Mutation Research 751–752 (2013) 8–14

Contents lists available at ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

Analysis of mutant frequencies and mutation spectra in hMTH1 knockdown TK6 cells exposed to UV radiation

Asal Fotouhi[a], Winta Woldai Hagos[b], Marina Ilic[a], Andrzej Wojcik[a], Mats Harms-Ringdahl[a], Frank de Gruijl[c], Leon Mullenders[b], Jacob G. Jansen[b], Siamak Haghdoost[a,*]

[a] Center for Radiation Protection Research, Department of Molecular Biosciences, Wenner-Gren Institute, Stockholm University, Sweden
[b] Department of Toxicogenetics, Leiden University Medical Center, Leiden, The Netherlands
[c] Department of Dermatology, Leiden University Medical Center, Leiden, The Netherlands

ARTICLE INFO

Article history:
Received 23 August 2013
Received in revised form 4 October 2013
Accepted 10 October 2013
Available online 19 October 2013

Keywords:
Ultraviolet radiation
Oxidative stress
Nucleotide pool
hMTH1
8-Oxo-dG
2-OH-dA

ABSTRACT

Ultraviolet radiation is a highly mutagenic agent that damages the DNA by the formation of mutagenic photoproducts at dipyrimidine sites and by oxidative DNA damages via reactive oxygen species (ROS). ROS can also give rise to mutations via oxidation of dNTPs in the nucleotide pool, e.g. 8-oxo-dGTP and 2-OH-dATP and subsequent incorporation during DNA replication. Here we show that expression of human MutT homolog 1 (hMTH1) which sanitizes the nucleotide pool by dephosphorylating oxidized dNTPs, protects against mutagenesis induced by long wave UVA light and by UVB light but not by short wave UVC light. Mutational spectra analyses of UVA-induced mutations at the endogenous *Thymidine kinase* gene in human lymphoblastoid cells revealed that hMTH1 mainly protects cells from transitions at GC and AT base pairs.

1. Introduction

Ultraviolet radiation (UVR) emitted from the sun induces both beneficial and harmful effects. Overexposure of UVR leads to immediate adverse effects, such as sunburn, and long-term effects, such as skin cancer (including malignant melanomas) [1]. The UVR emitted from sun can be divided into 3 types based on their wavelength: UVA (315–400 nm), UVB (280–315 nm) and UVC (200–280 nm). UVB and UVC can exert its genotoxic effects through direct excitation of the DNA molecule, giving rise to cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4 PP). The most commonly observed UV photolesions are induced at thymine–thymine dimers and thymine–cytosine dimers. In addition to these lesions UVR is known to induce a much wider range of DNA damage such as protein–DNA crosslinks, oxidative base damage (8-hydroxy-2-deoxyguanosine, 8-oxo-dG), single-stranded breaks and clustered lesions [2,3] depending on the wavelength of UVR. In particular, UVA can cause DNA damage in an indirect fashion by the production of reactive oxygen species (ROS) which lead to oxidation of nuclear DNA as well as oxidation of nucleotides (dNTP) in the nucleotide pool [4,5]. To eliminate oxidized nucleotides from the nucleotide pool, and thereby prevent incorporation of oxidized nucleotides in the DNA during replication, cells are equipped with MutT homolog 1 (hMTH1), a major cellular 8-oxo-dGTPase [6]. hMTH1 hydrolyzes oxidized nucleoside triphosphates, such as 8-oxo-dGTP, 2-hydroxy (OH)-dATP and 8-oxo-dATP to their respective monophosphate forms, thereby lowering the levels of oxidized dNTP [7]. Thus, hMTH1 sanitizes the nucleotide pool by eliminating oxidized nucleotides. It has previously been suggested that reduced expression of hMTH1 leads to an increase in oxidized purines concentrations in the nucleotide pool [5,8–10]. We recently reported that, compared to human Tk6 lymphoblastoid cells expressing normal levels of hMTH1 (hMTH1-WT cells), cells expressing reduced levels of hMTH1 (hMTH1-KD cells) display a significant increase of intracellular 8-oxo-dG as well as increased mutagenesis following exposure to UVA [5].

Abbreviations: ROS, reactive oxygen species; dNTP, deoxyribonucleotide triphosphate; 8-oxo-dG, 8-oxo-2′-deoxyguanosine; 8-oxo-dGTP, 8-oxo-2′-deoxyguanosine-5′-triphosphate; 2-OH-dA, 2-hydroxydeoxyadenosine; 2-OH-dATP, 2-hydroxydeoxyadenosine 5′-triphosphate; hMTH1, human MutT homolog protein; UVR, ultraviolet radiation; CPD, cyclobutane pyrimidine dimer; 6-4PP, pyrimidine (6-4) pyrimidone photoproduct; KD, knockdown; WT, wildtype; *Tk*, *Thymidine kinase*; TFT, trifluorothymidine; TK6, human B lymphoblastoid cells.

* Corresponding author. Tel.: +46 8 164064.
E-mail address: Siamak.Haghdoost@su.se (S. Haghdoost).

0027-5107/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.mrfmmm.2013.10.001

Analysis of mutation spectra induced by UVR is a powerful tool to delineate the contribution of different types of DNA lesions contributing to mutagenesis [11,12]. Published data on the spectra of UVA-induced mutations, however, provide contradicting results [13]. Some mutation spectra suggest a role of oxidative DNA damage, whereas other spectra indicate a strong contribution of CPDs in mutation induction [11,12]. These opposing results may be explained by differences in test assays, UV sources and other experimental conditions reviewed by Runger et al. [11]. The major aim of the present study is to investigate the contribution of oxidized nucleotides in the nucleotide pool to UVA-induced mutagenesis by determining the mutation spectra of hMTH1-WT and hMTH1-KD cells exposed to UVA. We used the *Thymidine kinase* (*Tk*) gene as a target gene for mutational analysis, since (i) Tk mutants can be selected easily using trifluorthymidine (TFT) and (ii) the *Tk* gene is a suitable model to study base pair substitutions, intragenic deletions and insertions, frameshift mutations, and loss of heterozygosity (LOH) events [14]. Here we report on the cytotoxicity and induction of mutants in hMTH1-KD and hMTH1-WT cells following exposure to UVB and UVC as well as investigation of mutation spectra induced by UVA in order to investigate the role of dNTP oxidation in the cytotoxicity and mutagenicity of UVA, UVB and UVC. Our results suggest that UVA and UVB induce mutations, at least partly, via oxidation of dNTP (particularly dATP) and subsequent incorporation in the DNA during DNA synthesis. The results indicate that hMTH1 protects against UVA and UVB mediated mutagenesis most likely by reducing the level of oxidized dNTPs.

2. Materials and methods

2.1. Cell culture

The generation of human lymphoblastoid TK6 cells, heterozygous for the *Tk* gene, that express reduced levels of hMTH1 (hMTH1-KD) was described previously and same cells were used in the present investigation [5]. Cells expressing normal levels of hMTH1 (hMTH1-WT cells) and hMTH1-KD cells were cultured in RPMI-1460 medium supplemented with 10% bovine calf serum (Hyclone, USA), 1% Pest (Invitrogen, UK) and 10 mM HEPES. The cells were grown in 75 cm^2 (T75) flasks at 37 °C and 5% CO_2. The cell density was kept between 1×10^5 and 1.5×10^5 cells/ml during the whole experiment. The cell concentration and viability was determined with an automated cell counter (Cell countess, Invitrogen, UK) with trypan blue staining.

2.2. Irradiation with UVR

Prior to irradiation, the cells were washed with RPMI without phenol red (RPMI w/o) and the cells were exposed to UVB and UVC in complete RPMI medium without phenol red. For clonogenic survival, 0.5×10^6 cells in 0.5 ml medium were exposed in a 35 mm × 10 mm petri dish. For the mutant frequency assay (UVB and UVC) and for studying mutation spectra (only UVA), 1×10^6 cells in 0.5 ml medium were exposed to UVR in a 35 mm × 10 mm petri dish. The cells were always kept on ice prior, during and after irradiation to avoid heating by the UV light. The UVA source was an Osram UltraMed 400 W lamp with 4.5 mm sekuritglas, heatfilter and blueglassfilter (typeUG1). The fluence was 122 W/m^2, corresponding to 122 J/s/m^2. For irradiation with UVB, a corona mini dose UV240 T lamp, 230 V 50 Hz, 70 W, with a dose rate of 1.4 W/m^2 was used. Irradiation with UVC was performed using a low-pressure mercury lamp (Philips UV, 15 W) with more than 80% output at 254 nm at a dose rate of 0.18 W/m^2, monitored by a radiometer (Ultra-Violet Products, Inc., model J-260 digital radiometer, with a calibrated probe).

2.3. Clonogenic survival

The clonogenic survival of the cells was investigated after 0, 20, 40, 60, and 80 J/m^2 for UVB irradiation and after 0, 1, 5, 10 and 15 J/m^2 for UVC irradiation. After irradiation, a defined number of cells (50–800 cells/well) were casted into a 6-well plate with 1.5 ml low gelling agarose prepared in complete RPMI. The plate was kept at 4 °C for approximately 15 min to let the agarose solidify, followed by incubation at 37 °C in 5% CO_2 for 10 days. Then, the number of colonies was determined and the relative cell survival was calculated.

2.4. Mutant frequency

The mutant frequency induced by UVB and UVC in the hMTH1-KD and hMTH1-WT cells was investigated. We exposed cells to 50 J/m^2 with UVB and 7 J/m^2 with UVC, which resulted in 50% cell clonogenic survival. For each experiment a sham-irradiated sample was prepared to determine the background mutant frequency in the cells. After irradiation, the cells were cultured in T75 flasks with complete RPMI for 10–14 days at 37 °C in 5% CO_2. The cells were subcultured every second day in order to maintain optimal concentration and viability. Thereafter, a defined number of cells (4×10^5–1×10^6 cells/well) was casted into 4 wells of a 6-well plate with 1.5 ml low gelling agarose prepared in complete RPMI supplemented with 5 μg/ml TFT for selection of the mutants. The clone forming ability was estimated in the remaining 2 wells. The plate was incubated in a cell culture incubator (37 °C in 5% CO_2) for 10 days to allow colony formation. The number of mutants per 10^5 surviving cells was calculated as $[(M/N)/CE] \times 10^5$ where M is the number of mutants, N is the total number of casted cells which was incubated with TFT and CE (%) represents the cloning efficiency of the cells in the absence of TFT.

2.5. Isolation of Tk mutant cells

After mock treatment or UVA irradiation (32 kJ/m^2), the cultures of hMTH1-KD and hMTH1-WT cells were divided into 12 T75 flasks and cultured in complete RPMI medium for 10 days. Thereafter, cells from each flask were casted in 6-well plates that contained low gelling agarose in medium with TFT. After formation of TFT-resistant colonies, a single colony was isolated from each well and put into a T25 flask with RPMI for culturing. Approximately 50 mutant colonies were collected from each condition.

2.6. RNA isolation, Tk cDNA synthesis and PCR

Total RNA was isolated from 5 to 10×10^6 cells using TRIzol® reagent according to the manufacturer's procedure (Invitrogen/Life Technologies). First-strand cDNA synthesis was performed according to the protocol of the manufacturer (Invitrogen/Life technologies). Briefly, 2 μg of total RNA together with 1 μl Oligo-dT oligonucleotides and 1 μl 2.5 mM dNTPs were mixed in a total volume of 10 μl and incubated at 65 °C for 5 min. Following incubation on ice for at least 1 min, 4 μl first strand buffer (5×), 4 μl 25 mM $MgCl_2$, 2 μl 0.1 M DTT, 1 μl RNaseOUT (40 U/μl) and 1 μl superscript III reverse transcriptase were added. cDNA synthesis was performed at 50 °C for 50 min and the reaction was inactivated at 85 °C for 5 min. *Tk* cDNA was amplified by PCR using two different protocols. A first set of cDNAs was amplified by PCR according to Grosovsky et al. [14]. The protocol consisted of two rounds of PCR of which the second round included a pair of nested *Tk* oligonucleotides. Since this protocol was time consuming and often resulted in low yields of PCR product, we optimized the protocol in order to amplify a second set of cDNAs. To that end, a single PCR reaction using