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Impaired NHEJ function in multiple myeloma☆

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ABSTRACT

Multiple myeloma (MM) is characterized by multiple chromosomal aberrations. To assess the contribution of DNA repair to this phenotype, ionizing radiation was used to induce DNA double strand breaks in three MM cell lines. Clonogenic survival assays showed U266 (SF4 = 15.3 + 6.4%) and RPMI 8226 (SF4 = 12.6.0 + 1.7%) were radiation sensitive while OPM2 was resistant (SF4 = 78.9 + 4.1%). Addition of the DNA-PK inhibitor NU7026 showed the expected suppression in radiation survival in OPM2 but increased survival in both radiation sensitive cell lines. To examine non-homologous end joining (NHEJ) repair in these lines, the ability of protein extracts to support in vitro DNA repair was measured. Among the three MM cell lines analyzed, RPMI 8226 demonstrated impaired blunt ended DNA ligation using a ligation-mediated PCR technique. In a bacterial based functional assay to rejoin a DNA break within the β-galactosidase gene, RPMI 8226 demonstrated a 4-fold reduction in rejoining fidelity compared to U266, with OPM2 showing an intermediate capacity. Ionizing radiation induced a robust γ-H2AX response in OPM2 but only a modest increase in each radiation sensitive cell line perhaps related to the high level of γ-H2AX in freshly plated cells. Examination of γ-H2AX foci in RPMI 8226 cells confirmed data from Western blots where a significant number of foci were present in freshly plated untreated cells which diminished over 24 h of culture. Based on the clonogenic survival and functional repair assays, all three cell lines exhibited corrupt NHEJ repair. We conclude that suppression of aberrant NHEJ function using the DNA-PK inhibitor NU7026 may facilitate access of DNA ends to an intact homologous recombination repair pathway, paradoxically increasing survival after irradiation. These data provide insight into the deregulation of DNA repair at the site of DNA breaks in MM that may underpin the characteristic genomic instability of this disease.

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1. Introduction

Malignant plasma cells in multiple myeloma (MM) often display aneuploidy and complex structural abnormalities associated with poor prognosis [1–4]. The immunoglobulin isotype switch region on chromosome 14q32 is frequently involved in translocations with several different partner chromosomes in MM [5,6]. The mechanism for this karyotypic instability is largely unknown. Recently, numeric and structural centrosomal abnormalities have been implicated in the development of karyotypic abnormalities in MM [7–10]. However abnormal DNA repair function provides an alternative explanation for aneuploidy and chromosomal rearrangements.

Under usual circumstances a DNA double strand break (DNA-DSB) sets into motion a cascade of events, collectively termed DNA damage response, DDR, which is initiated by the phosphorylation of the histone variant protein H2AX at serine 139, generating γ-H2AX which forms foci at the site of the DNA-DSB. H2AX is phosphorylated by the phosphatidylinositol-3-OH-kinase-like family of protein kinases, which includes the ataxia telangiectasia mutated (ATM) kinase as well as the DNA-protein kinase catalytic subunit. The γ-H2AX foci recruit the mediator of DNA damage checkpoint protein 1 (MDC1) which in turn interacts with several other down stream DDR mediators such as BRCA1, MRE11, RAD50 and Ku70 [11]. Once detected, DNA-DSB can be repaired by two distinct mechanisms, non-homologous end joining (NHEJ) which is predominant in the G_1/S phases of the cell cycle and homologous recombination (HR) which is most active in the G_2/M phases [12–14]. DNA-DSB with blunt ends as well as with 3′ and 5′ overhangs can be repaired by the NHEJ mechanism without requiring extensive

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inter-strand homology. NHEJ is also involved in the religation step of breaks introduced by the RAG endonucleases during VDJ rearrangement of the immunoglobulin heavy and light chain loci and during immunoglobulin isotype switching in B cells [15]. NHEJ is mediated by the recruitment of Ku70 and Ku80 to the DNA-DSB, which facilitates DNA-protein kinase catalytic subunit (DNA-PKcs) binding that in the presence of XRCC4/DNA-ligase IV and Artemis, executes repair in an ATP dependent reaction [16,17]. HR, on the other hand, requires extensive inter-strand homology between the broken DNA double strand and the one serving as a repair template and is mediated by RAD51 and its paralogs. HR resolves DNA-DSBs sustained during meiosis and during DNA replication [18]. The two DNA-DSB repair pathways have been shown to compensate and cooperate in various systems in order to maintain genome integrity [19–22].

Marked genomic instability and aneuploidy is seen in NHEJ and HR core protein gene knockout mouse models [23–28]. These Ku80$^{-/-}$p53$^{-/-}$, XRCC4$^{-/-}$p53$^{-/-}$, Lig4$^{-/-}$p53$^{-/-}$, Ku70, RAD51, XRCC2 deficient mice exhibit chromosomal translocations and enhanced radiation sensitivity. The NHEJ deficient mice also have abnormal lymphoid tissue development and an increased propensity to develop B and T cell lymphomas. We hypothesized that chromosomal rearrangement in MM is impacted by either inefficient or otherwise impaired DNA-DSB repair and this altered DNA-DSB repair capacity contributes to the overall aberrant karyotype observed in this disease. To explore this hypothesis we used ionizing radiation to introduce DNA-DSBs in a series of MM cell lines and analyzed their clonogenic response using a potent inhibitor of the NHEJ repair pathway, NU7026. Subsequently, the differences observed in cell survival were contrasted with a range of functional DNA repair assays measuring both intrinsic DNA rejoining and fidelity. From these results it is concluded that the MM cell lines studied are variably impaired in both DNA rejoining efficiency and fidelity. Further the defect in repair may relate to an inability to correctly assemble NHEJ repair proteins at sites of damaged DNA.

2. Materials and methods

2.1. Cell lines

MM cell lines studied included RPMI 8226, U266 (American Type Culture Collection), and OPM2 (kind gift of Dr. Lionel Coignet, Loyola University Medical Center, Maywood, IL). The cell lines possess variable amounts of karyotypic instability that is characteristic of multiple myeloma (Table 1). These cell lines were maintained in RPMI 1640 with 10% fetal calf serum, L-glutamine, and antibiotics under standard cell culture conditions at 37 °C. The DNA-PK mutant human glioblastoma cell line MO59J (NHEJ impaired) and DNA-PK wild-type cell line MO59K (NHEJ competent) were used as controls. These were cultured in DMEM:F12 (1:1) medium with 2.5 mM L-glutamine adjusted to contain 15 mM HEPES, 0.5 mM sodium pyruvate, and 1.2 g/L sodium bicarbonate supplemented with 0.05 mM non-essential amino acids and 10% fetal bovine serum.

2.2. Limiting dilution assay

Survival of all cells (±NU7026) after radiation exposure was determined by limiting dilution assay. At ~75% confluency, cells were counted, serially diluted, and plated using two different cell densities per dose point into 96-well plates. Concentrations ranged from 256 to 0.5 cell/well, depending on the dose of irradiation used. Cells were treated with NU7026 [2-(morpholin-4-yl)-benzo[h]chomen-4-one] (Sigma–Aldrich, St. Louis, MI), a DNA-PK inhibitor, diluted in DMSO such that the final concentrations were 2.5 and 10 μM. The control group was treated with DMSO only. After 2 h of drug treatment the plates were irradiated with 0, 2, 4, 6 and 8 Gy. Following irradiation, plates were placed in 37 °C incubator for 14 days, and 50 μl of fresh media was added every 4 days. Growth was assessed in an "all-or-nothing" (positive or negative) manner using a phase contrast microscope. Wells were scored positive for colony formation if they contained one or more colonies of 50 or more cells. The surviving fraction was determined by the formula given below whereby plating efficiencies of control and treated cells were compared [29].

$$\text{Plating efficiency} = \frac{-\ln[\text{number of negative wells/total number of wells}]}{\text{number of cells plated}}$$

2.3. In vitro functional NHEJ assays using cellular protein extracts

Isolation of total cellular protein extracts from 1×10^7 cells utilized a modification of a previously published protocol [12]. Briefly, the cell pellet was suspended with hypotonic lysis buffer and centrifuged at $700 \times g$; the supernatant was removed. The pellet was resuspended with 2 volumes of hypotonic lysis buffer and incubated at −20 °C for 20 min. Cells were lysed with a Dounce homogenizer, protease inhibitor cocktail was added with high salt buffer and the reaction mix was incubated on ice for 20 min. The extract was centrifuged at 42,000 rpm for 3 h at 4 °C in a Beckman Ultracentrifuge (Beckman, Palo Alto, CA). The supernatant was dialyzed against buffer E [20 mM Tris–HCl, pH 8.0/0.1 M KOAc/20% (vol/vol) glycerol/0.5 mM EDTA/1 mM DTT] for 3 h at 4 °C. Extracts were then snap-frozen in liquid nitrogen and stored at −80 °C. Protein concentration was determined using the BioRad Protein Quantitation Assay (BioRad, Hercules, CA). Equal protein concentrations were used in all the experiments.

2.4. DNA repair assay: ligation-mediated PCR (LM-PCR)

Five-microgram pCR-Script Amp vector was digested and linearized with 2 Units *Srf*I (Stratagene, La Jolla, CA). After heat inactivation, this linearized vector was purified using a Qiaquick PCR Purification kit (Qiagen, Valencia, CA). A double-stranded linker was constructed by annealing 500 pmole of linker-25 oligo (5′-GCG GTG ACC CGG GAG ATC TGA ATT C-3′) with 500 pmole linker-11 oligo (5′-GAA TTC AGA TC-3′) in the presence of 1× T4 DNA ligase ligation buffer (Promega, Madison, WI) and H_2O to a total volume of 5 μl. The reaction was incubated at 95 °C for 5 min, then cooled to 70 °C for 1 s and then the temperature was lowered from 70 to 25 °C over 1 h. After 1 h incubation at 25 °C the temperature was lowered to 4 °C over 1 h. The reaction mix was stored at −20 °C. The repair reaction was carried out for 2 h at 37 °C using 100 ng linearized pCR-Script, 100 pmole (1 μl) double-stranded linker, reaction buffer [Tris–HCl (pH 7.5), magnesium acetate, potassium acetate, ATP, DTT, BSA] and 10 μg protein extract with H_2O to 10 μl. For samples treated with wortmannin, a PI3 kinase inhibitor, the reaction was pre-treated with wortmannin to a final concentration of 25 μM and incubated at 37 °C for 30 min. Linearized pCR-Script was added subsequent to pre-treatment and the reaction conducted as stated.

The capacity of cellular extracts to support ligation of the linker to the linearized vector was determined by PCR using linker and vector specific primers (5′-GGA GCC CCC GAT TTA GAG CTT GAC G-3′). PCR amplification followed a previous protocol [30]. Briefly, PCR amplification was carried out using 1× PCR buffer, 5.0 μl of the repair reaction, dNTPs, 0.5 μM linker-25 oligo, 0.5 μM rep1.1 oligo (5′-GGA GCC CCC GAT TTA GAG CTT GAC G-3′) and H_2O to 25 μl. The reaction was incubated at 72 °C for 3 min then 2.5 U *Taq* Polymerase (MBI Fermentas, Hanover, MD) was added with a further incubation at 72 °C for 5 min. The reaction mix was then denatured at 95 °C for 4 min initially and then 30 cycles of denaturing at 95 °C for 45 s, annealing at 60 °C for 60 s, elongation at 72 °C for 45 s were performed, with a final 10 min elongation cycle at 72 °C. The products were size-fractionated on a 2.0% agarose gel and visualized by ethidium bromide staining.

2.5. Repair fidelity assay

Cellular protein extracts were incubated with a pUC-18 plasmid containing an Eco RI-mediated single DNA-DSB within the β-galactosidase gene. Extracts that support NHEJ will result in the re-circularization of the pUC-18 plasmid with restoration of enzymatic activity [31]. For this assay cell extracts were prepared as detailed above. These protein extracts were then used to rejoin the single DNA-DSB placed within the pUC-18 plasmid. The repair reaction was similar to the one detailed above

Table 1
Karyotypic analysis of cell lines used in this study.

	Addition (whole chromosome)	Addition (partial chromosome)	Deletion (whole chromosome)	Deletion (partial chromosome)	Translocation	Derivative	Total
OPM2	3	0	2	4	4	0	13
RPMI 8226	7	6	9	9	4	2	37
U266	0	8	4	0	3	0	15

Additions and deletions involved either whole chromosomes or parts of chromosomes. *Source*: HyperCLDB (http://www.biotech.ist.unige.it/cldb/indexes.html).