGTAGATGACAATGAG
	R AGAAGTTTCAGGTGACTGAG
HO-1	F ATGACACCAAGGACCAGAGC
	R GGGCAGAATCTTGCACTTTG
GCLM	F TGCAGTTGACATGGCCTGTT
	R TCACAGAATCCAGCTGTGCAA
ATF4	F CCTAGGTCTCTTAGATGATTACC
	R CAAGTCGAACTCCTTCAAATC
CHOP	F CTTTCCAGACTGATCCAAC
	R GATTCTTCCTCTTCATTTCCAG

Protein extraction/SDS-PAGE analysis

Radioimmunoprecipitation assay buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, phosphatase inhibitor cocktail tablet and protease inhibitor cocktail tablet from Roche) was used to extract whole cell lysates. NE-PER nuclear and cytoplasmic extraction reagents (Thermo scientific) were used to extract nuclear lysates. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins, then proteins were transferred to polyvinylidene difluoride membrane and Western blot analysis performed with the indicated antisera according to the manufacturer's guidelines. All images are representative of a minimum of three independent experiments. Detection was performed by electrochemical luminescence (ECL Chemdoc-It2 Imager (UVP).

Lentiviral knockdown

Plasmid containing MISSION® shRNA NRF2 (NRF2-KD) were purchased from Sigma-Aldrich and transduced into 293 T cells. MISSION pLKO.1-puro Control Vector, was used as the lentivirus control (Con-KD). Control and target lentivirus stocks were produced as previously described [24].

Promoter assay

The HO-1 promoter construct (pHO-1Luc4.0 and pHO-1mut ARE) was a kind gift from X. Chen, Baylor institute of Medicine, Houston. For the reporter assays a total of 0.5 μg of reporter plasmids and pRL-CMV control constructs were co-transfected into U226. Transfected cells were incubated for 48 h before the indicated treatments. For reporter assay, cells were treated with Dual-Luciferase Reporter Assay System (Promega).

ER-stress detection

ER-Tracker™ Red (BODIPY® TR Glibenclamide, Thermo Scientific) was purchased from Invitrogen. The live cellular ER-stress levels were determined according the manufacturer's guidelines by flow cytometry.

GSH assay

GSH-Glo™ Assay was purchased from Promega. The cellular GSH levels were determined according the manufacturer's guidelines by flow cytometry.

Statistical analysis

The Student's T test was used to compare results in control to treated groups. Results with $p < 0.05$ were considered statistically significant (*). We also use the Two-way ANOVA with Sidak's post-test. Results with $p < 0.05$ were considered statistically significant (*). Results represent the mean ± SD of 4 independent experiments. For Western blotting, data are representative images of 3 independent experiments. We generated statistics with Graphpad Prism 5 software (Graphpad, San Diego, CA, USA).

Results

Increased NRF2 activity in MM is pro-tumoral

NRF2 has been shown to be constitutively activated in various cancers [25–27]. Therefore, we first evaluated the basal expression of NRF2 in MM cell lines and primary cells. NRF2 is highly expressed in all MM cell lines and 4/8 primary MM tested (Fig. 1A). The functional consequence of high NRF2 was examined using NRF2 targeted shRNA in MM1s (low NRF2 expression) and U226 (high NRF2). Fig. 1B shows that MM1s and U226 infected with lentivirus targeted to NRF2 have reduced NRF2 RNA expression. Fig. 1C shows that targeted NRF2-KD inhibits HO-1 and GCLM mRNA expression. Furthermore targeted NRF2-KD significantly reduces the viability of U226 and MM1s (Fig. 1D). Finally, the NRF2 inhibitor brusotal inhibits cell viability of both MM#9 and MM1s (Fig. 1E). These results suggest that NRF2 is critical to the survival of a subset of MM.

Proteasome inhibition induces NRF2 activity in MM

Bortezomib and carfilzomib are proteasome inhibitors widely used in the treatment of MM. We therefore evaluated the nuclear NRF2 expression in proteasome inhibitor treated MM cell lines. Bortezomib and carfilzomib induced NRF2 protein in nuclear extracts (Fig. 2A) in all MM cell lines. Fig. 2B shows that NRF2

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