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Bifunctional alkylating agent-mediated MGMT-DNA cross-linking and its proteolytic cleavage in 16HBE cells

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ABSTRACT

Nitrogen mustard (NM), a bifunctional alkylating agent (BAA), contains two alkyl arms and can act as a cross-linking bridge between DNA and protein to form a DNA-protein cross-link (DPC). O^6-methylguanine–DNA methyltransferase (MGMT), a DNA repair enzyme for alkyl adducts removal, is found to enhance cell sensitivity to BAAs and to promote damage, possibly due to its stable covalent cross-linking with DNA mediated by BAAs. To investigate MGMT-DNA cross-link (mDPC) formation and its possible dual roles in NM exposure, human bronchial epithelial cell line 16HBE was subjected to different concentrations of HN2, a kind of NM, and we found mDPC was induced by HN2 in a concentration-dependent manner, but the mRNA and total protein of MGMT were suppressed. As early as 1 h after HN2 treatment, high mDPC was achieved and the level maintained for up to 24 h. Quick total DPC (tDPC) and γ-H2AX accumulation were observed. To evaluate the effect of newly predicted protease DVC1 on DPC cleavage, we applied siRNA of *MGMT* and *DVC1*, MG132 (proteasome inhibitor), and NMS-873 (p97 inhibitor) and found that proteolysis plays a role. DVC1 was proven to be more important in the cleavage of mDPC than tDPC in a p97-dependent manner. HN2 exposure induced DVC1 upregulation, which was at least partially contributed to MGMT cleavage by proteolysis because HN2-induced mDPC level and DNA damage was closely related with DVC1 expression. Homologous recombination (HR) was also activated. Our findings demonstrated that MGMT might turn into a DNA damage promoter by forming DPC when exposed to HN2. Proteolysis, especially DVC1, plays a crucial role in mDPC repair.

1. Introduction

Initially, nitrogen mustards (NM) are used as an important military vesicant agent. NM can cause skin inflammation, blisters, and ulcers, as well as eye and respiratory tract damage, with no effective treatment. One of the commonly accepted mechanisms of NMs that causes tissue damage is an active DNA alkylating ability. As a kind of bifunctional alkylating agent (BAA), NM contains two functional *N*-chloroethyl groups, which can react with nucleophilic groups within DNA or proteins to cause a DNA–DNA or DNA–protein cross-link (DPC) (Loeber et al., 2009). Based on the alkylating effect on biomolecules, many kinds of NM derivatives including *N*-methyl-2.2-di(chloroethyl)amine (HN2), also called mechlorethamine, chlorambucil, and melphalan are widely used clinically against various tumors including lymphoma, leukemia, and multiple myeloma. However, little is known about the details of DPCs in terms of their levels or their relationship with DNA damage repair.

Various endogenous and exogenous agents including irradiation, chemotherapy drugs, and cytotoxic agents can induce DNA damage (Loeber et al., 2009). DPC is a type of DNA damage when a protein covalently binds to DNA to form an adduct (Barker et al., 2005). Compared with DNA damages like double-strand break (DSB) and single-strand break (SSB), much less attention has been paid to DPCs and a poor understanding remains because of the low abundance and structural complexity of DPCs (Wong et al., 2012). In fact, bulky adducts and helix-distorting lesions have lethal effects on DNA replication, transcription, recombination, and chromatin remodeling (Barker et al., 2005). In addition, DPC lesions can prevent DNA repair proteins from binding to the damaged nucleobase, thus promoting subsequent failure of the DNA repair process. Both of these effects may lead to the cytotoxicity and genotoxicity of DPCs. However, because of the difficulty in establishing a model uniquely inducing DPC rather than other types of DNA damage and in accurately detecting different kinds of DPCs, the mechanisms of formation, repair, and effect on cell activity of DPCs are not yet well understood (Stingele et al., 2015; Stingele and Jentsch, 2015).

O^6-methylguanine–DNA methyltransferase (MGMT), also called O^6-alkylguanine-DNA alkyltransferase (AGT), is a DNA-repair protein that transfers alkyl adducts from the O^6-position of guanine to the 145

Abbreviations: NM, nitrogen mustard; MGMT, O^6-methylguanine–DNA methyltransferase; BAA, bifunctional alkylating agents; DPC, DNA-protein cross-link; DSB, double strand break; SSB, single strand break; NER, nucleotide excision repair; HR, homologous recombination.

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cysteine residue (Cys145) of MGMT meanwhile irreversibly inactivating itself. Then, the inactivated MGMT is ubiquitinated and degraded by proteasomes (Cabrini et al., 2015). Normally, the MGMT-mediated irreversible alkyl transfer prevents gene mutations and cell apoptosis resulting from alkylating and cross-linking damages commonly induced by environmental toxicants (Srivenugopal et al., 2016). On the other hand, because of the capability of alkyl adduct removal, MGMT may cause drug resistance of tumor cells to chemotherapy by upregulated *MGMT* expression. However, some recent studies find that MGMT may enhance the cytotoxicity and mutagenicity of several bis-electrophiles, which usually have two symmetrical reactive sites readily for substrate DNA or protein (Loeber et al., 2006; Kalapila and Pegg, 2010; Pegg, 2011). Definite evidence shows that DNA lesions are formed in NM-treated neurons (Kisby et al., 2009). Overexpressing *MGMT* in CHO cells can aggravate the cytotoxicity of bifunctional alkylating agents (Kalapila and Pegg, 2010; Pegg, 2011). It was proposed that MGMT increases the cytotoxic and mutagenic effects of NM and its analogues by reacting with one active arm of NM at the cysteine site of MGMT to form a half-mustard, which can either be cleaved via proteasome pathway or react further with DNA via another arm of MN to generate a MGMT-DNA cross-link (mDPC) (Pegg, 2011; Casorelli et al., 2012). We proposed that MGMT might play a different role in BAA-treated cells in addition to its original DNA repair function.

Many DNA repair pathways are candidates for DPC repair. Nucleotide excision repair (NER) is widely involved in both bacteria and eukaryotes in removing cross-linked protein with low molecular weights (<11 kDa) (Nakano et al., 2007; Ide et al., 2011). Homologous recombination (HR) is also involved in some types of DPC (especially >11 kDa) (Ide et al., 2011). The predominant pathway chosen by cells may also depend on the type of damage. For example, HR deletion caused the greatest sensitivity under low-dose, chronic exposure to formaldehyde, while NER conferred only low-to-moderate sensitivity under the same exposure. However, following high-dose acute exposure, NER conferred maximal survival, with little contribution of HR (de Graaf et al., 2009). Recently, a process of removing covalently linked proteins by proteolysis in a replication-dependent manner was revealed (Duxin et al., 2014; Stingele et al., 2014). Proteolysis is hypothesized to play a key role in DPC repair by enzymatic digestion. After reducing the size of adducted protein, the bulky protein yields a smaller substrate for canonical DNA repair pathways. Recently, Wss1 has been identified as a member of a protease family for DPC repair in yeast. Stingele et al. argue that DVC1 (DNA damage protein targeting VCP), also called Spartan (SprT-like domain-containing protein), could be a representative ortholog of Wss1 in mammals due to their sequence and motif homology. Primary data confirmed that DVC1 mediates DNA repair by ubiquitinating target proteins independent of HR (Stingele et al., 2014; Stingele et al., 2015). Up to now, the newly found DVC1 with its effect of proteolysis in DPC repair has not been reported.

The rapid development of methods for protein labeling and immunodetection provides the possibility to directly detect DPC in living cells with high accuracy and specificity, even to detect a specific protein in the DPC complex (Shoulkamy et al., 2012; Kiianitsa and Maizels, 2013). In this study, harnessing the methodological advance, we focused on NM-induced DNA damage and DPC (mDPC) formation to reveal their characteristics, and then studied the repair manner of DPC. Our results demonstrated that the formation of MGMT-DNA complex in NM-treated cells showed a dose- and time-effect relationship and that proteolysis as well as HR was involved in DPC repair.

2. Materials and methods

2.1. Cell culture and treatment

Human bronchial epithelial cell line 16HBE was cultured in MEM medium supplemented with 10% fetal calf serum with a seeding number of 1×10^6 per 60-mm dish. Two days later, cells with 80% confluence were treated with HN2, a kind of NM (DB, China), which was freshly dissolved in PBS at a concentration of 10 mM. In dose-response experiment, cells were treated with HN2 at a final concentration of 20 μM, 50 μM, 100 μM, and 200 μM in culture medium for 1 h before being placed in normal medium. The concentration of HN2 in other experiments was 50 μM. For protein inhibition assay, 2 μM of proteasome inhibitor MG132 (Selleck, USA) and 0.5 μM of p97 inhibitor NMS-873 (Selleck, USA) were added after HN2 exposure and incubated for 3 h before the shift to normal culture medium.

2.2. Transient transfection

Cells with a total number of 1×10^6 were seeded into a 60-mm dish. The next day, synthesized *MGMT* siRNA (5′-AAGCTGGAGCTGTCTGGTTGT-3′) and *DVC1* siRNA 5′-UCAAGUACCACCUGUAUUA-3′ (Invitrogen, USA) were transfected to cells with Lipofectamine2000 (Invitrogen, USA) according to the protocol. Cells were treated with HN2 2 days after the transfection.

2.3. Cell lysis and DPC isolation

Cell lysis and DPC isolation methods were referred to Kiianitsa's protocol with modification (Kiianitsa and Maizels, 2013). In brief, cells were lysed by adding DNAiso (TaKaRa, Japan) (not to exceed 2×10^6 cells per 1 ml lysis solution). Five minutes later, nucleic acids and DPC were precipitated by adding of 0.5 volume of 100% ethanol followed by centrifugation, and the precipitate was washed twice with 75% ethanol. The precipitate was resuspended in 200 μl TE buffer or Milli-Q water after completing ethanol volatilization, and DNA concentration quantified.

2.4. DNA quantification

SYBR Green binds to double-stranded DNA (dsDNA) and the amount of dsDNA is reflected by detecting fluorescence intensity emitted by the dye. Standard DNA from calf thymus (Sigma, USA) was diluted with TE to a final concentration of 8 ng/μl, 4 ng/μl, 2 ng/μl, 1 ng/μl, and 0.5 ng/μl. DPC sample harvested from a 100-mm dish was diluted to 1:1000. Into each well of a 96-well plate, 100 μl calf thymus DNA or DPC sample was added followed by the addition of an equal volume of SYBR Green solution (Rebio, China). After 15 min of incubation in the dark, fluorescence intensity was measured and the DNA concentration of the DPC sample was calculated according to the standard curve determined by calf-thymus DNA.

2.5. FITC labeling and total-DPC (tDPC) measurement

Shoulkamy's protocol with modification was carried out (Shoulkamy et al., 2012). In brief, 10 mM FITC stock solution in dimethylformamide was added to DPC solution (10 μg) to a final concentration of 0.1 mM and incubated at room temperature for 1 h to label the cross-linked proteins. The labeled DPC then was precipitated by 100% ethanol, and then washed twice with 75% ethanol, air dried, and dissolved in TE buffer. The fluorescence strength was measured on a fluorescence spectrophotometer to qualify the level of FITC-labeled protein. The loading amount of DPC samples could be adjusted according to the protein contents.

2.6. Immunological detection of mDPC

The study method was based mainly on Kiianitsa's report (Kiianitsa and Maizels, 2013). Briefly, slot-blot wells were washed with TBS before use. Equal amounts of DNA samples in 200 μl were slot-blotted onto a nitrocellulose membrane with a vacuum according to the manufacturer's instruction of the slot blotter, then washed with TBS three times. The membrane was blocked with 1% BSA and incubated with anti-MGMT primary antibody (Santa Cruz, USA). The membrane