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Sub-lethal ultraviolet B irradiation and Poly I:C treatment synergistically induced apoptosis of HaCaT cells through NF-κB pathway

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ABSTRACT

Ultraviolet B (UVB) irradiation exerts multiple effects on skin cells, inducing apoptosis, senescence and carcinogenesis. Toll-like receptor 3, a member of pattern recognition receptors, is reported to initiate inflammation by recognizing double-strand RNA (dsRNA) released from UVB-irradiated cells. It has not been studied, however, whether apoptosis induction in UVB irradiation is attributed to TLR3 activation. Here, we report on the pro-apoptotic role of TLR3 in UVB-irradiated epidermal cells. Poly I:C, an analogue of dsRNA that activates TLR3, was used in combination with sub-lethal UVB (4.8 mJ/cm^2) irradiation for investigating the effects of TLR3 activation on human immortalized keratinocyte HaCaT cells. Although sub-lethal dose of either Poly I:C or UVB alone did not induce cell death, UVB-Poly I:C co-treatment synergistically induced cell death by activation of caspase-3 and cleavages of ICAD and PARP, with apoptotic features when stained with Annexin V/PI or Hoechst 33342. Treatment with pan-caspase inhibitor, Z-VAD, attenuated UVB-Poly I:C-induced cell death. Silencing TLR3 by siRNA rescued HaCaT cells from UVB-Poly I:C-induced apoptosis. NF-κB, a major downstream component of TLR3 pathway, that usually negatively regulates the classical TLR3 apoptotic pathway, was analyzed by western blotting and immunofluorescence confocal microscopy. The results indicate to our surprise that NF-κB is translocated to nucleus in the cells co-treated with UVB-Poly I:C. The nuclear translocation of NF-κB is attenuated by TLR3 silencing. Treatment with BAY, an inhibitor of NF-κB pathway, blocked UVB-Poly I:C-induced apoptosis. Therefore, we conclude that NF-κB pathway plays a cytotoxic role in UVB-Poly I:C-treated HaCaT cells, mediating TLR3-related apoptosis.

1. Introduction

Exposure to UVB results in skin damages and has significant negative effects on human health. Numerous researches have confirmed that UVB irradiation causes programmed death of epidermal cells (Batista et al., 2009; Deshmukh et al., 2017; Moriyama et al., 2017). Revealing the precise mechanism of programmed cell death initiation and the events taking place following UVB exposure would help us to understand skin homeostasis and thus to make strategy for minimizing damages.

Keratinocytes represent the major cell population of human epidermis and provide first line defense barrier for the host, responding to invading pathogen. Like most of the host innate immune cells, keratinocytes also express functional Toll-like receptors (TLRs) (Kollisch et al., 2005), detecting pathogen-associated molecular patterns (PAMPs). Toll like receptor-3 (TLR3) detects viral double-strand RNA and self-noncoding RNA released from damaged cells. The functions of TLR3 activation in keratinocytes have been frequently reported in recent years. Interestingly, non-irradiated keratinocytes recognized the dsRNA released from UVB-irradiated cells, resulting in release of pro-inflammatory cytokines, such as TNFα and IL-6 (Bernard et al., 2012). However, the reaction of UVB-irradiated keratinocytes to dsRNA has not been investigated. Since skin is frequently exposed to continuous environmental UVB irradiation and may be simultaneously affected by dsRNA released before, the outcome of TLR3 activation in those UVB-irradiated keratinocytes should be considered. Production of TNFα is reported to mediate UVB-induced apoptosis (Schwarz et al., 1995), but UVB-induced apoptosis in skin is multifactorial event that still remains to be elucidated. It is reported that TLR3 induces programmed cell death in keratinocytes through a complex formed by assembly of TRIF

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(Toll/interleukin-1 receptor domain-containing adaptor-inducing interferon-β), RIP (receptor interacting protein)-1 and caspase-8 (Feoktistova et al., 2011; Kaiser et al., 2013; Weber et al., 2010). Whether TLR3 activation contributes to UVB-induced apoptosis has not been investigated yet. Therefore, we treated immortalized human keratinocyte HaCaT cells with sub-lethal doses of UVB (to mimic the environmental UVB) and Poly I:C, a synthetic TLR3 ligand, to explore whether activation of TLR3 has a role in programmed cell death of epidermal cells irradiated with sub-lethal dose of UVB.

NF-κB pathway responds to TLR3 activated with Poly I:C, inducing the production of pro-inflammatory cytokines (Alexopoulou et al., 2001; Han et al., 2004; Narayanan and Park, 2015). NF-κB is a transcription factor involved in many cellular responses and mostly exerts pro-survival effect through up-regulating some target genes (Bcl-xL, xIAP, cIAP-1/2, FLIP and A20) (Pahl, 1999). However, in the response to DNA damage, NF-κB activation is reported to be pro-apoptotic through induction of pro-apoptotic genes, such as TNFα, TRAIL and Fas Ligand (Jennewein et al., 2012; Karl et al., 2009) as well as repression of anti-apoptotic genes (Campbell et al., 2004). Therefore, it is worth revealing the involvement of NF-κB activation in the cells with activated TLR3 and UVB-irradiation.

2. Methods and materials

2.1. Cells and culture

Human immortalized keratinocyte HaCaT cells (CLS Cell Lines Service, 300,493) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco by life technology, USA) supplemented with 10% fetal bovine serum (Certified FBS, TBD0110HYT, Tianjin, China), 100 μg/mL of streptomycin and 100 U/mL of penicillin. Cells were incubated at 37 °C with 5% CO_2 in a humidified atmosphere.

2.2. Reagents

Polyinosinic-polycytidylic acid (Poly I:C), purchased from Sigma-Aldrich (St Louis, MO, USA), was dissolved with phosphate buffer solution (PBS) to the concentrations of 10 mg/mL as a long-term stock solution and of 100 μg/mL as a short-term stock solution. The short-term stock solution was then diluted with DMEM to the indicated concentrations.

Methylthiazolyldiphenyl-tetrazoliumbromide (MTT), Hoechst 33342, necrostatin-1 (Nec1) and 2′,7′-dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma Chemical (St Louis, MO, USA). Annexin V-FITC apoptosis detection kit was purchased form Bimake (Houston, TX, US). Primary antibodies against caspase-3, ICAD (inhibitor of caspase activated DNase), PARP (poly ADP-ribose polymerase), p-p53 (ser-15), TLR3 (Toll-like receptor 3), NF-κB (p65 subunit), IκBα, β-actin, and horseradish-peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibody against Lamin B was purchased from Proteintech (Suite 300 Rosemont, IL, USA). Z-VAD-fmk (Z-VAD), Ac-LEHD-CMK (LEHD) was obtained from Merck (Darmstadt, Germany). BAY 11-7082 (BAY), Z-IETD-FMK (IETD) was purchased from MedChem Express (Monmouth Junction, NJ, USA).

2.3. UVB exposure and Poly I:C treatment

HaCaT cells were irradiated with UVB at doses of 2.4, 4.8, 7.2 or 9.6 mJ/cm^2. UVB lamps (Beijing lighting research institute, Beijing, China) emitted UVB radiation wavelength from 280 to 340 nm, with a peak at 314 nm. UVB intensity was measured by using a UVB spectra radiometer (Photoelectric Instrument Factory of Beijing Normal University, Beijing, China). To avoid possible UVB absorption by the proteins and other components in the medium, cell layers were washed by PBS once and covered by PBS instead of medium with 50 μL of per well for 96-well plates, 250 μL of PBS for 24-well plates and 1 mL of PBS for 6-well plates when exposed to UVB irradiation. Poly I:C was dissolved in fresh DMEM culture medium, and added to the cell layers immediately after irradiation. Chemicals such as caspase inhibitor was added 1 h prior to UVB irradiation.

2.4. Cell viability assay

HaCaT cells were seeded into 96-well plates at the density of 1×10^4 cells per well and cultured for 24 h. Then the cells were subjected to different treatments for 6 h or the indicated time periods. Afterwards, cells were washed once with PBS, and incubated with 100 μL of 0.5 mg/mL MTT dissolved in medium at 37 °C for 2 h. 150 μL of dimethylsulfoxide (DMSO) was added to each well after removing the supernatant, and absorbance at the wavelength of 490 nm (A_{490}) was measured with a microplate reader. Cell viability was calculated using the formula below:

$$\text{Cell Viability } (\%) = 100 \times (A_{490,\ \text{sample}} - A_{490,\ \text{blank}})/(A_{490,\ \text{control}} - A_{490,\ \text{blank}})$$

2.5. Phase contrast microscopy

HaCaT cells (1×10^5/well) were seeded in 24-well plates to culture for 24 h, and then exposed to the indicated treatments for 6 h. The morphological changes of cells were observed with a phase contrast microscope (Leica, Nussloch, Germany).

2.6. Hoechst 33342 staining

HaCaT cells (1×10^5/well) were cultured in 24-well plates. After 24 h of incubation, the cells were subjected to the indicated treatments for 6 h. The cells were then incubated with 100 μL of Hoechst 33342 at 37 °C for 30 min in the dark. Nuclear images were observed with a fluorescence microscope (Olympus, Tokyo, Japan).

2.7. Immunofluorescence confocal microscopy

HaCaT cells were seeded into 6-well plates (6×10^5 cells/well), in which coverslips (20 × 20 mm) were placed, and cultured for 24 h. Then cells were treated with indicated treatments for 6 h. After that, cells on coverslips were washed three times with 2 mL PBS, fixed with 2 mL of 4% paraformaldehyde in PBS, pH 7.4, at room temperature for 10 min, washed three times with PBS, and permeabilized with 0.15% Triton X for 20 min. After washing with PBS three times, the cells on coverslips were incubated with 2 mL blocking solution (PBS with 10% fetal bovine serum and 0.1% Tween 20) for 30 min and then incubated with the indicated primary antibodies at 4 °C overnight. The cells on coverslips were washed with PBS three times and incubated with 100 μL of fluorochrome-conjugated secondary antibodies in blocking buffer for 2 h. After washing with PBS three times, the cells were incubated for 7 min with DAPI to stain the cell nuclei. The cells on coverslips were finally washed with PBS three times and mounted on microscopic slides (75 × 25 × 1 mm) with a drop of mounting medium. After sealing the edges with nail polish, slides were stored in the dark at 4 °C. Antibody localization was visualized with a confocal microscope (Nikon C2 plus, Tokyo, Japan).

2.8. Nuclear and cytoplasmic protein extraction

Cells were collected 6 h after the indicated treatments, and lysed with nuclear and cytoplasmic protein extraction kit purchased from Wanleibio (Shenyang, Liaoning, China). Harvested cells were first suspended with 100 μL cytoplasmic protein extraction reagent A supplemented with 1 mM phenylmethysulfonyl fluoride (PMSF) and 1 mM