Toxicology Reports 4 (2017) 441–449

Contents lists available at ScienceDirect

Toxicology Reports

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

Full length article

UVB damage onset and progression 24 h post exposure in human-derived skin cells

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ARTICLE INFO

Keywords:
HaCaT
UVB
MTS
Neutral red
LDH
Apoptosis
Comet assay
MAPK pathway

ABSTRACT

The focus of this research was on UVB radiation (280–320 nm) responsible for cellular changes in skin of acute and chronically exposed individuals. This study investigated the acute cellular damages triggered by UVB exposure of cultured human fibroblasts and keratinocyte cells immediately and 24 h post exposure in order to understand damage onset and progression. The study evaluated a number of cellular parameters including mitochondria, lysosomes, cell membrane, DNA damages as well as pro and anti-apoptotic protein expression levels. Cellular organelle damages were assessed by a battery of *in vitro* toxicological assays using MTS and Neutral red cytotoxicity assays. Cell membrane damages were also assessed by measuring lactate dehydrogenase (LDH) enzyme leakage from UVB exposed cells. Lastly DNA damages was assessed using the comet assay while protein expression was evaluated using Western Blot.

In this study we reported in all our assay systems (MTS, NR and LDH) that cellular damages were UVB dose dependent with damages amplified 24 h post exposure. Our results also indicated that incubation of exposed cells for a period of 24 h increased the sensitivity of the assay systems used. The increased sensitivity in detecting early cytotoxic damages was manifested though organelle damage measurement at very low doses which were not manifested immediately post exposure. The data also indicated that HaCaT cells were most sensitive in detecting UVB triggered damages immediately and 24 h post exposure using the MTS assay. We also established upregulation and downregulation of various apoptotic proteins at various time points post exposure. The presented data clearly indicated the need for a comprehensive assessment of UVB damages 4 and 24 h post exposure due to the different assay sensitivities in addition to various signaling mechanisms activated at different time points post exposure.

1. Introduction

Ultraviolet radiation represents one of the important contributing factors to cellular damages and cancer onset in human exposed skin. UV exposure especially in the UVB (280–320 nm) range is the main cause for malignant cancer developments and is responsible for many deaths worldwide [1]. UVB radiation can penetrate the upper layers of the skin and reach the dermis [2,3]. This ability to penetrate skin layers contributes to damaging major skin cell populations from keratinocytes to fibroblasts. Strong evidence suggests that UVB induces damage, resulting in skin cell loss and/or apoptosis [4,5]. It is assumed that keratinocytes are the most numerous cells in human skin and likely the first cells to be damaged by UV radiation [6]. Previous studies in the literature also indicated that exposure of skin cells to UVB radiation induced various cell modifications including formation of reactive oxygen species (ROS) [3,7], cell cycle arrest and activation of numerous cell genes and cell markers [8]. ROS generation induces membrane disruption as well as nuclear DNA damage leading to apoptotic cell death [9]. Mounting evidence also suggested that UVB-induced apoptosis in keratinocytes is mediated through several independent signaling pathways [3] mainly death receptor activation (extrinsic pathway) [10,11], mitochondrial intrinsic pathway [12] and mitogen-activated protein kinases (MAPKs) [13].

We have previously reported in [14] a good correlation between the MTS and NR assays in measuring UVB damages in exposed cells immediately post exposure. This correlation could not be observed 24 h post exposure hence the need for further investigations to elucidate the underlying mechanisms of UVB induced cellular damages. The present study represents a novel approach in assessing cellular organelles specific damages triggered by UVB exposure. The aim of this study is to elucidate cellular damages onset immediately post exposure and its progression 24 h post exposure. This was achieved by using a range of

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http://dx.doi.org/10.1016/j.toxrep.2017.07.008
Received 20 June 2017; Received in revised form 21 July 2017; Accepted 22 July 2017
Available online 01 August 2017

cytotoxicity techniques investigating a number of cell endpoints, ranging from membrane damages, mitochondrial dehydrogenase activity, lysosomal disruption, DNA damages.

2. Material and methods

2.1. Cell cultures and their maintenance

Cell cultures (passage number 30–35) were maintained in DMEM (Dulbecco's modified essential medium; Gibco, USA) medium supplemented with 5% (v/v) fetal calf serum (JS Bioscience, Australia), L-glutamine (2 mM), penicillin (100 Uml-1) and streptomycin (0.1 mg ml-1; Sigma, USA). Culture cells were kept at 37 °C in a humidified 5% CO_2 incubator. Confluent cells were washed with Hank's Balanced Salt Solution (HBSS) (Gibco), tripsinized, counted and then seeded at densities of (5×10^3 cells/ml). The 24 h assessment consisted in adding fresh media upon UVB exposure and incubating the cells for 24 h in a CO_2 incubator at 37°. This was followed by organelles damage assessment using the three cytotoxicity assays (NR, LDH and MTS).

Human Skin Fibroblasts were commercial cell cultures derived from human skin fibroblasts cells (GM05399). These cells were isolated from skin and maintained in cell culture. They were described as healthy non-fetal tissue derived from a 1-year-old male.

Non-tumorigenic Human Keratinocytes (HaCaT) were derived from normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line [15].

2.2. Experimental design

Confluent cells in log phase of growth were released from the bottom of the culture flask using Trypsin EDTA, and then washed three times with cell culture medium. This was followed by a cell count before the cells were seeded on 24 well plates and incubated overnight to allow cells to reattach to the bottom of plates before UVB irradiation.

2.3. UVB exposure

The UVB irradiation procedure consisted in replacing culture medium with HBSS, the coverlids of the 24 well microtiter plate removed and cells exposed to UVB irradiation (3.92×10^{-4} W/cm^2) from a 6 lamps (FS40212) supplied by Wayne Electronics (Somerby, Australia) in a UV irradiation chamber (Fig. 1). The lamp output was measured by an IL-1700 research radiometer (International Light, Newburry Port, MA). Cells were irradiated with UVB doses ranging from 0 to 5.6 J/cm^2 [14].

2.4. In vitro cytotoxicity assays

2.4.1. MTS cell proliferation assay

UVB irradiated cells mitochondrial damage was assessed using the MTS assay (CellTiter 96® Aqueous non-radioactive cell proliferation kit, Promega). The method consisted in incubating the cell cultures with MTS-PMS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt-phenazine methosulfate] solution at 37 °C for 4 h in the dark. Upon incubation 100 µl of the supernatant was removed and transferred to a 96-well plate for reading at 492 nm using a Labsystem Multiskan MS plate reader (Finland).

2.5. Neutral red uptake assay

The NR assay was performed in 24-well plates (NUNC, Denmark) at 37 °C. The NR dye was filter-sterilized prior to use to prevent crystal formation. The assay was undertaken using the method described in Soni et al., 2010. Briefly NR was diluted 10 fold (0.33%) and added to the cell cultures for 2 h in a humidified CO_2 (4%) incubator (Sanyo, Japan) at 37 °C. Upon incubation cells were washed with HBSS and treated with assay fixative (1% CaCl2:0.5% formaldehyde). The incorporated dye in the cells was dissolved in 300 ml of NRU assay solubilization solution (1% acetic acid: 50% ethanol) for 10 min. The colorimetric reaction was measured using the Labsystem, Multiskan MS reader at 540 nm. Background absorbance was measured (at 690 nm) and subtracted from the original reading to account for the corrected cell absorbance.

2.6. Lactate dehydrogenase assay

The assay measured the amount of lactate dehydrogenase released by the cells upon UVB insult (Promega Corporation). Released LDH in cultures was measured with a 30 min coupled enzymatic assay which resulted in the conversion of the tetrazolium salt into a red colored formazan product. The protocol supplied by Promega (Technical Bulletin 163) was followed without any alterations. 50 µl of the substances to be tested (HBSS in which the cells were exposed or the culture media where the cells were incubated for 24 h) was transferred to a 96 well plate. 12 ml of the assay buffer was mixed with the substrate mix and 50 µl of this solution added to each well of the plate. Cells were incubated for 30 min in the dark. This incubation period was followed by the addition of 50 µl of the stop solution (0.1 M HCl) to each well. The plates were spectrophotometrically read within 1 h at 492 nm.

2.7. Comet assay

The comet assay was performed using Trevigen comet assay silver staining kit. Briefly, Low melting agarose (LMA) container was uncapped and melted in a boiling water bath for 5 min before being placed at 37 °C water bath until ready for use. The prepared lysis solution was stored at 4 °C for 20 min before use. Cells at a density of 1×10^5 cells/ml were mixed with molten LMA (at 37 °C) at a ratio of 1:10 (v/v) and 75 ml immediately pipetted onto the comet slide, the side of the pipette tip was used to spread agarose and cells to ensure complete coverage of sample area. Slides were incubated at 4 °C in dark for 30 min for agarose solidification. Upon agarose solidification slides were immersed in the pre-chilled lysis solution at 4 °C for 60 min this was followed by slide tapped off to remove lysis solution before further immersion in freshly prepared alkaline solution for a period of 40 min at room temperature in dark. Slides were then gently taped and washed immersed in 1 X TBE buffer (pH 8.3) for 5 min prior to transfer to horizontal electrophoresis apparatus and aligned at equal distance from the electrodes. Slides immersed in Electrophoresis buffer (TBE, pH 8.3) were subjected to electrophoresis at 1 V cm^{-1} for 10 min. Upon electrophoresis completion, excess BE was tapped off and slide dipped in 70% ethanol for 5 min and air-dried to bring all cells in a single plan. Samples was desiccated overnight at room temperature for silver staining [6].

2.8. Comets silver staining

The silver staining method involved covering slide with 100 ml of fixation solution for 20 min at room temperature. The slide was then rinsed with deionized water for 30 min and sample area covered with 100 ml of staining solution for 5–20 min at room temperature until the comets acquired the desired intensity. The reaction was stopped by covering the samples with 100 ml of 5% acetic acid for 15 min followed by a deionized water wash. The slide was air-dried and stored in dark until ready for analysis.

2.9. Comets scoring and interpretation

The scoring of comet tail was based on DNA content (intensity) with untreated cells used as controls. DNA Silver staining generated a brown to black stain which could be observed under bright field microscopy. The DNA of untreated cells was confined to the nucleoid, as undamaged