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Research Paper

Cross-talk between two antioxidants, thioredoxin reductase and heme oxygenase-1, and therapeutic implications for multiple myeloma

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ABSTRACT

Multiple myeloma (MM) is characterized by an accumulation of abnormal clonal plasma cells in the bone marrow. Despite recent advancements in anti-myeloma therapies, MM remains an incurable disease. Antioxidant molecules are upregulated in many cancers, correlating with tumor proliferation, survival, and chemoresistance and therefore, have been suggested as potential therapeutic targets. This study investigated the cross-talk between two antioxidant molecules, thioredoxin reductase (TrxR) and heme oxygenase-1 (HO-1), and their therapeutic implications in MM. We found that although auranofin, a TrxR inhibitor, significantly inhibited TrxR activity by more than 50% at lower concentrations, myeloma cell proliferation was only inhibited at higher concentrations of auranofin. Inhibition of TrxR using lower auranofin concentrations induced HO-1 protein expression in myeloma cells. Using a sub-lethal concentration of auranofin to inhibit TrxR activity in conjunction with HO-1 inhibition significantly decreased myeloma cell growth and induced apoptosis. TrxR was shown to regulate HO-1 *via* the Nrf2 signaling pathway in a ROS-dependent manner. Increased HO-1 mRNA levels were observed in bortezomib-resistant myeloma cells compared to parent cells and HO-1 inhibition restored the sensitivity to bortezomib in bortezomib-resistant myeloma cells. These findings indicate that concurrent inhibition of HO-1 with either a TrxR inhibitor or with bortezomib would improve therapeutic outcomes in MM patients. Hence, our findings further support the need to target multiple antioxidant systems alone or in combination with other therapeutics to improve therapeutic outcomes in MM patients.

1. Introduction

Multiple myeloma (MM) is a malignant neoplasm of plasma cells characterized by an aberrant accumulation of plasma cells in the bone marrow (BM). In recent years, new therapeutics including proteasome inhibitors, bortezomib [1] and carfilzomib [2], and immunomodulatory drugs thalidomide [3] and lenalidomide [4] significantly improve MM patient outcome. Despite such developments in MM treatment, relapse is inevitable, and MM remains an incurable disease with the median survival rate of 3–5 years. Thus, better understanding of the myeloma biology and the mechanisms underlying chemoresistance can help to develop new therapeutic modalities with the potential to cure MM.

Heme oxygenase-1 (HO-1) is a cytoprotective enzyme catalyzing the conversion of intracellular heme into biliverdin, free iron, and carbon monoxide [5]. Biliverdin reductase further reduces biliverdin into a potent antioxidant bilirubin [6,7], which possesses anti-inflammatory, anti-oxidative, and anti-apoptosis properties [8,9]. Elevated HO-1 expression and activity have been observed in various cancer types including renal cell carcinoma [10], prostate cancer [11], lymphosarcoma [12], melanoma [13], chronic myeloid leukemia (CML) [14], and chronic lymphocytic leukemia (CLL) [15]. Moreover, HO-1 expression has been shown to increase in response to the treatment with chemotherapeutic agents in acute myeloid leukemia (AML) [16], CML [17], pancreatic cancer [11], and MM [18]. Inhibition of upregulated HO-1 has been demonstrated to reverse the chemoresistance and resensitizes cancer cells to the chemotherapeutic agents in many human cancer types [11,16,17]. Thus, HO-1 serves as a potential therapeutic target in cancer either

Abbreviations: AF, Auranofin; AML, Acute myeloid leukemia; ANOVA, Analysis of variance; AP-1, Activator protein-1; ARE, Antioxidant response element; BM, Bone marrow; Btz, Bortezomib; CLL, Chronic lymphocytic leukemia; CML, Chronic myeloid leukemia; CuPP IX, Copper Protoporphyrin IX; HIF-1β, Hypoxia inducible factor-1 beta; HO-1, Heme oxygenase-1; MM, Multiple myeloma; NF-κβ, Nuclear factor kappa beta; Nrf2, Nuclear factor-E2-related factor 2; PBMCs, Peripheral blood mononuclear cells; ROS, Reactive oxygen species; Trx, Thioredoxin; TrxR, Thioredoxin reductase; ZnPP IX, Zinc Protoporphyrin IX

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alone or in conjunction with conventional chemotherapeutic agents. Although bortezomib treatment increased HO-1 mRNA levels in MM [18], the functional role of HO-1 in myeloma cell survival, growth, and bortezomib resistance has not been established. The expression of HO-1 is regulated by many transcription factors including nuclear factor-κβ (NF-κβ) [19,20], NF-E2-related factor 2 (Nrf2) [21], activator protein-1 (AP-1) [22], and Bach-1 [23]. However, how HO-1 is regulated in myeloma cells is unclear.

The thioredoxin (Trx) system is one of the major cellular antioxidant systems and is comprised of thioredoxin (Trx), thioredoxin reductase (TrxR), and NADPH [24]. Both Trx1 and TrxR1 have been shown to be upregulated in many human cancer types including MM, and correlated with cancer cell survival, growth, resistance to apoptosis, and drug resistance [25,26]. Although the Trx system is a major antioxidant system involved in multiple redox-regulated signaling pathways in cancer [27,28], it also interacts with other antioxidant systems. Upon the loss of one antioxidant system, the cell may activate another antioxidant or stress molecule to compensate for the loss and protect themselves against increased oxidative stress and chemotherapeutic drugs [29–31]. Several lines of evidence indicate the involvement of the Trx/TrxR system in the regulation of HO-1 expression [31–33]. Inhibition of TrxR using pharmacological inhibitors, such as aurothioglucose, induces HO-1 expression and activity. Moreover, TrxR inhibition using auranofin has also increased HO-1 expression in an oxidative stress-dependent manner in CLL cells [34]. However, the role of TrxR in regulating HO-1 and the therapeutic implications of targeting both TrxR and HO-1 together in MM remains unclear.

The present study was designed to study the cross-talk between the two antioxidants, TrxR and HO-1 in MM, and the therapeutic implication of targeting both in conjunction in myeloma cells. Our results show that TrxR inhibition induces HO-1 expression through the Nrf2 signaling pathway. We show that HO-1 inhibition using a pharmacological inhibitor, Zinc Protoporphyrin IX (ZnPP IX), sensitizes myeloma cells to undergo apoptosis in response to TrxR inhibition at the lower concentrations of auranofin. Thus, HO-1 acts as a secondary anti-apoptotic mechanism and is upregulated to compensate for the loss of TrxR functions. Our data also show that HO-1 expression is increased in bortezomib-resistant myeloma cells and its inhibition restores the sensitivity to bortezomib. These findings suggest concurrent inhibition of HO-1 with either a TrxR inhibitor or with bortezomib would improve therapeutic outcomes in MM patients.

2. Materials and methods

2.1. Cells and reagents

The standard human myeloma cell lines (RPMI8226, U266, and OPM2) were obtained from Dr. Slavica Vuckovic (QIMR Berghofer Medical Research Institute). RPMI8226 cells were originally derived from the peripheral blood of a 61-year-old male with multiple myeloma (IgG lambda-type) [35]. U266 cells were originally derived from the peripheral blood of a 53-year-old male with IgE-secreting myeloma (refractory) [36]. OPM2 cells were originally derived from the peripheral blood of 56-year-old woman with multiple myeloma in leukemic phase (relapse) [37]. Human peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood of healthy volunteers and were collected under the ethical approval BPS/08/14/HREC. Cells were cultured in RPMI-1640 medium (Gibco) containing 10% (v/v) fetal bovine serum (FBS) (Bovagen), 200 mM L-glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). The monoclonal anti-HO-1 antibody was purchased from R & D Systems. The monoclonal anti-Nrf2 antibody, anti-Lamin B1, and anti-β-tubulin antibodies were purchased from Abcam. The monoclonal anti-HIF-1β antibody was purchased from BD Biosciences. The TrxR1 inhibitor auranofin was purchased from Sigma. An HO-1 inhibitor ZnPP IX and its control compound CuPP IX were purchased from Enzo Life Sciences. The dominant negative-Nrf2 plasmid was kindly provided by Dr. Xilin Chen (Atherogenics, USA) [38].

2.2. TrxR activity assay

RPMI8226, U266, and OPM2 cells were treated with auranofin (0–2 μM) for 24 h in 6-wells plate. The TrxR activity assays were performed as described previously [39]. Briefly, treated and untreated cells were lysed using 0.5% (v/v) Nonidet P-40 cell lysis buffer (150 mM NaCl, 50 mM Tris–Cl, pH 8; 0.5% (v/v) Nonidet P-50, 0.5 mM EDTA, 2 mM PMSF, 1 μl/ml protease inhibitor cocktail VI, 1 × PBS). To omit non-TrxR1-specific DTNB reduction, cell lysates were treated with or without 8 μM auranofin for 30 min at room temperature. The TrxR activity was measured using a buffer containing 0.5 M potassium phosphate, 200 mM EDTA, 20 mM NADPH, and 125 mM DTNB. TNB production was measured by following an increase in absorbance at 412 nm for 10 min. The reaction rates obtained in the presence of auranofin were subtracted from the reaction rates obtained in the absence of auranofin to give the final corrected TrxR rates. Units of TrxR activity (μmoles of TNB produced/minute) were calculated using an extinction coefficient of $13.6 \times 10^3\ M^{-1}$ of TNB at 412 nm. The specific thioredoxin reductase activity was determined using the following equation: Specific activity (U/mg)=U/total protein.

2.3. Cell proliferation assay

0.5×10^6 cells were treated with the appropriate drugs for indicated period of time in 24-wells plate. Relative cell proliferation was assayed using the CellTiter-Blue Cell Viability Assay (Promega), as per the manufacturer's instructions.

2.4. Western blot analysis

Whole cell extracts were prepared using 0.5% (v/v) Nonidet P-40 cell lysis buffer. Nuclear and cytosolic fractions were prepared using Nuclear Protein Extraction Kit (Cayman Chemicals) according to the manufacturer's guidelines. Western immunoblotting analysis was performed as described previously [40]. Blots were probed with various specific antibodies (HO-1, Nrf2, Lamin B1, HIF-1β, and β-tubulin) and ECL detection was done using GE ECL Western Blotting Substrate (GE Healthcare).

2.5. Caspase-3 activity assay

0.5×10^6 cells were treated with the appropriate drugs for indicated period of time in 24-wells plate. Caspase-3 activity within the treated and untreated myeloma cell lines and PBMCs was determined as described previously following the cleavage of Ac-DEVD-AMC (Enzo Life Sciences, NY, USA), a caspase-3 substrate [26]. Briefly, treated or untreated cells (0.5×10^6 cells) were pelleted, washed with PBS, re-suspended in 10–15 μl of PBS, and transferred to black-walled 96-wells plate. 90 μl of caspase assay buffer (5 mM dithiothreitol, 100 mM HEPES, 10% (w/v) sucrose, 0.1% NP-40 at pH 7.25) containing 50 μM Ac-DEVD-AMC was added to the samples and the amount of AMC cleaved by caspase-3 was measured at 37 °C by measuring the fluorescence at excitation wavelength of 370 nm and emission of 445 nm using SpectraMax plate reader.