DNA Repair 28 (2015) 14–20

Contents lists available at ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair

Brief Communication

The bacterial alkyltransferase-like (eATL) protein protects mammalian cells against methylating agent-induced toxicity

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ARTICLE INFO

Article history:
Received 20 August 2014
Received in revised form
13 December 2014
Accepted 22 January 2015
Available online 31 January 2015

Keywords:
Alkyltransferase
MGMT
ATL
O6MeG
Killing protection
Alkylating agents

ABSTRACT

In both pro- and eukaryotes, the mutagenic and toxic DNA adduct O^6-methylguanine (O^6MeG) is subject to repair by alkyltransferase proteins *via* methyl group transfer. In addition, in prokaryotes, there are proteins with sequence homology to alkyltransferases, collectively designated as alkyltransferase-like (ATL) proteins, which bind to O^6-alkylguanine adducts and mediate resistance to alkylating agents. Whether such proteins might enable similar protection in higher eukaryotes is unknown. Here we expressed the ATL protein of *Escherichia coli* (eATL) in mammalian cells and addressed the question whether it is able to protect them against the cytotoxic effects of alkylating agents. The Chinese hamster cell line CHO-9, the nucleotide excision repair (NER) deficient derivative 43-3B and the DNA mismatch repair (MMR) impaired derivative Tk22-C1 were transfected with eATL cloned in an expression plasmid and the sensitivity to N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) was determined in reproductive survival, DNA double-strand break (DSB) and apoptosis assays. The results indicate that eATL expression is tolerated in mammalian cells and confers protection against killing by MNNG in both wild-type and 43-3B cells, but not in the MMR-impaired cell line. The protection effect was dependent on the expression level of eATL and was completely ablated in cells co-expressing the human O^6-methylguanine-DNA methyltransferase (MGMT). eATL did not protect against cytotoxicity induced by the chloroethylating agent lomustine, suggesting that O^6-chloroethylguanine adducts are not target of eATL. To investigate the mechanism of protection, we determined O^6MeG levels in DNA after MNNG treatment and found that eATL did not cause removal of the adduct. However, eATL expression resulted in a significantly lower level of DSBs in MNNG-treated cells, and this was concomitant with attenuation of G2 blockage and a lower level of apoptosis. The results suggest that eATL confers protection against methylating agents by masking O^6MeG/thymine mispaired adducts, preventing them from becoming a substrate for mismatch repair-mediated DSB formation and cell death.

1. Introduction

DNA is known to contain various types of alkylation damage that are presumed to arise from exposure to exogenous chemical agents or endogenously as by-products of cellular metabolism [1]. One of the lesions found in DNA as a result of the reaction with methylating species is O^6-methylguanine (O^6MeG), a highly mutagenic and cytotoxic DNA lesion [2]. O^6MeG exerts its mutagenic effect through mispairing with thymine during DNA replication, leading after a further round of DNA replication to G:C to A:T transition mutations [3,4]. The cytotoxic effect of O^6MeG stems from the binding of the mismatch repair (MMR) protein MutSα, a dimer composed of MSH2 and MSH6, to O^6MeG:T mispairs. This initiates binding of other MMR proteins and the excision of the misincorporated thymine, but due to the mispairing properties of O^6MeG, thymine is reinserted opposite O^6MeG, resulting in futile cycles of MMR along with extended DNA single-strand gaps [5]. Replication of DNA containing these structures generates DNA double-strand breaks (DSBs), which trigger cell death by activating apoptosis pathways [6–8]. The more complex DNA

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http://dx.doi.org/10.1016/j.dnarep.2015.01.009
1568-7864/

adduct, O^6-chloroethylguanine (O^6ClEtG), which is generated by chemotherapeutic chloroethylnitrosoureas (CNUs) such as lomustine (CCNU), is also cytotoxic. However, induction of cell death following CNUs occurs in an MMR independent manner [9]. Thus, intramolecular rearrangement of the O^6ClEtG adduct leads ultimately to the formation of an N1-guanine-N3-cytosine interstrand crosslink (ICL) [10], which blocks replication, resulting in collapse of replication forks and the formation of DSBs [11].

The potential lethal effects of O^6-guanine alkylation damage have likely resulted in the evolution and conservation in pro- and eukaryotes of various strategies to eliminate the damage from DNA. One such mechanism, in all species except plants, involves the removal of the alkyl group by alkyltransferases (for human cells O^6-methylguanine-DNA methyltransferase (MGMT) [9]). These proteins bind to O^6-alkylguanine adducts in DNA and transfer the alkyl group to an internal cysteine residue in an autoinactivating stoichiometric process leading ultimately to proteasomal degradation [12]. In addition, in bacteria and yeast, there are proteins with some sequence homology to AGTs, collectively designated as alkyltransferase-like (ATL) proteins. Where examined, these proteins exhibit no alkyltransferase activity [13,14], but are able to bind to a wide variety of O^6-alkylguanine lesions [13,15] and confer resistance to the toxic and mutagenic effects of alkylating agents [16,17].

The protective effect of ATL proteins has been attributed to binding to the damaged DNA strand, which results in DNA bending and base flipping and the recruitment of nucleotide excision repair (NER) proteins that eliminate the O^6-alkylguanine adduct [18,19]. In *Schizosaccharomyces pombe*, the alkyltransferase-like protein 1 (Atl1) targets O^6MeG for global genome NER, whereas transcription-coupled NER participates in the repair of more complex adducts [20]. The ATL of *Escherichia coli*, initially described as ybaZ [21], was not only reported to initiate NER [17], but also to mask DNA damage and thus prevents the conversion of certain O^6-alkylguanines to toxic lesions by the MMR system [22]. This process was shown to reduce the transforming effect in *E. coli* of a plasmid containing O^6-hydroxyethyl-, O^6-1-hydroxypropyl- and O^6-2-hydroxypropylguanine, but not O^6MeG, albeit at the cost of a higher mutation frequency [22]. It was therefore proposed to be a lethality avoidance mechanism by damage tolerance.

ATL proteins, that is, O^6-alkylguanine sensing proteins that have no inherent catalytic activity but support the repair of O^6-alkylguanine adducts, have not so far been reported in mammalian cells. O^6MeG is a highly mutagenic and cytotoxic adduct, and, given that prokaryotes have evolved different strategies for removing this lesion from DNA, it is remarkable that mammalian cells appear to have only a single protein, MGMT, responsible for its repair. It might be speculated that mammalian cells either do not need a backup system for repairing O^6MeG (in which ATL is involved) or do not tolerate protein(s) with ATL function that probe the DNA for the presence of O^6-alkylguanines and support their repair. It was therefore of interest to investigate whether the expression of ATL in mammalian cells is tolerable and whether it impacts the effects of simple alkylating agents in cells that do not express MGMT. To examine the possible effects of ATL expression in mammalian cells, we introduced the ATL protein of *E. coli*, in the following referred to as eATL, into Chinese hamster cells and exposed them to alkylating agents. We show that expression of eATL is well tolerated, having no impact on cell proliferation and survival. It protects against cytotoxicity induced by N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), but not lomustine (CCNU). Intriguingly, eATL expressing cells did not demonstrate increased capacity to eliminate O^6MeG from DNA. However, they showed a reduced number of DSBs, attenuated G2 arrest and apoptosis following MNNG indicating that protection against killing occurred by preventing the action of MMR on O^6MeG:T mispairs induced in replicating cells.

2. Materials and methods

2.1. Cell culture and drug treatment

The wild-type Chinese hamster cell line, CHO-9, the corresponding ERCC1 mutant 43-3B (NER-deficient) [23,24], the MMR-impaired derivative Tk22-C1 (designated originally as strain Tk22cos9/5-1/2-C1 [25]) and a CHO-9 derivative stably transfected with the human MGMT cDNA [26] were used in this study. All cell lines were cultured with Dulbecco's MEM/F-12 medium (1:1) containing 5% foetal calf serum (FCS) and penicillin (100 unit/ml) and streptomycin (100 μg/ml) in a humidified atmosphere in 7% CO_2 at 37 °C. MNNG (Sigma, Munich, Germany) was dissolved in dimethylsulfoxide (DMSO) and then diluted with sterile water to a final concentration of 10 mM. A solution of 10 mM lomustine [1-(2-chloroethyl)-3-cyclohexyl-L-nitrosourea; CCNU] (Sigma, Munich, Germany) was prepared by dissolving in ethanol. Aliquots of MNNG and CCNU were stored at −80 °C and −20 °C, respectively. For the treatment of exponentially growing cells MNNG or CCNU was added directly to the medium.

2.2. Plasmid constructions and transfection

The eATL pcDNA3.1 vector (containing a neomycin resistance cassette) was generated using the pcDNA3.1/V5 His Topo TA Expression Kit (Invitrogen, Darmstadt, Germany) according to the provided protocol. The eATL cassette was isolated by PCR using the vector pMBP-YbaZ [22] as template and eATL forward: 5′-GCCATGCGACTTCACTCGGGC-3′; reverse: 5′-TCAGTAGTTCCAGCGATAACG-3′ primers. The version of eATL pcDNA3.1 containing a hygromycin resistance cassette was obtained by excision of the eATL sequence with Hind III and Xho I from the eATL pcDNA3.1 (neomycin) vector and insertion into the Hind III-Xho I digested pcDNA3.1 (hygromycin) vector. The Effectene transfection kit (Qiagen, Hilden, Germany) was used for transient transfection of Tk22-C1 cells and to stably transfect CHO-9 and 43-3B cells with the eATL pcDNA.3.1 neomycin vector, as well as AT17-C3 cells with the eATL pcDNA.3.1 hygromycin vector. After transient transfection Tk22-C1 cells were allowed to regenerate before initiation of experiments. To obtained stably transfected clones cells were grown in media containing 1.5 mg/ml G418 (CHO-9, 43-3B) or 0.8 mg/ml hygromycin B and resistant clones were selected. Transfectants were routinely cultured in selective media, but the selective agents were omitted during the experiments.

2.3. Polymerase chain reaction

Total RNA from cultured cells was isolated using the Nucleospin RNA II Kit (Macherey-Nagel, Düren, Germany). One microgram RNA was transcribed into cDNA using the Verso cDNA Kit (Thermo Scientific, St. Leon-Rot, Germany). PCR amplification was performed using Red-Taq Ready Mix (Sigma–Aldrich, Taufkirchen, Germany) and primers for eATL (forward: 5′-TCGCCACGGCACAATTTCGC-3′, reverse: 5′-AGTCGATTTGCCCGCTTCCCG-3′) and β-Actin (forward: 5′-GCTCTTTTCCAGCCTTCCTT-3′, reverse: 5′-GAGCCAGAGCAGTG ATCTCC-3′).

2.4. Antibody production

Anti-eATL polyclonal antibodies were obtained by immunisation of rabbits (Eurogentec) with recombinant MBP-eATL and purified by protein A affinity chromatography.

2.5. Preparation of cell extracts and western blotting

To achieve a probable accumulation of eATL protein in the nucleus (through binding of eATL to O^6MeG), cells were treated