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Short communication

Cells deficient in PARP-1 show an accelerated accumulation of DNA single strand breaks, but not AP sites, over the PARP-1-proficient cells exposed to MMS

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ABSTRACT

Poly(ADP-ribose) polymerase-1 (PARP-1) is a base excision repair (BER) protein that binds to DNA single strand breaks (SSBs) and subsequently synthesizes and transfers poly(ADP-ribose) polymers to various nuclear proteins. Numerous biochemical studies have implicated PARP-1 as a modulator of BER; however, the role of PARP-1 in BER in living cells remains unclear partly due to lack of accurate quantitation of BER intermediates existing in cells. Since DT40 cells, chicken B lymphocytes, naturally lack PARP-2, DT40 cells allow for the investigation of the PARP-1 null phenotype without confounding by PARP-2. To test the hypothesis that PARP-1 is necessary for efficient BER during methylmethane sulfonate (MMS) exposure in vertebrate cells, intact DT40 cells and their isogenic PARP-1 null counterparts were challenged with different exposure scenarios for phenotypic characterization. With chronic exposure, PARP-1 null cells exhibited sensitivity to MMS but with an acute exposure did not accumulate base lesions or AP sites to a greater extent than wild-type cells. However, an increase in SSB content in PARP-1 null cell DNA, as indicated by glyoxal gel electrophoresis under neutral conditions, suggested the presence of BER intermediates. These data suggest that during exposure, PARP-1 impacts the stage of BER after excision of the deoxyribosephosphate moiety from the 5′ end of DNA strand breaks by polymerase β.

1. Introduction

Base excision repair (BER) limits DNA damage formed through spontaneous or oxidative processes associated with endogenous metabolism [1]. Additionally, BER can act upon non-bulky base damage, such as N3-methyladenine, and the depurination product of N7-methylguanine (N7-meG) caused by exposure to mono-functional alkylating agents [2]. With formation of such alkylative damage, entry into BER can proceed with the removal of the adducted base from the DNA strand via spontaneous depurination (e.g., N7-meG) or by the mono-functional methyl purine glycosylase (e.g., N3-methyladenine). The resulting intact apurinic (AP) site is incised by AP endonuclease (APE), thereby generating a single strand break (SSB) with a 5′-deoxyribosephosphate (5′-dRP) terminus. Subsequently, polymerase β (POLβ) removes the 5′-dRP moiety and replaces the appropriate nucleotide to the DNA sequence. DNA ligase IIIα (LIG IIIα) finally seals the DNA strand to complete this sequence of events, which is commonly referred to as short-patch (SP)-BER. Alternatively, the long-patch (LP)-BER, which consists of a different complement of enzymes, can also operate to remove 5′-dRP residues and ligate DNA. Following the binding of proliferating cell nuclear antigen, POLβ or the replicative polymerases δ or ε participate in strand displacement synthesis creating a 2–8 nucleotide flap that is excised from DNA by flap endonuclease-1 (FEN-1). DNA ligase I subsequently closes the DNA strand [3].

Poly(ADP-ribosyl)ation is a ubiquitous protein modification involved in the regulation of transcription, cell proliferation, differentiation, DNA methylation, and apoptosis [4,5]. Of the 17 human poly(ADP-ribose) polymerase (PARP) enzymes, both PARP-1 and PARP-2 have been proposed to play an important role in DNA single strand break and base excision repair pathways [4]. In the process of these DNA repair pathways, a posttranslation modification believed to limit genotoxic stress is the synthesis and covalent addition of poly(ADP-ribose) (PAR) polymers to acceptor proteins associated with DNA metabolism [4]. These ribosylation reactions are largely attributed to PARP-1, the archetypal member of a diverse family of proteins capable of such reactions [6]. PARP-1 surveys DNA for strand disruptions, binds to them, and synthesizes PAR polymers,

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through NAD^+ consumption, for attachment to itself and other proteins such as histones. While PAR polymers have a transient existence due to degradation by poly(ADP-ribose) glycohydrolase (PARG), ribosylation reactions influence chromatin structure and protein activity. Additionally, charge repulsion causes the dissociation of polyribosylated PARP-1 from DNA with the subsequent cessation of PAR synthesis.

The development of viable *Parp-1* knockout mice provided a model from which subsequent investigations could elucidate the necessity of PARP-1 in DNA repair. Cells from these animals are hypersensitive to alkylating agents and ionizing radiation, suggesting the participation of PARP-1 in BER [7]. Furthermore, mouse embryonic fibroblasts deficient in PARP-1 showed a delayed repair of SSBs caused by methylating agents as determined by a weak alkaline comet analysis [8]. As determined by the comet analysis under strong alkaline conditions, PARP-1 knock-down by siRNA also introduces a persistence of SSBs/alkaline labile sites in human primary fibroblasts and HeLa cells, leading to γH2AX foci formation [9]. PARP-1 can physically interact with and recruit X-ray cross complementing group 1 (XRCC1) to SSBs [10,11]. Since interactions of XRCC1 with POLβ and LIGIIIα have also been demonstrated, a model has emerged where PARP-1 activity could lead to the formation of a repair complex at SSBs, which consists of XRCC1, POLβ, and LIGIIIα [12,13]. PARP-1 also heterodimerizes with PARP-2, a functional homolog that possesses similar interaction capabilities, but lacks the affinity for SSBs and the capacity for PAR synthesis [4,14]. However, the requirement for PARP-1 in the processing of BER related damage still remains tenuous due to the existence of conflicting observations [8,15]. In addition, it is not well characterized regarding which BER steps are influenced by PARP-1 in living cells. This is partly due to a lack of accurate quantitation of BER intermediates existing in cells using an adequate analysis. In an attempt to further solidify a requirement for PARP-1 in BER and address which BER process is most affected by PARP-1, we assessed the PARP-1 null phenotype in intact cells. DT40 chicken cells (chicken B lymphocytes) and isogenic PARP-1 null cells were used for this study. Although the chicken genome has major PARP enzymes (e.g., PARP-1, -3, -4, -6, -8, -9, -11, -12, -14, and -16, TIPARP, TNKS, and TNKS2) [16–18], these chicken cells naturally lacks PARP-2, allowing for an investigation without the contribution of this PARP-1 homolog to the genotoxic response [18]. Cell lines were challenged under different MMS exposure scenarios for subsequent evaluation of endpoints, including survival and the accumulation of BER substrates throughout this pathway. We observed an accelerated accumulation of DNA single strand breaks, but not AP sites, in PARP-1 deficient DT40 cells over the PARP1-proficient cells exposed to MMS.

2. Materials and methods

2.1. Culture conditions and dish exposures

The generation of and culture conditions for DT40 and PARP-1 null cells and PARP-1 null cells stably expressing human PARP-1 were described previously [18,19]. For chemical exposure, wild-type (PARP-1 proficient) and mutant DT40 (PARP-1 deficient) cells were seeded into 10 cm dishes with complete medium and allowed to incubate overnight to obtain the desired cell density ($1 \times 10^6\ mL^{-1}$). Without changing medium, MMS (Aldrich) dosing solution (100×) was added to the cultures and cells were incubated at 39.5 °C for appropriate time points. After exposure, cells were harvested, washed with cold 1× PBS, pelleted, and then stored at −80 °C until DNA isolation.

2.2. Cytotoxicity assay

Colony formation was determined in medium containing methylcellulose as described previously [19].

2.3. DNA extraction

DNA isolation was performed with modification to the PureGene DNA extraction kit (Gentra Systems Inc., Minneapolis, MN, USA) as described previously [20].

2.4. Immuno-slot blot for ring opened N7-meG

Levels of N7-meG were measured based on the alkaline conversion of the adduct to 2,6-diamino-4-hydroxy-5-*N*-methyl-formamidopyrimidine (roN7-meG) with subsequent immuno-slot blot analysis [21,22].

2.5. AP site assay

AP sites were measured as previously described by aldehyde reactive probe (ARP, Dojindo Molecular Technology, Gaithersburg, MD, USA) labeling and slot blot analysis [23].

2.6. NAD(P)H depletion assay

During continuous MMS exposure, an imbalance in BER for DT40 cell lines was assessed in real-time by a colorimetric assay monitoring intracellular NAD(P)H [19]. NAD(P)H depletion served as a proxy for NAD^+ consumption, an indicator of PARP-1 activation from SSB accumulation [24]. To confirm the activation of PARP-1 during continuous MMS exposure, cells were also co-exposed in the presence of the PARP inhibitor 3-aminobenzamide (3-AB, 10 mM, Sigma).

2.7. Glyoxal gel electrophoresis assay

To qualitatively assay the extent of SSB formation in genomic DNA from exposed cells, single stranded DNA was fractionated by neutral electrophoresis as previously described with modification [25]. Briefly, equal amounts of DNA (3–10 μg) samples to be compared were first denatured in 1.5 M glyoxal (Fluka), DMSO (50% (v/v); Sigma), and 10 mM sodium phosphate (pH 7) for 1 h at 50 °C. Loading buffer, which consisted of 50% glycerol (Fisher), 0.01% bromophenol blue (Sigma), 0.01% xylene cyanol (Sigma), and 10 mM sodium phosphate (pH 7), was added to each sample prior to loading and separation of the DNA fragments on 0.7% agarose gels (Fisher) in 10 mM sodium phosphate (pH 7) for 16 h (30 V) at 4 °C. Gels were stained with acridine orange (5 μg/mL; Fisher) for 1 h and then destained in deionized water for subsequent visualization.

With GGE analysis of DNA from MMS treated DT40 cells, the resulting DNA migration pattern within a gel lane approximated the images normally obtained from the Comet assay. Because of this similarity, our numerical assessment of the GGE experiments was based on image analysis associated with the Comet assay (CometScore Version 1.5 from Tritek). We equated the high molecular weight DNA retained above the 23.1 kb marker in the GGE analysis with the high molecular weight DNA retained in the head of the comet [26]. Similarly, the DNA smear produced during GGE represented a comet tail, and the magnitude of DNA migration in both approaches is ultimately predicated by the extent of SSB content. Tail moment was selected to express SSB content revealed by the GGE experiments; this metric was calculated as the product of tail length and percentage of DNA in the tail. Accordingly, a higher tail moment suggested increased DNA damage, in this case SSBs.

2.8. Statistical analyses

Adduct and AP site data were log transformed to approximate linearity. Analysis of covariance (ANCOVA) was then performed to test for differences in the mean intercept and in the slopes of the linear dose–response curves between DT40 and PARP-1 null cells.

3. Results

3.1. Influence of PARP-1 on cell survival during MMS exposure

In this study, DT40 cells and their isogenic PARP-1 null counterparts served as an experimental model to investigate the *in vivo* role of PARP-1 in various aspects of BER. Since they lack PARP-2, DT40 cells allow for the investigation of the PARP-1 null phenotype without confounding by PARP-2 [18]. When challenged with MMS for 10 days, PARP-1 null cells exhibited extreme hypersensitivity to cell killing (Fig. 1). The consistency between this observation with previous analyses in vertebrate and mammalian cell models reaffirmed the role of PARP-1 as a survival factor after alkylative stress [8,18]. The hypersensitivity was complemented by ectopic expression of human PARP-1 (Fig. 1). Therefore, the hypersensitivity of PARP-1 null cells to MMS is due to the lack of PARP-1.

3.2. roN7-meG as an exposure marker

Subsequent experiments aimed to identify any BER defects in PARP-1 null cells, which may allow for the accumulation of repair