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DNA damage, signalling and repair after exposure of cells to the sulphur mustard analogue 2-chloroethyl ethyl sulphide

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ABSTRACT

Sulphur mustard (SM) is a blistering agent that is directly toxic to the skin and mucosal surfaces of the eye and respiratory system. Symptoms take several hours to develop and the mechanism of action is poorly understood although SM is able to alkylate nucleic acids and proteins. The ability of SM to form adducts with DNA has been documented, although there are limited data demonstrating how cells respond to this insult to repair the damage. This study used the sulphur mustard surrogate 2-chloroethyl ethyl sulphide (CEES) to identify DNA damage repair pathways and signalling events that are activated after exposure to the agent. A dose-dependent increase in DNA damage was observed in TK6 lymphoblastoid cells, which was associated with a loss of cell viability. Using both model human lymphoblastoid cell lines and pharmacological inhibitors, it was found that DNA damage induced by CEES was repaired by base excision repair (BER) and nucleotide excision repair (NER) pathways. Finally, CEES was found to induce the phosphorylation of p53 and Chk2 and these events were mediated by both the ATM ataxia telangiectasia mutated and ATR (ATM and Rad-3 related) protein kinases.

1. Introduction

Sulphur mustard [SM, 2(bis-chloroethyl)sulphide)] is a vesicating agent that causes debilitating damage to the skin, eyes and respiratory system upon exposure (Balali-Mood and Hefazi, 2005; Saladi et al., 2006). In cases of severe exposure, immunodepletion can occur, as well as death normally due to secondary infections. Although the first known use of SM was in military conflict over 100 years ago, there are still no effective treatments or preventative measures. Instead, exposed individuals are treated symptomatically. The lack of effective treatments is likely due to insufficient knowledge on the mechanism of action of SM and how cells respond to exposure and repair any damage.

The toxicity of SM is thought to be mediated by the alkylation of nucleic acids and proteins, although the exact mechanisms are not clear (Papirmeister et al., 1985; Ribeiro et al., 1991). Several studies have demonstrated that SM is able to modify DNA via the formation of DNA mono-adducts and crosslinks. The most abundant DNA lesion after SM exposure is the mono-adduct N7-hydroxyethyl thioethyl guanine (N7-HETE-G), constituting around 60% of total DNA adducts (Fidder et al., 1994), although other adducts are also formed. DNA crosslinks caused by SM constitute approximately 16% of total DNA lesions. As SM is able to cause different forms of DNA damage, it is likely that the repair of these lesions will involve different DNA repair pathways. To date, only nucleotide excision repair (NER) has been implicated in the repair of SM-induced damage in experiments using non-human models (Matijasevic et al., 2001; Matijasevic and Volkert, 2007).

Cells respond to genotoxic damage by initiating DNA damage signalling cascades and utilising specific DNA repair pathways to repair the DNA lesions (Kao et al., 2005; Ljungman, 2005; Niida and Nakanishi, 2006). DNA damage signalling is mediated by the ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia related) protein kinases. These kinases respond to different forms of DNA damage, for example ATM is normally activated in the presence of DNA double strand breaks, whereas ATR is activated by various forms of DNA damage, including DNA adducts and crosslinks (Abraham, 2004; Hurley and Bunz, 2007). Once activated, these kinases phosphorylate a wide range of target proteins, including the checkpoint kinases (Chk1 and Chk2), the tumour suppressor p53 and the histone variant H2AX. These target proteins, along with many others, act to slow cell cycle progression, regulate transcription, enhance the DNA repair capacity of cells or to direct cells to die via apoptosis if the damage encountered is too great.

As well as mechanisms to slow the cell cycle, thus preventing propagation of DNA damage, cells also possess a variety of DNA repair pathways that are able to respond to specific forms of DNA damage (Kao et al., 2005). For example, simple DNA adducts such as methylated bases and those caused by oxidative damage to DNA are recognised and repaired by the base excision repair (BER)

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pathway (Wilson and Bohr, 2007). This pathway utilises specific DNA glycosylases to detect DNA lesions before removing the damaged base. The DNA backbone is broken by an endonuclease to produce a single strand break, followed by DNA synthesis and ligation. The DNA repair enzyme poly(ADP-ribose) polymerase-1 (PARP-1) plays a crucial role in BER, as part of a complex containing XRCC1 and DNA ligase III. Cells lacking PARP-1 protein or activity are hypersensitive to chemicals that induce alkylation or oxidative damage to DNA (Dantzer et al., 2000; Masson et al., 1998).

More bulky DNA adducts as well as DNA crosslinks, such as pyrimidine dimers induced by exposure to UV radiation, are repaired by the NER pathway. In this pathway, lesion recognition and excision (several nucleotides are excised) are carried out by a family of proteins called XP proteins, followed by DNA synthesis and ligation. The term XP is derived from the human genetic condition Xeroderma Pigmentosa, which predisposes to cancer due to mutations in various XP proteins leading to defects in NER (Lichon and Khachemoune, 2007). Other major DNA repair pathways in human cells include non-homologous end joining (NHEJ), where double strand breaks (DSB's) are directly ligated together This process is mediated by the action of the ku70/80 and DNA-dependent protein kinase (DNA-PK) complex, which is necessary for the phosphorylation of XRCC4 (X-ray repair cross-complementing protein 4) and subsequent recruitment of DNA ligase IV, allowing ligation of the DNA double strand break (Burma et al., 2006). DSB's are also repaired by homologous recombination, although this is a relatively minor pathway in human cells.

In order to develop better treatments/preventative measures for exposure to SM, it is necessary to have a thorough understanding of biochemical changes/signalling events that are induced after exposure to the agent. It is also important to know how the cell responds to and repairs any damage induced. This study aimed to investigate DNA damage signalling and repair pathways after exposure to the sulphur mustard surrogate compound 2-chloroethyl ethyl sulphide (CEES). This compound is able to produce DNA mono-adducts with the chemical structure DNA–CH_2–CH_2–S–CH_2–CH_3 that are very similar in structure to those induced by SM (DNA–CH_2–CH_2–S–CH_2–CH_2–OH). Due to the similarity between the adducts, it is likely that our data with CEES is directly relevant to the most abundant DNA lesion (mono-adducts) induced by SM. DNA repair pathways were inhibited using a combination of specific inhibitors and model cell lines followed by exposure to CEES and measurement of cell viability and levels of endogenous DNA damage. We investigated the roles of BER and NER, which both respond to and repair DNA adducts. As a consequence of the excision repair pathways, a single strand break in the DNA is generated as a repair intermediate prior to DNA synthesis/ligation. If DNA adducts occur in close proximity on opposite strands of DNA, there is a potential for double strand break generation. We therefore also investigated the role of NHEJ in the cellular response to CEES. DNA damage signalling pathways were investigated by using inhibitors of the ATM/ATR protein kinases as well as ATM/ATR-deficient cell lines. Data is presented showing that CEES causes DNA damage that is repaired by both the BER and NER pathways. In addition, DNA damage signalling pathways activated after CEES, including phosphorylation of p53 and Chk2, are mediated by a combination of the ATM and ATR protein kinases.

2. Materials and methods

2.1. Cell lines, chemicals and treatments

TK6 (normal), DK0064 (seckel syndrome) LB707 (XPA heterozygote mutant) and LB708 (XPA homozygote mutant, defective in NER) are lymphoblastoid cell lines and were obtained from the European Collection of Cell Cultures (ECACC). The XPA heterozygote cells are from the father of the XPA homozygote. The ATM null cells (Vw lymphoblastoid) were kindly provided by Prof. Malcolm Taylor (University of Birmingham). Lymphoblastoid cells were maintained as exponentially growing cultures in RPMI 1640 medium supplemented with 15% FBS, 2 mM L-glutamine and penicillin/streptomycin (all reagents were from Sigma). Hela cells were obtained from ECACC and maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine and penicillin/streptomycin. GM04312 and GM15876 fibroblasts were obtained from the Coriell Institute for Medical Research and maintained in DMEM, as above. CEES (Sigma) was stored at 4 °C. On the day of use, a 200 mM stock was prepared in isopropanol. This stock was used to prepare serial dilutions, also in isopropanol. CEES was added to cells such that the final isopropanol concentration in culture medium did not exceed 1%. Stock solutions of CEES were handled in a fume cupboard, with waste being inactivated with 20% alcoholic potassium hydroxide for 24 h prior to disposal. The DNA-PK inhibitor NU7026 (Boulton et al., 1999; Veuger et al., 2003), the ATM/ATR inhibitor wortmannin (WM) (Powis et al., 1994), the PARP-1 inhibitor NU1025 (Boulton et al., 1995; Bowman et al., 1998) and the BER inhibitor methoxyamine hydrochloride (MX) (Fishel et al., 2007) were obtained from Sigma and the ATM inhibitor KU55933 (Hickson et al., 2004) was obtained from Merck. NU7026, WM, NU1025 and KU55933 were prepared at stock concentrations of 10 mM, 20 mM, 100 mM and 10 mM, respectively, in DMSO. When used in experiments (see below), inhibitors were added at the indicated concentrations, with DMSO concentrations being maintained at <1%. MX was prepared at a stock concentration of 5 M in phosphate buffered saline (PBS) before dilution to 20 mM in culture medium and addition to cells. The pH of this medium was adjusted to 7.0 using sodium hydroxide prior to use. Inhibitors were added to cells 1 h prior to treatment with CEES. For treatment with hydrogen peroxide, serial dilutions of hydrogen peroxide were prepared in PBS before addition to cells. For UV treatment, cells were placed in 60 mm tissue culture dishes and treated with UV doses ranging from 2 to 12 J/m^2 using an Amersham UV crosslinker. Finally, cells were treated with 1–5 Gy γ-radiation using a Gammacell Irradiator.

2.2. Western blotting

After the indicated treatments, cells were washed in cold PBS before lysis in cold native lysis buffer (50 mM Tris pH 7.4, 0.27 M sucrose and 1% Triton X-100) supplemented with Halt™ protease and phosphatase inhibitors (Pierce Biotechnology). Lysates were cleared by centrifugation at 13,000 rpm for 5 min at 4 °C. Protein concentrations were determined using Coomassie Bradford Reagent (Pierce Biotechnology) prior to denaturing of the sample in an equal volume of 2× LDS sample buffer (Invitrogen) containing 10% 2-mercaptoethanol (Sigma). Proteins (25 μg) were separated on 4–12% Bis–Tris gels (Invitrogen) before transfer to Hybond-C nitrocellulose membrane (Amersham Biosciences). Membranes were probed using standard protocols, with the following primary antibodies: anti-p53 (DO-7, Novocastra), anti-Chk2, anti-Chk2 phospho-Thr68, anti-p53 phospho-Ser15 (all from Cell Signalling Technologies) and anti-GAPDH (ab9485, Abcam). Membranes were then washed in TBST (50 mM Tris pH 7.6, 150 mM NaCl and 0.2% Tween20) followed by incubation with the relevant secondary antibody. Membranes were washed thoroughly with TBST and visualised using chemiluminescence (ECL Plus, GE Biosciences).

2.3. Cell viability assay

The toxicity of CEES was assessed using an MTT-based assay (TOX-1 *in vitro* toxicology kit, Sigma). For lymphoblastoid cells, cell suspensions were prepared at 2×10^5 cells per ml. After the indicated treatments (see Fig. 2), 100 μl of cell suspension was transferred into three wells of a 96-well plate. To each well, 10 μl of MTT reagent was added followed by gentle agitation. Cells were incubated at 37 °C for 2 h before lysis of the cells and solubilisation of the precipitate formed using solubilisation solution (part of TOX-1 kit). The absorbance of each well was measured at 570 nm and 690 nm. Cell proliferation/viability is directly proportional to the difference between these values ($A_{570} - A_{690}$). For adherent cells, the assay was performed in 24-well plates. Data was plotted on graphs, with relative cell viability at each dose of CEES being calculated as a % relative to untreated cells.

2.4. Comet assay

Cell suspensions were prepared at 1×10^6 cells per ml and treated as indicated (Figs. 1 and 3). Cells were then washed twice in cold PBS. Cells were resuspended in 1 ml of cold PBS and 10 μl added to 90 μl of 1.1% molten agarose (low melting point agarose, equilibrated to 37 °C in a waterbath). After mixing, the cell suspension in agarose was added to a CometSlide (Trevigen) and the agarose spread over the defined area. The slide was immediately transferred to an ice-cold aluminium tray sitting on a bed of ice. After 10 min, slides were submerged in lysis buffer (10 mM Tris, 2.5 M NaCl and 100 mM EDTA pH 10.0) for 1 h at 4 °C. Just prior to use the lysis buffer was chilled on ice and supplemented with 1% Triton X-100 and 1% DMSO. After 1 h, slides were rinsed in ice-cold PBS and placed in an electrophoresis tank containing cold alkali buffer (0.3 M NaOH, 1 mM EDTA) for 30 min prior to electrophoresis. Electrophoresis was performed at 22 V for 30 min. Slides were then submerged in neutralisation buffer (0.5 M Tris pH 7.5) for 15 min prior to being rinsed in PBS. Excess PBS was removed from the slides before staining with SYBR Gold (1:10,000 in PBS) for 10 min. Slides were then briefly submerged in ultra-pure water to remove excess stain prior to drying in the dark overnight. Slides were analysed using a fluorescent microscope and Komet IV software (Kinetic Imaging). DNA damage was expressed as the mean Olive tail moment (OTM) from 50 individual scored cells.