Troxerutin induces protective effects against ultraviolet B radiation through the alteration of microRNA expression in human HaCaT keratinocyte cells

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Received September 17, 2013; Accepted January 20, 2014

DOI: 10.3892/ijmm.2014.1641

Abstract. Ultraviolet light B (UVB), contained in sunlight, induces damaging effects on skin by impairing cells in the epidermis and dermis. In particular, keratinocytes in the epidermis are those cells which are mainly affected by UVB light. UVB radiation induces cell death, growth arrest, DNA damage and restricts cell migration. Various phytochemicals have been shown to alleviate UVB-induced cellular damage. Troxerutin is a natural flavonoid rutin mainly found in extracts of *Sophora japonica*, and is a well-known antioxidant and anti-inflammatory compound used in experimental mouse models. In this study, we examined the effects of troxerutin on UVB-induced damage in HaCaT cells. HaCaT cells were pre-treated with troxerutin (0-10 μM) and then exposed to UVB radiation (50 mJ/cm^2). Cell viability, cell cycle and migration assays were performed to determine the protective effects of troxerutin on the cells. DNA repair activity was also measured. Troxerutin protected the cells against UVB-induced damage, such as cell death, growth arrest, restriction of cell migration and decreased DNA repair activity in HaCaT cells. Analyses of microRNA (miRNA) expression demonstrated that the protective effects of troxerutin correlated with alterations in miRNA expression, as indicated by Gene Ontology analyses of putative target genes. Overall, our data demonstrate that troxerutin exerts protective effects against UVB-induced damage by regulating miRNA expression.

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Key words: troxerutin, keratinocyte, ultraviolet B, microRNA, cell death

Introduction

Ultraviolet (UV) radiation found in sunlight is the major environmental cause of skin aging and skin disorders (1). There are three different wavelengths of UV radiation, subdivided as UVC (290-320 nm), UVB (290-320 nm) and UVA (320-400 nm) (2). UVC radiation contains the highest energy, which can induce skin damage. However, the majority of UVC radiation is blocked by the atmosphere. By contrast, UVA radiation is, on average, 1,000-fold lower in energy than UVB radiation and its transmission through the atmosphere is greater (3). UVB radiation reaches the dermis and epidermis when penetrating the atmosphere. UVB radiation is absorbed by the epidermis and dermis and causes damage to various cells and to the extracellular matrix of the skin (4-6). Keratinocytes are particularly influenced by UVB radiation due to their outermost cutaneous location (7). Keratinocytes, mainly contained in the epidermis, have been implicated in its development via differentiation (8). In keratinocytes, UVB radiation induces the formation of 'sunburn cells' (keratinocytes undergoing apoptosis) and may result in skin cancer (5). UVB induces keratinocyte apoptosis by promoting DNA damage, death receptor activation and the production of reactive oxygen species (ROS) (9). UVB radiation can stimulate UVB-induced signaling pathways involving p53 and MAP kinases (JNK and p38), and can alter gene expression and subsequently induce cell cycle arrest, apoptosis or cellular senescence (10-14). Recently, microRNAs (miRNAs) have been implicated in UVB responses in keratinocytes (15-16). The miRNA, miR-23a, regulates DNA damage repair and apoptosis by targeting topoisomerase-1, caspase-7 and serine/threonine kinase 4 (STK4) (17). In addition, UVB radiation induces apoptosis by upregulating miR-141, which then leads to the suppression of phosphatase and tensin homolog (PTEN) in keratinocytes (18). Aberrantly expressed miRNAs have been linked to apoptosis and cell cycle arrest in UVB-exposed

keratinocytes, where they can be used as regulators of UVB responses (19). In the current study, we identified a novel phytochemical that protect against UVB-induced cell death through the regulation of miRNA expression.

Troxerutin, {vitamin P4; 3',4',7'-Tris[O-(2-hydroxyethyl)] rutin} is a natural flavonoid rutin mainly found in extracts of *Sophora japonica*, and is a well-known antioxidant and anti-inflammatory compound used in experimental mouse models (20-24). In addition, troxerutin has been shown to improve capillary function by suppressing capillary fragility and abnormal leakage (25). It has been shown to inhibit erythrolysis and exert anti-thrombotic, fibrinolytic, edema-protective and rheological effects in a model of chronic venous insufficiency (26).

In the present study, we demonstrate that troxerutin exerts protective effects against UVB radiation by regulating miRNA expression. The current data may enhance our understanding of the protective mechanisms of troxerutin in UVB-exposed skin.

Materials and methods

Cell culture and reagents. HaCaT cells, an immortalized human keratinocyte cell line, were grown as monolayers at 37˚C, in a 5% CO_2 atmosphere, in DMEM medium (Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin/streptomycin solution (Gibco-Invitrogen). Viability assays were carried out in 96-well microplates seeded with $4x10^4$ HaCaT cells. Cell cycle analyses, DNA damage assays, and microarray analyses were performed in 60-mm culture dishes seeded with $7x10^5$ HaCaT cells. Troxerutin was purchased from Sigma-Aldrich. Hydrogen peroxide (30%) was purchased from BioShop Canada Inc. (Burlington, ON, Canada).

Exposure of HaCaT cells to UVB radiation. The HaCaT cells were exposed to UVB radiation using a G8T5E lamp (Sankyo Denki, Toshima-ku, Japan). The UVB radiation intensity was measured by a UV light meter (UV-340; Lutron, Taiwan, Taipei). The cells were washed and resuspended in phosphate-buffered saline (PBS) prior to exposure to UVB radiation. Non-exposed control samples were maintained in the dark under the same conditions. Following exposure to UVB radiation, the cells were grown in fresh medium.

Cell viability assay. Cell viability was measured by a water-soluble tetrazolium salt (WST-1) assay. HaCaT cells were seeded at $4x10^4$ cells/well in 96-well microplates and incubated for 24 h. In order to measure troxerutin toxicity, the cells were treated with the indicated concentrations of troxerutin for 24 h. In order to determine the protective effects of troxerutin against UVB radiation, the cells were incubated with the indicated concentrations of troxerutin for 4, 8 and 12 h. The troxerutin-pre-treated HaCaT cells were then exposed to 50 mJ/cm^2 of UVB radiation followed by incubation for 24 h. WST-1 solution (EZ-Cytox Cell Viability Assay kit; ITSBio, Seoul, Korea) was then added to the cells for 1 h. The absorbance of each sample was measured using a microplate reader (iMark; Bio-Rad Laboratories, Hercules, CA, USA) with filters at 450 nm and a reference wavelength at 620 nm. The results were presented as the relative absorbance [optical density (OD)] compared with untreated HaCaT cells.

Cell cycle analysis. Cell cycle distribution was measured by propidium iodide (PI; Sigma-Aldrich) staining. HaCaT cells were seeded at a density of $7x10^5$ cells in 60-mm culture dishes, and incubated for 24 h. The cells were pre-treated with 5 μM troxerutin for 8 h, and the troxerutin-pre-treated HaCaT cells were then exposed to 50 mJ/cm^2 of UVB radiation and incubated for 24 h. The cells were fixed in 70% ethanol at 4˚C for 3 h, and stained with PI staining solution containing 50 μg/ml PI (Sigma-Aldrich), 0.5% Triton X-100 and 100 μg/ml RNase (both from BioShop Canada Inc.) at 37˚C for 1 h. The fluorescence intensity of each cell sample was detected by the FL2-H channel of the FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Migration assay. Migration activity in the cells was measured by a scratch wound assay. The HaCaT cells were seeded at a density of $7x10^5$ cells in 60-mm culture dishes, and cultured to >90% confluency. The cells were pre-treated with 5 μM troxerutin for 8 h, and the troxerutin-pre-treated HaCaT cells were then exposed to 50 mJ/cm^2 of UVB radiation and scratched using a 200 μl micropipette tip. At 0 and 48 h, images were acquired of the scratch wound using a phase contrast microscope.

DNA repair activity assay. DNA repair activity was measured by using an impaired plasmid. HaCaT cells were seeded at a density of $7x10^5$ cells in 60-mm culture dishes, and incubated for 24 h. The cells were transiently transfected with damaged (due to impaired exposure response to UVB) pGL3 Luciferase reporter vectors. pSV-β-galactosidase is a positive control vector that was used for monitoring the transfection efficiency. The transfected cells were pre-treated with 5 μM troxerutin for 24 h. After 24 h, luciferase activity was measured using the Luciferase assay system (Promega, Fitchburg, WI, USA) as described in the instruction manual. The normalized results were presented as relative percentages of the experimental control values.

Detection of global miRNA expression levels. Alterations in global miRNA expression levels were measured using a SurePrint G3 Human v16.0 miRNA microarray kit (based on the miRBase release 19.0; Agilent Technologies, Santa Clara, CA, USA). Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, while RNA stability and purity were measured using the Bioanalyzer 2100 (Agilent Technologies), and calculated as $A_{260/A280}$ and $A_{260/A230}$ ratios using a MaestroNano Spectrophotometer (Maestrogen, Las Vegas, NV, USA). The total RNA eliminated phosphate was measured using calf intestine alkaline phosphatase (CIP), and was then labeled with pCp-Cy3, using T4 RNA ligase (all from Agilent Technologies). The labeled total RNA was hybridized to the miRNA microarray. Microarrays were scanned using an Agilent microarray scanner. The miRNA microarray data were normalized and analyzed using GeneSpring GX version 11.5 software (Agilent Technologies).

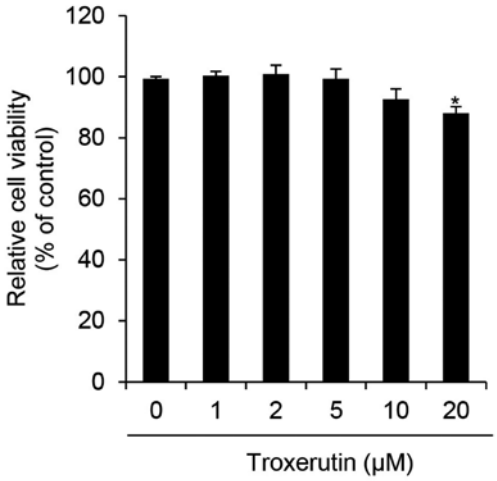

Figure 1. Effects of troxerutin on HaCaT cell viability. HaCaT cells ($4x10^4$ cells) were seeded in 96-well plates and treated with indicated concentrations of troxerutin for 24 h. Cell viability was measured by the water-soluble tetrazolium salt (WST-1) assay as described in Material and methods. The results are presented as the means ± standard error (SE) of the percentage of the control optical density (OD) in triplicate. *p<0.05.

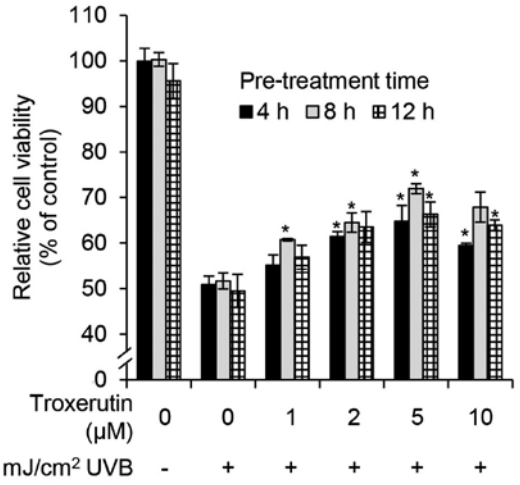

Figure 2. Protective effects of troxerutin on cell viability in ultraviolet B (UVB)-exposed HaCaT cells. HaCaT cells ($4x10^4$ cells) were seeded in 96-well plates, and pre-incubated with indicated concentrations of troxerutin for 4, 8 and 12 h. Following pre-treatment, the HaCaT cells were exposed to 50 mJ/cm^2 of UVB and incubated for 24 h. Cell viability was measured by the water-soluble tetrazolium salt (WST-1) assay as described in Materials and methods. Results are presented in triplicate, as the means ± SE of the percentage of the control optical density (OD). Asterisks denote a significant difference compared with HaCaT cells treated with UVB alone (*p<0.05).

miRNA target gene prediction and Gene Ontology (GO) analysis. A list of target genes for the selected miRNAs was extracted by TargetScan (http://www.targetscan.org). The biological functions of putative target genes were classified into different biological processes (GO categories). Enrichment analysis of GO categories was performed using DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/). We selected significant GO categories using several parameters (count >600 and p-value <0.01).

Statistical analysis. Statistical significance was determined using a Student's t-test. Values of p<0.05 were considered to indicate statistically significant differences.

Results

Troxerutin reduces UVB-induced cytotoxicity in HaCaT cells. We first determined the cytotoxicity of troxerutin in HaCaT cells by performing WST-1 assays. The HaCaT cells were treated with the indicated concentrations of troxerutin and then incubated for 24 h. Cytotoxicity in HaCaT cells was significantly decreased at 20 μM troxerutin (Fig. 1). In a previous study, troxerutin was shown to exert limited, if any, cytotoxic effects at a dose of 20 μg/ml (approximately 26.93 μM) in V79 Chinese hamster lung fibroblasts (27). Therefore, a similar concentration range of troxerutin (0-10 μM) was used in the current study. To determine whether troxerutin protects against UVB-induced growth arrest in HaCaT cells, we performed cell viability assays. The HaCaT cells were pre-treated with the indicated concentrations of troxerutin for 4, 8 and 12 h, and then exposed to 50 mJ/cm^2 of UVB radiation and incubated for 24 h. Troxerutin prevented UVB-mediated growth arrest in a dose-dependent manner (Fig. 2). Troxerutin (5 μM) effectively protected the cells against UVB-mediated growth arrest. Notably, pre-treatment with 5 μM troxerutin for 8 h increased cell viability by 20.27% compared with untreated UVB-exposed HaCaT cells (Fig. 2). Overall, pre-treatment with troxerutin protected the cells from UVB-mediated growth arrest.

Troxerutin reduces UVB-induced death in HaCaT cells. As growth arrest and cell death often cause a decrease in cell viability, in the current study, we determined whether the troxerutin-mediated protective effects against UVB exposure were due to the suppression of growth arrest and cell death. In normal HaCaT cells, 50 mJ/cm^2 of UVB radiation caused an increase in the number of cells in the sub-G1 phase (1.49-11.49%). However, in the HaCaT cells pre-treated with troxerutin, the UVB-mediated increase in the number of sub-G1 phase cells decreased to 4.51% (Fig. 3). As the sub-G1 phase indicates a population of dead cells, the troxerutin-mediated recovery of cell viability may involve the blockade of cell death-associated pathways.

Troxerutin enhances migration activity in HaCaT cells. We then determined whether troxerutin affects migration activity in HaCaT cells. To assess migration activity, the scratched wound healing assay was used as described in Materials and methods. The migration activity was increased by treatment with troxerutin in HaCaT cells as compared to the untreated cells. Exposure to UVB radiation attenuated migration in HaCaT cells, whereas troxerutin restored the migration activity to the control levels (Fig. 4).

Troxerutin induces DNA repair activity in HaCaT cells. To determine whether troxerutin increases DNA repair activity, we examined its effects using the damaged luciferase reporter vector. Troxerutin had no effects on luciferase activity in non-damaged pGL3 vector-transfected HaCaT cells (Fig. 5).

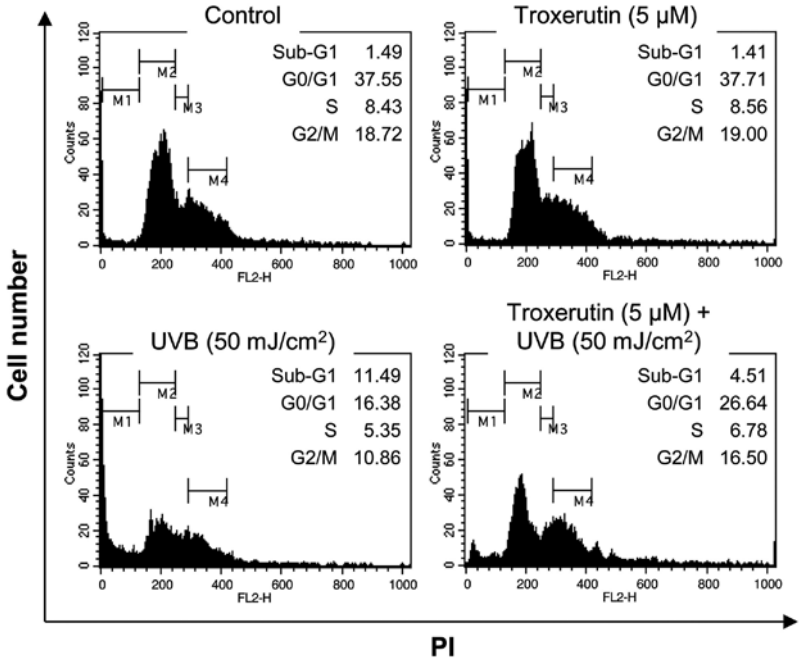

Figure 3. Protective effects of troxerutin on cell cycle in ultraviolet B (UVB)-exposed HaCaT cells. HaCaT cells ($7x10^5$ cells) were seeded in 60-mm culture dishes and pre-incubated with 5 μM troxerutin for 8 h. Following pre-treatment, the HaCaT cells were exposed to 50 mJ/cm^2 of UVB and incubated for 24 h. The cell cycle was measured by propidium iodide (PI) staining. Cell distribution measured by fluorescence intensity in the sub-G1 phase is presented as a histogram plot. Each cell cycle population (G1, S, G2/M phase) was measured using gates.

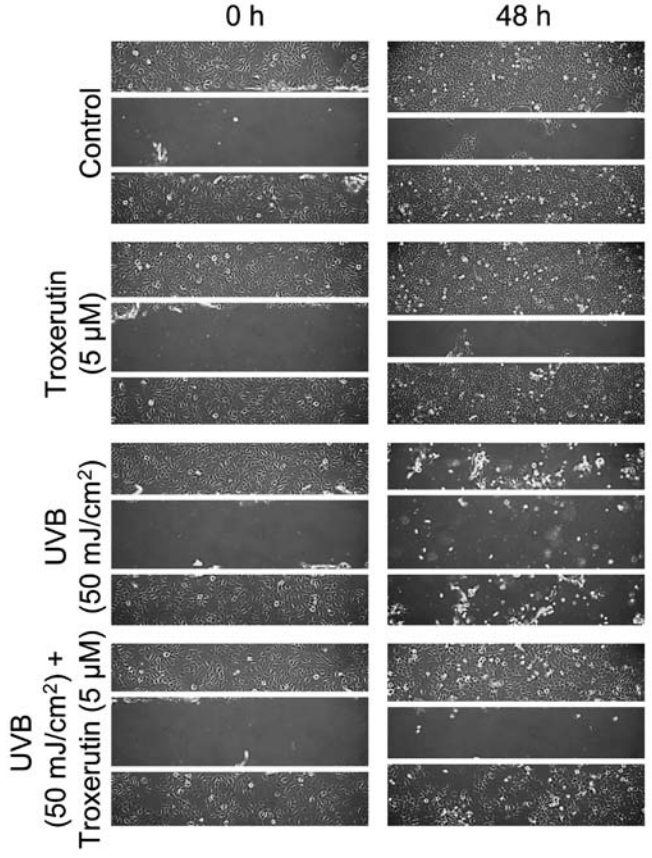

Figure 4. Protective effects of troxerutin on migration in ultraviolet B (UVB)-exposed HaCaT cells. HaCaT cells ($7x10^5$ cells) were seeded in 60-mm culture dishes, and pre-incubated with 5 μM troxerutin for 8 h. Following pre-treatment, the HaCaT cells were exposed to 50 mJ/cm^2 of UVB and scratched using a pipet tip. Cells were then incubated for 48 h. The left panel shows a representative image of indicated conditions immediately after scratching (0 h). The right panel shows a representative image of indicated conditions at 48 h after scratching.

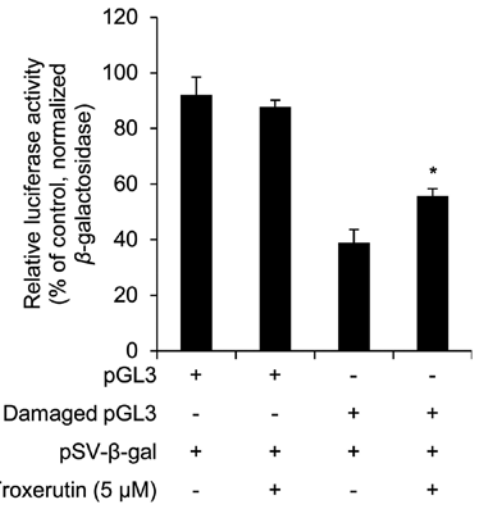

Figure 5. Protective effects of troxerutin on DNA repair activity. HaCaT cells ($7x10^5$ cells) were seeded in 60-mm culture dishes and transfected with damaged pGL3 and pSV-β-galactosidase. Following transfection, the cells were pre-incubated with 5 μM troxerutin for 8 h. Following pre-treatment, the HaCaT cells were exposed to 50 mJ/cm^2 of ultraviolet B (UVB) and incubated for 24 h. DNA repair activity is presented as luciferase activity. The results are presented in triplicate, as the means ± standard error (SE) of the percentage of luciferase activity. Asterisks denote a significant difference compared with damaged HaCaT cells transfected with pGL3 alone ($^*p<0.05$).

However, in the damaged-pGL3 vector-transfected HaCaT cells, troxerutin increased luciferase activity (Fig. 5). The increased luciferase activity was used to measure DNA repair activity; troxerutin increased DNA repair activity in HaCaT cells. Similarly, in a previous study, Maurya *et al* (28) demonstrated that troxerutin exerted protective effects against gamma radiation-induced damage in mouse cellular DNA.

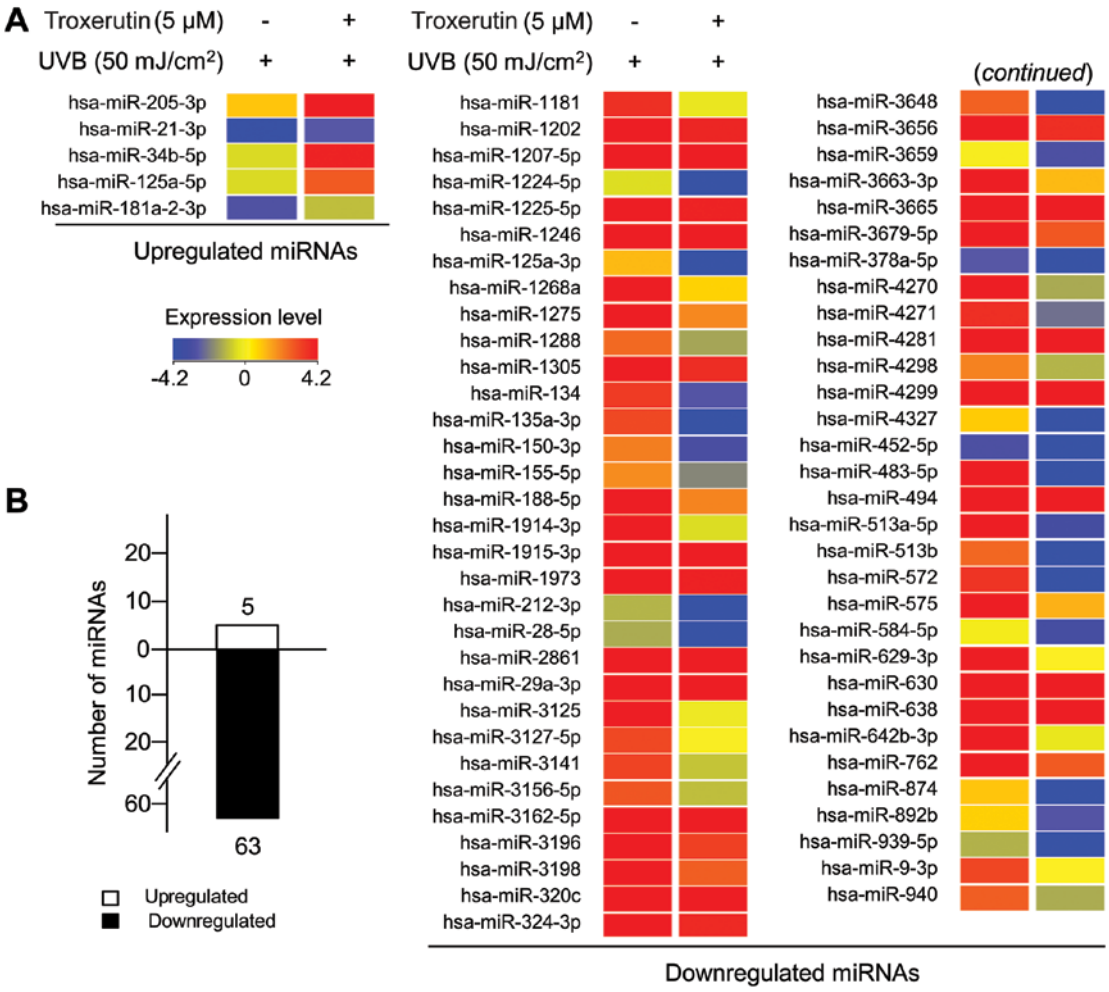

Figure 6. Troxerutin-induced expression profiles of microRNAs (miRNAs) in ultraviolet B (UVB)-exposed HaCaT cells. (A) Heat map analysis of miRNAs upregulated and downregulated with a ≥2-fold change in expression in UVB-irradiated HaCaT cells. HaCaT cells ($7x10^5$ cells) were seeded in 60-mm culture dishes, and pre-incubated with 5 μM troxerutin for 8 h. Following pre-treatment, the HaCaT cells were exposed to 50 mJ/cm^2 of UVB and incubated for 24 h. miRNA expression was determined using the SurePrint G3 Human v16.0 miRNA microarray kit. The color bars displaying fluorescence intensity correspond to each miRNA expression. Highly expressed miRNAs are shown in red, while those present at low levels are shown in blue. (B) Total number of miRNAs upregulated and downregulated by troxerutin.

Troxerutin alters miRNA expression profiles in UVB-exposed HaCaT cells. As described in the Introduction, miRNAs play a crucial role in UVB responses through specific binding to target genes (19). Therefore, in UVB-exposed HaCaT cells, we examined the effects of troxerutin on global miRNA expression levels. To analyze the global miRNA expression levels and identify putative alterations, we used miRNA gene microarrays, which contained 1,368 probes representing 1,205 human miRNAs. Total RNA, labeled with Cy3, a green fluorescent dye, was extracted from the HaCaT cells treated with or without troxerutin and then exposed to UVB radiation. Each Cy3-labeled sample was hybridized onto an miRNA microarray. Using analytical software (GeneSpring GX version 11.5; Agilent), we first normalized the data from each sample using global normalization. To obtain refined data, we did not take into consideration the absent and undetectable data in all samples. Lastly, significantly altered miRNAs were designated as miRNAs with a ≥2-fold change in expression. A total of 68 miRNAs, 5 upregulated miRNAs and 63 downregulated miRNAs, were selected for analyses (Fig. 6 and Table I). miR-205-3p expression was increased by 4.29-fold, whereas miR-513b, miR483-5p and miR-3648 expression was significantly decreased by 23.10-, 16.61- and 11.55-fold, respectively. Overall, our results demonstrate that troxerutin protects HaCaT cells from UVB-induced damage through the specific regulation of miRNA expression.

Analysis of putative target genes and GO analysis of troxerutin-specific miRNAs using bioinformatics. From the detection of global miRNA expression levels, our results identified 68 troxerutin-specific miRNAs. miRNAs regulate biological functions through the interference of their target genes (29). To identify biological functions of the miRNAs, we used TargetScan to predict putative target genes of troxerutin-specific miRNAs in UVB-exposed HaCaT cells. Using a seed sequence-based target prediction system (TargetScan), we analyzed the 5 miRNAs upregulated by troxerutin and identified 1,755 putative target genes. For the 63 downregulated miRNAs, 13,768 putative target genes were identified. We then analyzed the categorized functions, using GO analysis within the putative target pool. Following classification into GO categories, we selected significant GO categories using

Table I. Upregulated and downregulated miRNAs in UVB-exposed HaCaT cells pre-treated with troxerutin.

miRNA	Fold change	miRNA	Fold change	miRNA	Fold change
Upregulated miRNA					
hsa-miR-205-3p	4.29	hsa-miR-34b-5p	2.65	hsa-miR-181a-2-3p	2.00
hsa-miR-21-3p	2.97	hsa-miR-125a-5p	2.62		
Downregulated miRNA					
hsa-miR-513b	-23.10	hsa-miR-630	-4.88	hsa-miR-3141	-3.15
hsa-miR-483-5p	-16.61	hsa-miR-188-5p	-4.83	hsa-miR-1915-3p	-3.08
hsa-miR-3648	-11.55	hsa-miR-1202	-4.78	hsa-miR-1181	-3.02
hsa-miR-513a-5p	-9.66	hsa-miR-1224-5p	-4.66	hsa-miR-940	-2.98
hsa-miR-135a-3p	-9.49	hsa-miR-4299	-4.58	hsa-miR-320c	-2.97
hsa-miR-572	-9.26	hsa-miR-1207-5p	-4.45	hsa-miR-324-3p	-2.96
hsa-miR-575	-8.01	hsa-miR-3663-3p	-4.44	hsa-miR-3156-5p	-2.89
hsa-miR-4327	-7.32	hsa-miR-3162-5p	-4.35	hsa-miR-1288	-2.87
hsa-miR-874	-7.15	hsa-miR-212-3p	-4.14	hsa-miR-1305	-2.86
hsa-miR-638	-6.68	hsa-miR-3198	-4.08	hsa-miR-1973	-2.78
hsa-miR-4270	-6.44	hsa-miR-642b-3p	-3.96	hsa-miR-155-5p	-2.76
hsa-miR-1246	-6.27	hsa-miR-150-3p	-3.90	hsa-miR-28-5p	-2.59
hsa-miR-3679-5p	-5.78	hsa-miR-1914-3p	-3.77	hsa-miR-452-5p	-2.54
hsa-miR-1225-5p	-5.64	hsa-miR-762	-3.66	hsa-miR-378a-5p	-2.48
hsa-miR-3656	-5.60	hsa-miR-3196	-3.62	hsa-miR-892b	-2.47
hsa-miR-1275	-5.59	hsa-miR-3125	-3.43	hsa-miR-4298	-2.40
hsa-miR-494	-5.53	hsa-miR-1268a	-3.30	hsa-miR-3127-5p	-2.38
hsa-miR-2861	-5.44	hsa-miR-9-3p	-3.28	hsa-miR-939-5p	-2.33
hsa-miR-134	-5.18	hsa-miR-629-3p	-3.21	hsa-miR-29a-3p	-2.26
hsa-miR-4271	-5.03	hsa-miR-4281	-3.21	hsa-miR-584-5p	-2.16
hsa-miR-125a-3p	-4.91	hsa-miR-3665	-3.15	hsa-miR-3659	-2.08

miRNAs, microRNAs; UVB, ultraviolet B.

Table II. GO analysis of potential target genes of troxerutin-regulated miRNAs.

Accession no.	GO term	Count	Percentage (%)	p-value
GO:0045449	Regulation of transcription	2,050	15.01	4.83E-18
GO:0007242	Intracellular signaling cascade	1,021	7.48	6.80E-15
GO:0006793	Phosphate metabolic processes	792	5.80	6.75E-12
GO:0008104	Protein localization	712	5.21	1.74E-09
GO:0043067	Regulation of programmed cell death	653	4.78	2.87E-08
GO:0042127	Regulation of cell proliferation	642	4.70	4.15E-10
GO:0007049	Cell cycle regulation	611	4.47	1.69E-05
GO:0006811	Ion transport	603	4.42	3.54E-05

GO, gene ontology; miRNAs, microRNAs.

parameters, such as a count of >600, a percentage (%) of >4% and a p-value <0.01. Our analyses revealed the following distribution of biological processes: regulation of transcription (2,050; number of GO term-related genes in the putative target pool), intracellular signaling cascades (1,021), phosphate metabolic processes (792), protein localization (712), regulation of programmed cell death (653), regulation of cell proliferation (642), cell cycle (611) and ion transport (603) (Table II).

The aforementioned effects of troxerutin were further grouped into four cell functional groups: cell death and apoptosis, cell proliferation and the cell cycle, migration and DNA repair. These groups included the following: cell death and apoptosis, 16 GO terms; cell proliferation and cell cycle, 19 GO terms; migration, 8 GO terms; and DNA repair, 2 GO terms (Table III). Overall, these results demonstrated that troxerutin exerted protective effects against UVB-induced damage,

Table III. Groupring of GO terms into four cell functional groups reflecting the effects of troxerutin.

Accession no.	GO term	Count	Percentage (%)	p-value
Cell death and apoptosis (16 GO terms)				
GO:0043067	Regulation of programmed cell death	653	4.78	2.87E-08
GO:0010941	Regulation of cell death	653	4.78	8.51E-08
GO:0042981	Regulation of apoptosis	647	4.74	2.75E-08
GO:0016265	Death	569	4.17	4.95E-05
GO:0008219	Cell death	564	4.13	7.79E-05
GO:0012501	Programmed cell death	474	3.47	1.65E-03
GO:0006915	Apoptosis	466	3.41	2.44E-03
GO:0043068	Positive regulation of programmed cell death	344	2.52	4.31E-04
GO:0010942	Positive regulation of cell death	344	2.52	7.94E-04
GO:0043065	Positive regulation of apoptosis	341	2.50	5.82E-04
GO:0043069	Negative regulation of programmed cell death	289	2.12	2.55E-04
GO:0060548	Negative regulation of cell death	289	2.12	3.66E-04
GO:0012502	Induction of programmed cell death	251	1.84	1.16E-02
GO:0006917	Induction of apoptosis	250	1.83	1.27E-02
GO:0006916	Anti-apoptosis	162	1.19	3.08E-02
GO:0043524	Negative regulation of neuron apoptosis	49	0.36	8.44E-05
Cell proliferation and cell cycle (19 GO terms)				
GO:0042127	Regulation of cell proliferation	642	4.70	4.15E-10
GO:0007049	Cell cycle	611	4.47	1.69E-05
GO:0022402	Cell cycle process	442	3.24	7.41E-04
GO:0008283	Cell proliferation	345	2.53	7.21E-04
GO:0008284	Positive regulation of cell proliferation	336	2.46	2.11E-05
GO:0022403	Cell cycle phase	331	2.42	2.36E-04
GO:0008285	Negative regulation of cell proliferation	292	2.14	1.24E-04
GO:0040008	Regulation of growth	265	1.94	1.61E-02
GO:0000279	M phase	260	1.90	3.88E-03
GO:0051726	Regulation of cell cycle	260	1.90	6.85E-03
GO:0000087	M phase of mitotic cell cycle	173	1.27	6.99E-02
GO:0001558	Regulation of cell growth	151	1.11	6.26E-02
GO:0010564	Regulation of cell cycle process	96	0.70	3.86E-03
GO:0007050	Cell cycle arrest	87	0.64	5.25E-03
GO:0051329	Interphase of mitotic cell cycle	85	0.62	1.91E-02
GO:0051327	M phase of meiotic cell cycle	78	0.57	9.53E-02
GO:0045927	Positive regulation of growth	66	0.48	4.23E-02
GO:0000082	G1/S transition of mitotic cell cycle	49	0.36	1.34E-02
GO:0045787	Positive regulation of cell cycle	47	0.34	9.65E-02
Migration (8 GO terms)				
GO:0048870	Cell motility	244	1.79	3.03E-03
GO:0016477	Cell migration	227	1.66	1.11E-04
GO:0042060	Wound healing	151	1.11	2.78E-02
GO:0030334	Regulation of cell migration	143	1.05	2.08E-04
GO:0030335	Positive regulation of cell migration	79	0.58	3.86E-04
GO:0030336	Negative regulation of cell migration	48	0.35	5.24E-02
GO:0010595	Positive regulation of endothelial cell migration	13	0.10	8.71E-02
GO:0002685	Regulation of leukocyte migration	19	0.14	5.47E-02
DNA repair (2 GO terms)				
GO:0051052	Regulation of DNA metabolic process	90	0.66	9.55E-02
GO:0051054	Positive regulation of DNA metabolic process	47	0.35	5.15E-02

GO, gene ontology.

which was related to an alteration in cellular miRNA expression profiles.

Discussion

In the skin, UVB radiation is a crucial inducer of apoptosis and growth arrest (5). In the epidermis, the accumulation of UVB-induced damaged keratinocytes results in sunburn, psoriasis and skin cancer (30). Therefore, protection from UVB-induced keratinocyte damage, including cell death, growth arrest, DNA damage and decreased migration, is essential in order to maintain epidermal homeostasis.

In the present study, we demonstrated that troxerutin protected cells from UVB-induced decrease in cell growth through the suppression of apoptosis. Pre-treatment with troxerutin protected the HaCaT cells from UVB-induced growth arrest (Fig. 2) by preventing apoptosis (Fig. 3). Also, migration assay results deonstrated that troxerutin increased cell migration activity (Fig. 4). In previous studies, troxerutin-induced DNA repair enhanced its protective effects against gamma radiation (28,30). Similarly, in the present study, DNA repair activity was increased by troxerutin in HaCaT cells (Fig. 5). In addition, using miRNA microarray analysis and bioinformatics tools, we identified specific troxerutin-induced miRNAs and found a link between troxerutin-induced miRNAs, as well as anti-apoptotic, enhanced migration and DNA repair effects. In UVB-exposed HaCaT cells, troxerutin altered the expression of 68 miRNAs, with a ≥2-fold up- or downregulation (Fig. 3).

In our study, in UVB-exposed HaCaT cells, miR-181a-5p was upregulated 2-fold by troxerutin. The miR-181a, a mature form of miR-181a-1, and miR-181a-2, have been implicated in proliferation, migration and invasion, and target *BIM*, a member of the apoptotic BCL-2 family (32,33). In the present study, in the downregulated miRNA group, miR-513a-5p was markedly decreased by 9.66-fold in troxerutin-treated HaCaT cells. This miRNA induces apoptosis by targeting the X-linked inhibitor of apoptosis (XIAP) in endothelial cells (34). miR-874 was also decreased by 7.15-fold in troxerutin-treated HaCaT cells and has been shown to contribute to cell proliferation through its effects on histone deacetylase 1 (35). miR-874 has been implicated in cell proliferation and migration by targeting aquaporin-3 (36). In this study, the expression levels of miR-1246 and miR-494 were significantly decreased in troxerutin-pre-treated HaCaT cells exposed to UVB. The expression of miR-1246 has been reported to be regulated by p53, a crucial transcription factor in the DNA damage response, and can repress the translation of dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (DYRK1A), an inducer of proliferation (37). miR-494 has been implicated in TRAIL-induced apoptosis through the repression of *BIM* (38). In addition, miR-494 regulates three pro-apoptotic proteins [PTEN, Rho-associated, coiled-coil containing protein kinase 1 (ROCK1) and Ca^{2+}/calmodulin-dependent protein kinase δ CaMKIIδ] and two anti-apoptotic proteins [fibroblast growth factor receptor 2 (FGFR2) and leukemia inhibitory factor (LIF)] (39). Therefore, our results suggest that troxerutin protects cells from UVB-induced DNA damage and apoptosis by regulating miRNA expression.

Furthermore, we predicted target genes of troxerutin-regulated miRNAs and analyzed the GO terms of potential target genes using DAVID bioinformatics resources. DAVID functional annotation suggested that the target genes of troxerutin-regulated miRNAs have a regulatory role in transcription, intracellular signaling cascades, phosphate metabolic processes, protein localization, programmed cell death, cell proliferation, the cell cycle and ion transport (Table II). Indeed, we grouped the GO terms into four functional groups induced by troxerutin in UVB-damaged cells: cell death and apoptosis, cell proliferation and the cell cycle, cell migration and DNA repair (Table III).

Overall, the current study provides evidence of the protective effects of troxerutin against UVB-induced damage in HaCaT cells. The present study demonstrates a significant correlation between the four cell function groups with alterations in miRNA expression, troxerutin-regulated miRNA function, and GO analysis of the miRNA target genes.

Acknowledgements

We would like to thank all other members of Coreana Cosmetics Co., Ltd. for their support. This study was supported by the KU Research Professor Program of Konkuk University and a grant from the Ministry of Science, ICT and Future Planning (no. 20110028646) of the Republic of Korea.

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