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Investigation of micronucleus induction in MTH1 knockdown cells exposed to UVA, UVB or UVC

Asal Fotouhi, Nicola Cornella, Mehrafarin Ramezani, Andrzej Wojcik, Siamak Haghdoost*

Center for Radiation Protection Research, Department of Molecular Biosciences, Wenner-Gren Institute, Stockholm University, Sweden

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ABSTRACT

The longer wave parts of UVR can increase the production of reactive oxygen species (ROS) which can oxidize nucleotides in the DNA or in the nucleotide pool leading to mutations. Oxidized bases in the DNA are repaired mainly by the DNA base excision repair system and incorporation of oxidized nucleotides into newly synthesized DNA can be prevented by the enzyme MTH1. Here we hypothesize that the formation of several oxidized base damages (from pool and DNA) in close proximity, would cause a high number of base excision repair events, leading to DNA double strand breaks (DSB) and therefore giving rise to cytogenetic damage. If this hypothesis is true, cells with low levels of MTH1 will show higher cytogenetic damage after the longer wave parts of UVR. We analyzed micronuclei induction (MN) as an endpoint for cytogenetic damage in the human lymphoblastoid cell line, TK6, with a normal and a reduced level of MTH1 exposed to UVR. The results indicate a higher level of micronuclei at all incubation times after exposure to the longer wave parts of UVR. There is no significant difference between wildtype and MTH1-knockdown TK6 cells, indicating that MTH1 has no protective role in UVR-induced cytogenetic damage.

This indicates that DSBs induced by UV arise from damage forms by direct interaction of UV or ROS with the DNA rather than through oxidation of dNTP.

1. Introduction

Ultraviolet radiation (UVR) is a potent mutagen and carcinogen causing harmful effects such as sunburn and skin cancer [1]. The UVR wavelengths that reach the Earth are UVA (315–400 nm) and UVB (280–315 nm), while UVC (200–280 nm) is completely absorbed by the ozone layer [2]. The observation of a decreasing thickness of the ozone layer in some parts of the world is of concern because it leads to larger UV-doses to organisms on Earth [3,4]. Indeed, a relationship has been observed between ozone depletion and skin cancers [5].

UVR can react with DNA molecules and cause DNA damage, but the mode of action is strongly dependent on the UVR wavelength. UVC and the short wave part of UVB is directly absorbed by the DNA leading to photo-induced reactions that can cause DNA crosslinking such as cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs). In contrast, UVA and the long wave part of UVB induce DNA damage indirectly, via photosensitization reactions leading to the formation of reactive oxygen species (ROS) [6,7]. ROS can induce the formation of oxidized bases both in the DNA and in the nucleotide pool [8–10]. Moreover, formation of DNA base damage to the DNA can lead to single strand breaks during repair process that, when in close proximity to each other, can result in double strand breaks (DSB) and further cytogenetic damage [11].

Components of the nucleotide pool, deoxyribonucleoside triphosphate (dNTP) molecules, can be modified by ROS and incorporated into the DNA causing mutations. The dNTP sanitization enzyme, MTH1, inhibits the incorporation into the DNA of the modified bases 8-oxo-dGTP and 2-OH-dATP by dephosphorylating them to the monophosphate forms 8-oxo-dGMP and 2-OH-dAMP, respectively, which can then be excreted into the extracellular milieu. The monophosphate forms cannot be incorporated into

Abbreviation: ROS, reactive oxygen species; DSB, double strand breaks; MN, micronucleus; UVR, ultraviolet radiation; dNTP, deoxyribonucleoside triphosphate; 8-oxo-dGTP, 8-hydroxy-2′-deoxyguanosine-5′-triphosphate; MTH1, human *MutT* homolog protein.

* Corresponding author.
E-mail address: Siamak.Haghdoost@su.se (S. Haghdoost).

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the DNA during replication and thus possible mutations caused by modified bases are prevented [12,13].

In previous studies we could demonstrate that MTH1 protects cells against DNA point mutations induced by UVA and to a lower extent also by UVB in the *Thymidine kinase* (*Tk*) gene, indicating that the nucleotide pool is a significant target for UVA and UVB-induced oxidative damage [9,10]. An interesting question is how far MTH1 protects cells against UVR-induced cytogenetic damage, where DNA double strand breaks (DSB) are the ultimate lesion leading to its induction [14,15]. UVA and the long wave parts of UVB may induce DSB indirectly in the DNA via ROS. In contrast, UVC and the short wave parts of UVB induce DSB only when cells are treated in the G1 phase of the cell cycle and pyrimidine dimers are transformed into DSB during DNA replication in the S-phase of the cell cycle [16]. Do oxidative modifications of nucleotides in the nucleotide pool lead to the formation of DSB in the DNA? Does MTH1 protect cells from UVR-induced cytogenetic damage? The present investigation was carried out to answer these questions.

Human lymphoblastoid TK6 cells with a wildtype and reduced levels of MTH1 were exposed to UVA, UVB or UVC and harvested for micronuclei (MN) at three different incubation times in order to assess the level of cytogenetic damage in cells irradiated at different phases of the cell cycle. Cells were also exposed to gamma rays as positive control. The results show that UVA is a significant micronucleus inducer and that MTH1 plays no role in protecting the cells against UVR-induced cytogenetic damage.

2. Material and methods

2.1. Cell culture

Human lymphoblastoid cell lines, TK6, with normal and reduced level of MTH1 (stably transfected with shRNA) were used in the present project. These cell lines have previously been used in our studies [9,10]. The MTH1-knockdown and wildtype TK6 cells were cultured in complete RPMI-1460 without phenol red supplemented with 10% bovine calf serum (Hyclone, USA), 1% Pest (Invitrogen, UK) and 10 mM HEPES (Applichem GmbH, Darmstadt, Germany). The cells were grown in 25 cm^2 (T25) 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 UVA, UVB, UVC or γ-rays

All experiments were performed in triplicate. 5×10^5 cells in a volume of 500 μl were placed into a 35×10 mm petri dish for exposure. As described previously [9], the petri dishes with the cells were kept on ice prior to, and during exposure. The micronucleus frequency was investigated after doses of UVA, UVB, UVC and gamma rays corresponding to IC80 (inhibitory concentration—acquired from survival curves in previous publications) [9]. Doses applied were 73 kJ/m^2 for UVA, 124 J/m^2 for UVB, 18 J/m^2 for UVC, and 1 Gy for gamma rays. UVA source was an Osram UltraMed 400 W lamp with 4.5 mm sekuritglas, heat filter and blue glass filter (typeUG1) with a fluence of 107 W/m^2. For irradiation with UVB, a corona mini dose UV240T lamp, 230 V 50 Hz, 70 W, with a fluence 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 fluence of 0.18 W/m^2, monitored by a radiometer (Ultra-Violet Products, Inc., model J-260 digital radiometer, with a calibrated probe). As a positive control the cells were irradiated with gamma rays, as it is well known that they induce micronuclei [17,18]. A ^{137}Cs source with a dose rate of 0.4 Gy/min (Scanditronix, Uppsala, Sweden) was used for the exposures to gamma rays.

2.3. Cytokinesis-block micronucleus assay

Following irradiation 4×10^5 cells from each treatment sample were transferred to a T25 flask and diluted to a final volume of 5.4 ml with RPMI without phenol red that contained cytochalasin B (Cyt B, Sigma Aldrich, final concentration 5.6 μg/ml). Cells were then incubated for 24, 30, and 46 h at 37 °C and 5% CO_2. At the end of the incubation time the cells were collected into tubes and centrifuged where the supernatant was aspirated and the cells were re-suspended in approximately 0.5 ml medium.

Of note, the addition of all solutions was conducted dropwise with simultaneous vortexing. The cells were swelled with a hypotonic solution, 0.14 M KCl (Sigma–Aldrich), followed by an incubation for 4 min at room temperature. Thereafter the cells were centrifuged and the supernatant removed. The cells were fixed by addition of Fix I (methanol (VWR, France): 0.9% sodium chloride: acetic acid (VWR, France), 12:13:3) and incubated for 5 min at room temperature. Thereafter the samples were centrifuged and the supernatant removed. The fixation proceeded by addition of Fix II (methanol (VWR, France): acetic acid (VWR France), 4:1) with an incubation of 5 min at room temperature. The fixation step with Fix II was performed twice. After each step the cells were centrifuged and the supernatant removed. The cells were resuspended in Fix II and dropped onto slides (Menzel-Cläser, Braunschweig, Germany) that had been pre-washed with methanol. The slides with the cells were stained with 5% Giemsa (Merck KGaA, Darmstadt, Germany) in phosphate-buffered saline (PBS, Sigma–Aldrich) for 10 min. The slides were coded randomly so that scoring was carried out blind.

2.4. Micronucleus scoring criteria

The scoring criteria for MN were as described by Fenech et al. [14]. The slides were analyzed using a light microscope with a 40× objective. MN frequencies were estimated in 500 binucleated cells per treatment and experiment. In addition to scoring MN, the cell proliferation was estimated by analyzing the replication index (RI) according to Formula (1):

$$\mathrm{RI} = \frac{\mathrm{Mono} + (\mathrm{Bi} \times 2) + (\mathrm{Tri} \times 3) + (\mathrm{Tetra} \times 4) + (\mathrm{Penta} \times 5)}{N} \quad (1)$$

where Mono, Bi, Tri, Tetra and Penta indicate, respectively, mononucleated, binucleated, trinucleated, tetranucleated and penta + more nucleated cells, while N = number of scored cells.

2.5. Statistical analysis

Student's *t*-test was used to investigate the effect of the radiations and MTH1 on the induction of MN frequency and RI. The results obtained for these cell lines have been analyzed using *t*-test for paired samples and assuming an equal variance between the groups. The comparison between the different cell lines has been analyzed instead with a *t*-test for unpaired samples, but always assuming an equal variance between samples.

3. Results

3.1. UVA increased micronucleus induction at all incubation times

MTH1-knockdown and wildtype cells were exposed to an IC80 dose of UVA, UVB or UVC, and as a positive control to gamma rays. Cells were harvested after 24, 30 and 46 h post radiation. Following UVA and gamma radiation significantly enhanced MN frequencies were observed in cells fixed at all incubation times (Fig. 1), although