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

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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.

Keywords

NRF2 or Nuclear factor erythroid 2 [NF-E2]-related factor 2

Multiple myeloma

Oxidative stress

Endoplasmic reticulum

1. 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 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.

2. Materials and Methods

2.1. 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.

2.2. 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% CO2 and 95% relative humidity.

2.3. 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.

2.4. 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 minutes), the PCRs were amplified for 45 cycles (95°C for 15 seconds and 60°C for 10 seconds and 72°C for 10 seconds) on a 384-well LightCycler 480 (Roche, Burgess Hill, UK). Each mRNA expression was normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Sequences of real-time PCR primers (Sigma) used in this study are listed in table1.

2.5. 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).

2.6. Lentiviral Knockdown

Plasmid containing MISSION® shRNA NRF2 (NRF2-KD) were purchased from Sigma-Aldrich and transduced into 293T 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).

2.7. 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 hours before the indicated treatments.

For reporter assay, cells were treated with Dual-Luciferase Reporter Assay System (Promega).

2.8. 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.

2.9. GSH assay

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

2.10. 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).

3. Results

3.1. 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 (Figure 1A). The functional consequence of high NRF2 was examined using NRF2 targeted shRNA in MM1s (low NRF2 expression) and U226 (high NRF2). Figure 1B shows that MM1s and U226 infected with lentivirus targeted to NRF2 have reduced NRF2 RNA expression. Figure 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 (Figure 1D). Finally, the NRF2 inhibitor brusotal inhibits cell viability of both MM#9 and MM1s (Figure 1E). These results suggest that NRF2 is critical to the survival of a subset of MM.

3.2. 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 (Figure 2A) in all MM cell lines. Figure 2B shows that NRF2 accumulates and is present in the nucleus of primary MM cells treated with bortezomib and carfilzomib. Moreover, bortezomib and carfilzomib induced NRF2 regulated genes in U226 and MM1s cells and primary MM (Figure 2C and 2D).

Next we wanted to determine if NRF2 was active when MM cells are treated with bortezomib and carfilzomib. To do this we used the HO-1 promoter assay in which a wild type HO-1 promoter or a mutant HO-1 promoter (NRF2 antioxidant response element mutated; Figure 3A) were transfected into the MM cell line MM1s. Cells were then treated with bortezomib and carfilzomib and promoter activity was examined. Figure 3B shows that mutant HO-1 promoter had a significant reduction in activity compared to wildtype. Finally, we used NRF2-KD in MM1s and U266 cells and treated with bortezomib or carfilzomib. Figure 3C shows that bortezomib and carfilzomib induced NRF2 up-regulated is inhibited by NRF2-KD. Figure 3D shows that bortezomib induced NRF2 regulated genes were also inhibited by NRF2-KD in U266

cells. Together these data confirm that NRF2 induced transcription is activated by bortezomib and carfilzomib in MM.

3.3 NRF2 inhibition induces ER-stress associated apoptosis through up-regulation of CHOP.

MM cells have high levels of ER-stress as a consequence of the large amount of immunoglobulin they produce (9, 28, 29). Moreover, the addition of a proteasome inhibitor increases this stress response thus inducing apoptosis (30). We therefore wanted to explore if NRF2 regulates ER-stress associated apoptosis in response to proteasome inhibition. Initially we identified that in U226 and MM1s cells NRF2-KD induced CHOP expression, but not ATF4 expression (Figure 4A). Next we wanted to determine if ER-stress was increased in response to NRF2-KD. Figure 4B shows that using the ER tracker assay, NRF2-KD cells have a higher ER-stress compared to control-KD cells. Moreover, the addition of bortezomib or carfilzomib to the NRF2-KD cells further increased ER-stress (Figure 4C).

Next we examined if CHOP and ATF4 were increased in response to bortezomib or carfilzomib. Figure 4D shows that CHOP is increased in MM1s cells in response to proteasome inhibition. To determine if the ER-stress was an effect of increased CHOP expression we analysed CHOP mRNA expression in NRF2-KD cells when treated with bortezomib or carfilzomib. Figure 5A shows that NRF2-KD cells have a significant increase in CHOP expression when treated with bortezomib or carfilzomib as compared control-KD cells. Finally we confirmed the increase in CHOP using Western blotting in NRF2-KD cells treated with bortezomib and carfilzomib (Figure 5B and 5C).

3.4. NRF2 regulates ER-stress associated apoptosis in MM cells through its regulation of GSH synthesis.

CHOP mRNA expression has been shown to be controlled by direct NRF2 binding of the CHOP promoter or via an indirect NRF2 response (21). The indirect response is mediated through glutathione (GSH) whose synthesis is tightly controlled by NRF2 (31). To determine if GSH plays a role in regulating CHOP in MM we first analysed the GSH levels in MM cell lines treated with bortezomib. Figure 6A shows that bortezomib increases GSH levels in MM cells. Next we wanted to determine if this was regulated by NRF2. Figure 6B shows that GSH levels were not significantly increased in NRF2-

KD MM cells compared to Con-KD cells. Next we wanted to examine if the precursor to GSH, N Acetyl Cysteine (NAC) could inhibit bortezomib induced CHOP mRNA expression and ER-stress. Figure 6C and 6D show that NAC inhibits bortezomib induced CHOP and ER-stress responses. Next we wanted to detect if the glutathione (GSH) synthesis inhibitor, buthionine sulfoximine (BSO) increased bortezomib induced CHOP mRNA expression and ER-stress responses. Figure 6E and 6F show that BSO increases bortezomib induced CHOP and ER-stress responses. Finally, we show that bortezomib induced cell death in both MM1s and U226 is enhanced when NRF2 expression is inhibited (Figure 6E and F). Taken together, these results confirm that NRF2 regulates bortezomib induced CHOP mediated apoptosis in MM at least in part through the generation of GSH.

4. Discussion

Here we report that NRF2 supports survival and chemotherapy resistance in MM. We find that NRF2 is constitutively expressed in approximately 50% of primary MM samples tested and all MM cell lines. Subsequently, genetic or drug induced inhibition of NRF2 reduces survival of MM. We find that inhibiting NRF2 induces upregulation of the ER-stress response protein CHOP. Moreover, treatment with PI further increased expression and activity of NRF2, which inhibits CHOP and increases glutathione. Silencing NRF2 prevents PI induced glutathione, which regulates CHOP expression. Consequently, genetic inhibition of NRF2 increases MM sensitivity to PI.

Proteasome inhibitors are highly effective in MM, however patients will inevitably relapse following treatment through the emergence of drug resistant clones (4-7, 32). Here we report that proteasome inhibitor induced NRF2 activation supports the survival of MM. We find that inhibiting NRF2 induces upregulation of the ER-stress response protein CHOP, which increases PI induced associated apoptosis. Subsequently, we demonstrate that NRF2 regulates CHOP and ER-stress associated apoptosis via the regulation of GSH. Finally, we show that pharmacological inhibition of NRF2 induces MM apoptosis via the induction of CHOP.

The mechanisms of MM cellular resistance to PI include inherent or acquired mutation and inducible pro-survival signalling (8). *In vitro* studies show that BMSC protect MM cells from carfilzomib and bortezomib induced apoptosis (33), which is a consequence of MM cell migration and adhesion to the BMSC (34). In the present study we present data which suggest proteasome induced NRF2 activation protects the MM cells from carfilzomib and bortezomib induced apoptosis. The mechanism for this NRF2 acquired protection is the control of CHOP expression. This is because CHOP induction is associated with induced apoptosis. Thus the inhibition of this protein protects the MM cells from undergoing proteasome induced cell death.

Bortezomib has been shown to induce ER-stress response through the up-regulation of the unfolded protein response (UPR) (35). The results of which show that bortezomib at doses less than 10nM can induce transcriptional up-regulation of ATF4, an important effector of the UPR via its induction of CHOP induced apoptosis. In our experiments we show that bortezomib and carfilzomib at doses of 10nM cannot induce

ATF4 mRNA up-regulation. We observed, that NRF2-KD had no effect on ATF4 expression both alone or in combination with carfilzomib and bortezomib. Moreover, others have identified two distinct mechanism through which NRF2 regulates CHOP induction (21, 31). The first is through direct binding of the CHOP promoter which prevents ATF4 from binding and inducing expression of CHOP (21). The second is via a GSH regulated mechanism (31). In this study the authors suggest that exogenous GSH could prevent palmitate induced CHOP and associated ER-stress in adipocyte cultures. Moreover, others have shown that regulating redox homeostasis MM can enhance the sensitivity of MM to bortezomib through an NRF2 dependent mechanism (20). Here we add to these important studies and show proteasome induced NRF2 activation regulates CHOP in MM in a GSH driven mechanism.

We found that GSH is elevated in MM when treated with carfilzomib and bortezomib and this elevation was inhibited when we knockdown NRF2 expression. Moreover, others have shown that GSH synthesis is regulated by the NRF2 signalling pathway (36). This is through the regulation of multiple genes involved in regulating the synthesis and recycling of GSH. Our observations are consistent with the work of others who have previously reported the importance of GSH in regulating the survival of MM in response to proteasome inhibition and we extend that observation to place CHOP downstream of GSH

In summary, although NRF2 in the non-malignant cell is a regulator of endogenous ROS, in the context of MM and chemotherapy, NRF2 enhances the resistance of malignant cells via the up-regulation of the GSH. Here we report that PI induce the activation of the NRF2 pathway, which negatively regulates ER-stress via inhibition of CHOP expression. Specifically, NRF2 modulates the synthesis of GSH resulting in the suppression of CHOP induced apoptosis. Accordingly, we hypothesise that identification of a clinically relevant inhibitor of NRF2 will open up potential strategies in the treatment of MM.

5. Authorship Contributions

SAR, KMB and YS designed the research. YS and AAA performed the research. KMB provided essential reagents. SAR and YS performed essential analysis. YS, SAR and KMB wrote the paper. All authors reviewed the manuscript

6. Acknowledgements

The authors wish to thank the Norwich Medical School and The Ministry of Higher Education and Scientific Research of the State of Libya for funding. Additionally, we are grateful to Professor Richard Ball, Dr Mark Wilkinson and Mr Iain Sheriffs, Norwich Tissue Bank and Biorepository (UK) for help with sample collection and storage.

7. Disclosure

The authors declare no conflict of interest

8. References

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Legends

Figure 1. NRF2 expression in MM cell lines and primary MM cells. (A) Whole cell protein was extracted from MM cell lines and primary MM cells and Western blotting was performed for NRF2 protein expression. Blots were reprobed for β-actin to show loading across samples. (B-C) Lentiviral mediated shRNA knockdown (KD) of NRF2 in MM1S and U266 cells. (B) RNA was extracted and analysed using qRT-PCR for NRF2 expression. Gene expression was normalised to GAPDH. (C) HO-1 and GCLM expression was analysed and normalised to GAPDH. (D) Cell viability was analysed using flow cytometer with PI/Annexin V staining and Cell-TiterGlo. (E). MM1s cells and MM#9 cells were treated with 30nM brusatol for 24 hours and then analysed for cell viability using flow cytometer with PI/Annexin V staining and Cell-TiterGlo.

Figure 2. NRF2 is activated by proteasome inhibitors. (A) MM cell lines were treated with bortezomib (Bz, 10nM) and carfilzomib (Cfz, 10nM) for 4 hours. Nuclear protein was extracted and Western blotting was performed for NRF2 protein expression. Blots were reprobed for SAM68 and GAPDH to show loading across samples. (B) Primary MM was treated with Bz, 10nM and Cfz, 10nM for 4 hours. Cells were fixed and stained with NRF2 and DAPI and then analysed using fluorescence microscopy. Scale bar = 10uM. (C and D), MM cell lines and primary MM cells were treated with Bz and Cfz for 4 hours at 10nM. RNA was extracted and analysed using qRT-PCR for HO-1 and GCLM expression. Gene expression was normalised to GAPDH.

Figure 3. Bortezomib and carfilzomib induce NRF2 activity.
(A) Schematic of the human HO-1 promoter construct (pHO-1Luc4.0) or human HO-1 promoter with ARE mutation construct (pHO-1mutARE). (B) U266 cells were transfected with pHO-1Luc4.0 or pHO-1mutARE for 48 hours and then treated with Bz (10nM) or Cfz (10nM) for 24 hours. HO-1 promoter activation was measured by luciferase activity. (C and D) Lentiviral mediated shRNA knockdown (KD) of NRF2 in MM1S cells. (C) Cells were then treated with Bz (10nM) or Cfz (10nM) for 4 hours and whole cell protein was extracted and Western blotting was performed for NRF2 protein expression. Blots were reprobed for β-actin to show equal loading across

samples (D) RNA was extracted and analysed for HO1 and GCLM. Gene expression was normalised to GAPDH.

Figure 4. ER-stress is regulated by NRF2 in MM. (A) Lentiviral mediated shRNA knockdown (KD) of NRF2 in MM1s and U266 cells. RNA was extracted and analysed for ATF4 and CHOP. Gene expression was normalised to GAPDH. (B) Control-KD and NRF2-KD MM1S cells were incubated with the Hoechst 33342 and the ER Tracker and visualized by fluorescence microscopy. Scale bar = 20uM. (C) Control-KD and NRF2-KD MM1S cells were incubated with the ER Tracker and analysed by flow cytometry. Results expressed as median fluorescence intensity. (D) MM1s cells were treated with bortezomib and carfilizomib for 4 h and RNA was extracted and analysed for ATF4 and CHOP. Gene expression was normalised to GAPDH. **Figure 5. NRF2 regulate Chop expression in MM.** Lentiviral mediated shRNA knockdown (KD) of NRF2 in MM1S and U266 cells. Cells were treated with bortezomib and carfilzomib for 4 h. (A) MM1S cells RNA was extracted and analysed for ATF4 and CHOP. (B) Protein was extracted and Western blotting was performed for CHOP protein expression. Blots from Figure 3C were reprobed for CHOP protein expression.

Figure 6. NRF2 regulates ER-stress associated apoptosis in MM cells through its regulation of GSH synthesis. (A) MM1s cells were treated with Bz (10nM) for 4 hours. GSH assay was performed to detect GSH level. (B) Con-KD and NRF2-KD MM1s cells were treated with Bz (10nM) for 4 hours. GSH assay was performed to detect GSH level. (C) MM1s was treated with Bz (10nM) in combination with NAC (100uM) (C) or buthionine sulphoximine (BSO) 5uM (E) for 4 hours and then RNA was extracted and analysed using qRT-PCR for CHOP expression. Gene expression was normalised to GAPDH. MM1S treated with Bz (10nM) in combination with NAC (100uM) (D) or BSO 5uM (F) for 4 hours. Cells were incubated with the ER Tracker and analysed by flow cytometry. Results expressed as relative median fluorescence intensity. (G) Con-kD or NRF2-KD MM1S cells were incubated with Bz or Cfz for 36 hours then cell viability was analysed using flow cytometer with PI/Annexin V staining.

Figure 7. Schematic representation of NRF2 activity in MM.