Modification of gene expression by melatonin in UVB-irradiated HaCaT keratinocyte cell lines using a cDNA microarray

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Abstract. Excessive ultraviolet B (UVB) irradiation causes apoptotic cell death or induction of tumors in skin. Melatonin is a promising antioxidant and direct radical scavenger. Recently, it was reported that melatonin increases the survival of ultraviolet-B (UVB)-irradiated HaCaT keratinocyte cell lines. However, the precise molecular mechanisms underlying protective effect of melatonin on UVB damage are largely unknown. In this study, to gain more insight into the molecular mechanisms involved in melatonin-induced cell survival on UVB-irradiated HaCaT keratinocytes, we performed cDNA microarray analysis. HaCaT keratinocytes were incubated without or with melatonin at 100 nm for 30 min prior to UVB irradiation at 100 mJ/cm^2, and total RNA was isolated. Our data showed that the expression of apoptosis regulator genes (apoptosis related protein-3, apoptotic chromatin condensation inducer in the nucleus), cancer related genes (tumor suppressor deleted in oral cancer-related 1), cell cycle regulator (cyclin-dependent kinase 2 interacting protein), enzymes (glutathione peroxidase 1, ubiquitin-conjugating enzyme E2M), and signal transducer genes [fibroblast growth factor (acidic) intracellular binding protein, transforming growth factor ß-stimulated protein TSC-22] were decreased by melatonin treatment in the UVB-irradiated HaCaT keratinocyte cell lines, compared to that of UVB-irradiated HaCaT cells without melatonin. Thus, findings of the present study demonstrate that melatonin modulates the expression of apoptosis related genes in UVB-irradiated HaCaT cells, resulting in increasing cell survival, thereby suggesting that melatonin may be used as a promising sunscreen substance to reduce cell death of keratinocytes after excessive UVB irradiation.

Introduction

Ultraviolet B (UVB) causes many important biological changes in skin, including erythema, sunburn, photoaging, apoptosis, and skin tumors (1-3). These cellular events are mediated through the activation or suppression of variety of genes following UVB irradiation (4). To elucidate the molecular mechanisms underlying these events, especially focused on skin carcinogenesis by UVB, several research groups have investigated the expression transcriptional profiles in UVB-irradiated HaCaT keratinocytes using cDNA microarray (5,6). Despite intensive investigations into the regulation of gene expression in keratinocytes, most of the precise molecular events remain to be elucidated.

Melatonin (N-acetyl-5-methoxy-tryptamine) is synthesized by pinealocytes and retinal photoreceptors on a cyclic pattern, with highest levels occurring at night, implying melatonin plays key roles in sleeping (7). Interestingly, melatonin has been reported to exhibit UV-protective effects through scavenging or reduction of free radical formation, especially quenching hydroxyl radicals (8-12). In addition, experimental data indicated that melatonin protects human keratinocytes from UVB irradiation by light absorption (13,14). Moreover, in clinical studies, melatonin was shown to have strong UV-protective effects, evidenced by suppression of UV-induced erythema of skin (15-17). We previously reported that melatonin reduces UVB-induced cell damage and polyamine levels in human skin fibroblasts (18). Furthermore, recently, it was reported that melatonin increases survival of HaCaT keratinocytes by suppressing UV-induced apoptosis (19). However, the molecular mechanisms underlying protective effects of melatonin on human keratinocytes and human fibroblasts upon UVB induced-apoptotic cell death are largely unknown.

To elucidate the protective roles of melatonin in skin upon UVB irradiation, especially focused on apoptotic pathway, we have investigated the gene expression profiles using cDNA microarray in UVB-irradiated HaCaT keratinocytes with or without melatonin.

Materials and methods

Materials. Melatonin was purchased from Sigma-Aldrich (St. Louis, MO). cDNA microarray chip was purchased from Genomictree (Daejeon, South Korea). A total of 840 clones was printed on an aminosilane-coated CMT GAPS II slide.

Cell culture. Human keratinocyte cell line, HaCaT cells, were maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO_2 in Eagle's minimum essential medium supplemented

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with 10% heat inactivated fetal bovine serum, 2 mM glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin. For experiments, cells ($5x10^4$ cells/ml) were seeded in a culture dish, and maintained in the tissue culture incubator. Cells were preincubated with melatonin at 100 nm for 30 min prior to UVB irradiation at 100 mJ/cm^2.

UVB irradiation. UVB was supplied by a closely spaced array of seven Westinghouse FS-40 sunlamps, which delivered uniform irradiation at a distance of 38 cm. The energy output of UVB (290-320 nm) at 38 cm was measured with a UVB photometer (IL1350 photometer, International Light, Newburyport, MA). Cells were exposed for 0 and 90 sec of UVB, corresponding to doses of 0 and 100 mJ/cm^2. To prevent light absorption by tissue-culture medium, the culture medium was removed just prior to irradiation and replaced with a thin layer of phosphate-buffered saline (PBS) to cover the cells. After UVB irradiation, cells were fed with fresh growth medium. Cells were harvested at 12 h using a cell scraper.

RNA preparation. Total RNA was isolated from cells using the RNAzol™ B (Biotecx Laboratories, Houston, TX) according to the manufacturer's instructions. The amount and purity of each RNA sample were determined by spectrophotometry at 260/280 nm.

Microarray analysis. For hybridization, 100 μg of total RNA was labelled with Cy3 or Cy5 monoreactive dyes (Amersham, Little Chalfont, UK) using a Superscript cDNA synthesis system (Gibco-BRL). Labelled Cy3 and Cy5 cDNA probes were cleaned with Qiaquick nucleotide removal kit (Qiagen). The purified probes were dried and resuspended in 40 μl of hybridization buffer containing 5X sodium chloride, sodium citrate buffer (SSC), 0.1% sodium dodecyl sulphate (SDS), 20 μg Cot-1 DNA (Gibco-BRL), 20 μg poly(A) RNA (Promega, Madison, WI), and 20 μg yeast tRNA (Gibco-BRL). Hybridization was performed at 42˚C for 16 h, and the microarray slide was washed once with 2X SSC, 0.1% SDS at 42˚C for 4 min, once with 0.1X SSC, 0.1% SDS at room temperature for 10 min, and three times with 0.1X SSC at room temperature for 1 min. Then, the microarray slide was washed with distilled water and spin-dried.

Microarray scanning and normalization of the data were performed using GenePix 4000B scanner and GenePix Pro 3.0 software (Axon Instruments, Union City, CA). Poor quality spots were discarded from the raw data before analysis. For each hybridized spot, background intensity was subtracted, and a ratio of Cy5 and Cy3 fluorescence intensity was assigned as gene expression value (GEV) for determining the relative gene expression level. The cDNA microarray experiments were performed twice, and the mean of two GEVs for each gene was used for further analysis.

Measurement of ROS generation. The generation of ROS was measured by a flow cytometry analysis using DCFH-DA as a substrate. Briefly, after treatment, cells were harvested, washed twice with PBS, and suspended in PBS ($1x10^6$ cells/ml). The cell suspension (500 μl) was placed in a tube, loaded with DCFH-DA to a final concentration of 20 μM, and incubated at 37˚C for 15 min. Cells were then irradiated with UVB and the ROS generation was assessed by the DCF fluorescence intensity (FL-1, 530 nm) from 10,000 cells with a FACS Caliber flow cytometer (Becton-Dickinson).

Measurement of caspase-3 activity. Cells were collected, washed in PBS, and resuspended in a buffer [25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothreitol, 2 mM PMSF, 1 μg/ml aprotonin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin]. Cell lysates were clarified by centrifugation at 12,000 rpm for 10 min, and 50 μg of protein of the supernatant was incubated with 50 μM Ac-DEVD-*p*Na colorimetric substrate at 37˚C for 1 h. The optical density was measured at 405 nm.

Results

Genes regulated by melatonin in UVB-treated HaCaT keratinocyte cell lines. HaCaT keratinocyte cell lines were cultured as a model system for investigating UVB-induced gene expression profiles with or without melatonin. The purpose of this microarray survey was to identify candidate genes in the UVB-irradiated HaCaT keratinocytes that may be modified directly or indirectly by melatonin. Fig. 1 shows the expression pattern of untreated HaCaT cells (Fig. 1A), UVB-treated HaCaT cells (Fig. 1B), and UVB-treated HaCaT cells in the presence of melatonin. The borderline for discriminating differential expression was set at ±2-fold. As expected, UVB irradiation resulted in robust changes in the gene expression profile (Fig. 1B). The gene expression profile was modified in UVB-treated HaCaT cells in the presence of melatonin (Fig. 1C).

UVB treatment (100 mJ/cm^2) resulted in induction or repression of specific genes in HaCaT keratinocyte cell lines 12 h after UVB treatment (Table I). The genes that showed at least a 2-fold change were selected. Genotoxic stress following UVB treatment is known to increase the transcription of certain genes including stress response, cancer related genes, inflammation, and apoptosis regulators. In this study apoptosis regulator genes (apoptosis related protein APR-3, apoptotic chromatin condensation inducer in the nucleus), cancer related genes especially in RAS oncogene family, and oxidative stress response gene (glutathione peroxidase 1) were increased in UVB-irradiated HaCaT keratinocyte cell lines. Interestingly, signal transducer genes such as fibroblast growth factor intra-cellular binding protein, insulin-like growth factor binding protein 6, insulin-like growth factor 2 receptor, and nerve growth factor receptor were increased in UVB-irradiated HaCaT keratinocyte cell lines. In the presence of melatonin, apoptosis regulator genes (apoptosis related protein APR-3, apoptotic chromatin condensation inducer in the nucleus), cancer related genes (tumor suppressor deleted in oral cancer-related 1), cell cycle regulator (cyclin-dependent kinase 2 interacting protein), enzymes (glutathione peroxidase 1, ubiquitin-conjugating enzyme E2M), and signal transducer genes [fibroblast growth factor (acidic) intra-cellular binding protein, transforming growth factor ß-stimulated protein TSC-22] are down-regulated in the UVB-irradiated HaCaT keratinocyte cell lines.

Effect of melatonin on apoptosis and generation of reactive oxygen species (ROS). In this study, expression levels of apoptosis regulator genes (apoptosis related protein APR-3,

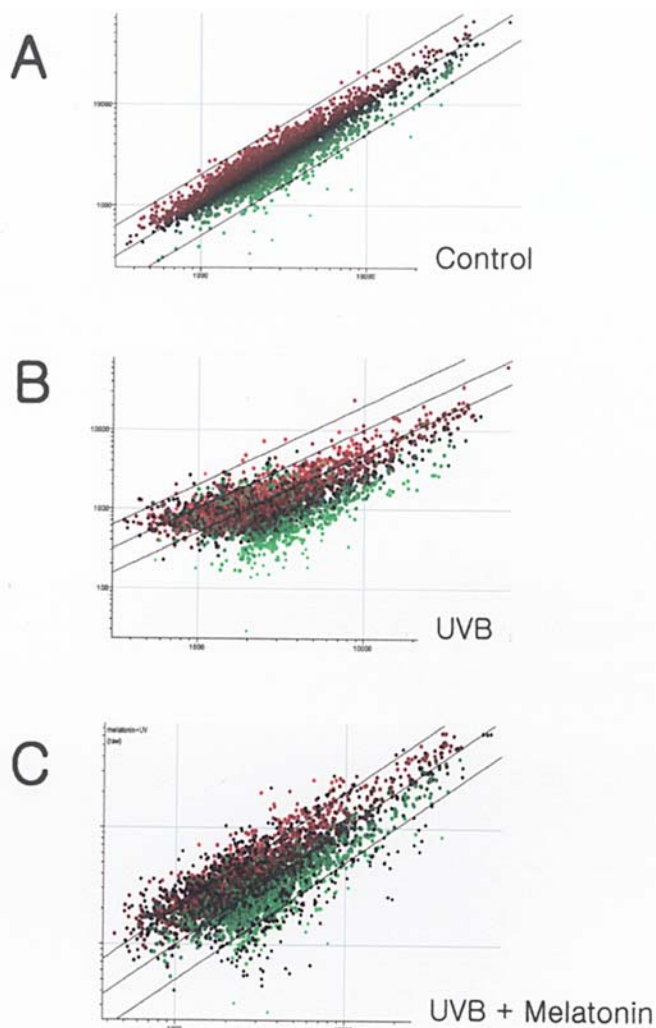

Figure 1. Analysis of the gene expression pattern in cells. The data are shown as log/log scatter plots of the expression values in untreated HaCaT cells (A), UVB-irradiated HaCaT cells without pretreatment of melatonin (B), and with pretreatment of melatonin (C).

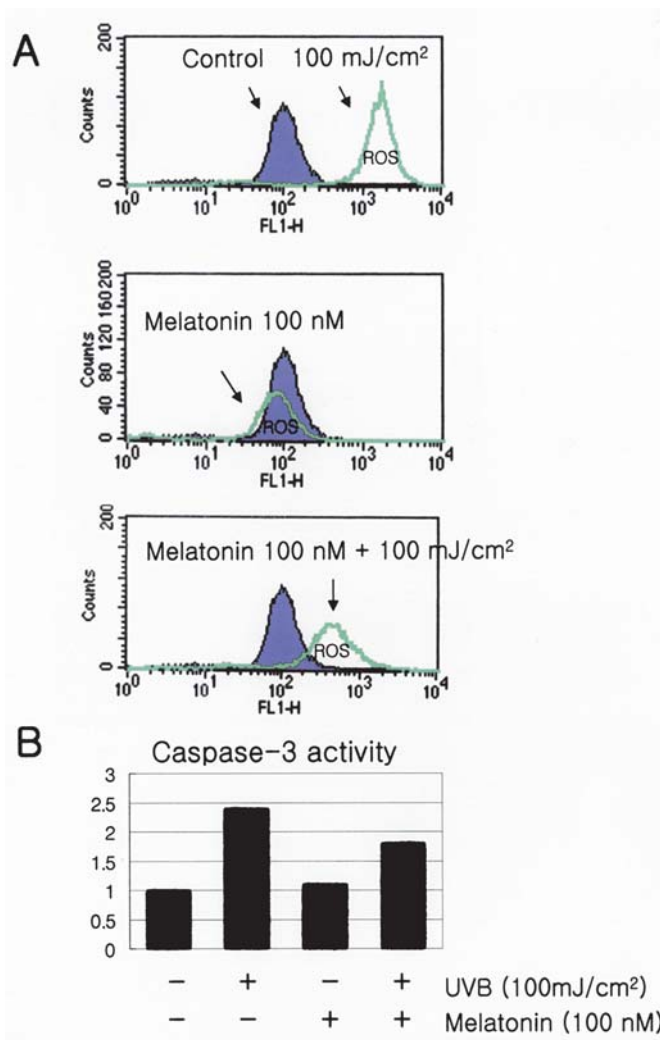

Figure 2. Inhibitory effects of melatonin on generation of ROS and caspase-3 activity in UVB-irradiated HaCaT cells. Measurement of ROS (A) and caspase-3 activity (B) was performed 24 h after UVB irradiation without or with melatonin pretreatment, respectively.

apoptotic chromatin condensation inducer in the nucleus) and enzymes (glutathione peroxidase 1) were decreased in melatonin-treated HaCaT cells after UVB irradiation compared to those of UVB-treated HaCaT cells without melatonin treatment. Down-regulation of these genes indicates that melatonin reduces the generation of ROS, thereby decreasing oxidative stress in UVB-irradiated HaCaT cells and, ultimately, reducing apoptotic cell death. To confirm the inhibitory effect of melatonin on ROS synthesis and apoptotic cell death, we measured the generation of ROS using DCFH-DA and activity of caspase-3 using Ac-DEVD-*p*Na synthetic peptide (Fig. 2). As shown in Fig. 2, generation of ROS (Fig. 2A) and caspase-3 activity (Fig. 2B) was attenuated in melatonin-treated HaCaT cells after UVB treatment, implying involvement of melatonin in attenuating apoptotic cell death through reducing generation of ROS.

Discussion

UVB irradiation causes damage at the molecular level to intra- and extracellular structure through formation of excessive amounts of free radicals (1-4). Melatonin protects cells from free radical damage by antioxidant properties; directly scavenging radicals and stimulating antioxidant enzymes (8,9,11-14). In this study, we investigated the genes responding to UVB irradiation of HaCaT keratinocytes using a cDNA microarray. Furthermore, to confirm the effect of melatonin on UVB-irradiated cells, the expression levels of genes were analyzed in melatonin-treated cells after UVB irradiation. UVB affects several biological processes, mainly being involved in the cell adhesion, signal transduction, proliferation, metabolism, apoptosis, and stress (3,4,20). In this study, our data showed that apoptosis regulator genes (apoptosis related protein APR-3, apoptotic chromatin condensation inducer in the nucleus), cancer related genes especially in RAS oncogene family, and oxidative stress response gene (glutathione peroxidase 1), signal transducer genes (fibroblast growth factor intracellular binding protein, insulin-like growth factor binding protein 6, insulin-like growth factor 2 receptor, nerve growth factor receptor) were increased in UVB-irradiated HaCaT keratinocyte cell lines.

The primary purpose of this microarray survey was to identify candidate genes, which are modulated by melatonin,

Table I. Genes differentially regulated by melatonin in UVB-irradiated HaCaT cells.

Genes (GeneBank accession number)	UVB only	UVB + melatonin
Apoptosis regulator		
1. Apoptosis related protein APR-3 (AA485898)	3.2	2.1
2. Apoptotic chromatin condensation inducer in the nucleus (H75862)	2.2	1.3
Cancer related genes		
1. Member RAS oncogene family, RAB34 (AA485449)	3.8	3.1
2. Tumor suppressor deleted in oral cancer-related 1 (AA457108)	3.3	2.1
3. Member RAS oncogene family (N93392 RAB25)	2.9	2.6
4. Member RAS oncogene family, RAB 1B (AA476287)	2.2	2.2
5. Member of RAS oncogene family, RAP 1A (AA279804)	0.3	0.4
6. FGFR1 oncogene partner (AI292342)	0.2	0.3
Cell cycle regulator		
1. Cyclin-dependent kinase 2 interacting protein (AI301694)	2.3	1.6
2. Cyclin-dependent kinase 4 (AA486208)	2.3	2.0
3. Cyclin L1 (AA465166)	0.3	0.3
4. Nuclear ubiquitous cyclin-dependent kinase substrate (AA137266)	0.3	0.3
Enzymes		
1. ATPase, H^+ transporting, lysosomal 14 kDa, V1 subunit (AA664077)	4.7	3.6
2. Cytochrome c oxidase subunit VIII (AA862813)	4.0	3.2
3. 3-Hydroxybutyrate dehydrogenase (heart, mitochondrial) (T67057)	3.8	2.4
4. Glutathione peroxidase 1 (AA485362)	3.6	2.8
5. Ubiquitin-conjugating enzyme E2M (AI971344)	3.5	2.3
6. Dermatan-4-sulfotransferase-1 (AA922082)	2.6	1.9
7. Cytochrome c oxidase subunit VIa polypeptide 1 (AA482243)	2.6	2.5
8. Glutathione S-transferase π (R33642)	2.3	2.2
9. PTK9 protein tyrosine kinase 9 (AA019459)	0.2	0.3
Signal transducer		
1. Fibroblast growth factor (acidic) intracellular binding protein (AA490046)	3.0	2.4
2. TNFAIP3 interacting protein 1 (T64483)	2.6	2.0
3. Insulin-like growth factor binding protein 6 (AA478724)	2.5	2.5
4. Transforming growth factor ß-stimulated protein TSC-22 (AA664389)	2.3	1.6
5. Insulin-like growth factor 2 receptor (T62547)	2.2	1.8
6. Nerve growth factor receptor (TNFR superfamily, member 16) (R55303)	2.2	1.8
7. Pentaxin-related gene, rapidly induced by IL-1ß (W48562)	2.2	1.7
8. Ephrin-A1 (AA857015)	2.1	1.5
9. TRAF and TNF receptor associated protein (AA488650)	0.4	0.7
10. EGF-containing fibulin-like extracellular matrix protein 1 (AA875933)	0.4	0.3
11. Phosphofructokinase, platelet (AI340203)	0.29	0.39
12. Dishevelled associated activator of morphogenesis 1 (AA487243)	0.3	0.4
13. FGFR1 oncogene partner (AI292342)	0.2	0.3
Structural protein		
1. Matrix Gla protein (AA155913)	3.1	1.7
2. EGF-containing fibulin-like extracellular matrix protein 1 (AA875933)	0.4	0.4

in UVB-irradiated HaCaT cells. Our data showed that the expressions of apoptosis regulator genes (apoptosis related protein-3, apoptotic chromatin condensation inducer in the nucleus), cancer related genes (tumor suppressor deleted in oral cancer-related 1), cell cycle regulator (cyclin-dependent kinase 2 interacting protein), enzymes (glutathione peroxidase 1,

ubiquitin-conjugating enzyme E2M), and signal transducer genes [fibroblast growth factor (acidic) intracellular binding protein, transforming growth factor ß-stimulated protein TSC-22] were decreased by melatonin treatment in the UVB-irradiated HaCaT keratinocyte cell lines, compared to that of UVB-irradiated HaCaT cells without melatonin.

Up-regulation of apoptosis related protein-3 is observed in apoptotic cells, implying functions of this gene relating to apoptosis (21). The nuclear events occurring during apoptosis include changes at the molecular level (i.e., DNA cleavage, modifications of nuclear polypeptides, and proteolysis of several proteins important for cell maintenance), and, consequently, alterations at the morphological level (i.e., chromatin condensation, nuclear shrinkage, DNA fragmentation and apoptotic body formation) may be involved in these processes after UVB irradiation (22). Accumulating data indicate that melatonin attenuates the ROS-induced apoptotic cell death through antioxidant activities (13,15,18,19). In this study, melatonin reduced the generation of ROS in UVB-irradiated HaCaT cells. Thus, expressions of apoptosis related protein-3 and apoptotic chromatin condensation inducer in the nucleus may be decreased by pretreatment of melatonin in UVB-irradiated HaCaT cells through direct or indirect inhibition of ROS generation. In addition, melatonin has been reported to act also directly as an antiapoptotic substance by inhibiting the apoptotic pathway in mitochondria at different levels (23-25). In this study, caspase-3 activity was attenuated by pretreatment of melatonin in UVB-irradiated HaCaT cells, compared to that of UVB-irradiated HaCaT cells without melatonin treatment. Furthermore, to elucidate melatonin-induced gene modification in HaCaT cells concerning resistant to apoptosis, we cultured HaCaT keratinocytes in cultured media with melatonin for 3 days, and performed the cDNA microarray analysis in melatonin-treated HaCaT keratinocytes. Unfortunately, we did not obtain useful data relating to antiapoptosis or ROS scavenger activity of melatonin in melatonin treated-HaCaT cells (data not shown).

Antioxidant enzymes, such as superoxide dismutase 1 and glutathione peroxidase 1, play key roles in attenuating genotoxic damage upon UVB irradiation (26,27). In this study, consistent with reducing ROS level in melatonin-treated HaCaT cells after UVB irradiation, expression of glutathione peroxidase 1 was decreased by melatonin pretreatment in UVB-irradiated HaCaT cells.

In conclusion, findings of the present study demonstrate that melatonin inhibits the expression of apoptosis related protein-3, apoptotic chromatin condensation inducer in the nucleus, and glutathione peroxidase 1 in UVB-irradiated HaCaT cells, and indicate that these inhibitory effect of melatonin upon UVB irradiation is likely to be, at least in part, associated with antioxidant capacity of melatonin, thereby suggesting that melatonin may be used as a promising sunscreen substance.

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