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Melatonin reduces ultraviolet-B induced cell damages and polyamine levels in human skin fibroblasts in culture

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ODC, ornithine decarboxylase

Abstract

UV radiation is known to cause photoaging of the skin and is considered one of the leading cause of developing skin carcinogenesis. Melatonin which has a highly lipophilic molecular structure facilitating penetration of cell membranes and serving as an extra- and intracellular free radical scavenger has been demonstrated to protect photodamage of skin affected by UV exposure. In this study, we have examined the role of melatonin in response to UVB induced photodamaging process, using human skin fibroblasts *in vitro*. Cell survival curves after UVB irradiation showed dose-dependent decrease. Only 60% of fibroblasts were survived at 140 mJ/cm^2 UVB irradiation. By pre-cultivation of cells with melatonin (100 nM), a significant number of cells remained unaffected. After UVB irradiation with 70 mJ/cm^2, the level of putrescine was 1.7±0.3 fold increased compared to melatonin pre-treated group. In Northern analyses, the transcriptional level of ornithine decarboxylase (ODC) gene expression was increased by UVB irradiation and prohibited by melatonin. These results indicated that melatonin was effectively able to neutralize membrane peroxidation when present in relevant concentration during UVB irradiation and diminishes the UVB-induced increase of polyamine synthesis and ODC gene expression. Collectively, ODC response to UVB induced changes are possibly involves a melatonin or antioxidant sensitive regulatory pathway in normal human skin fibroblast.

Keywords: antioxidants; cell death; melatonin; ornithine decarboxylase; polyamine; ultraviolet rays

Introduction

Exposure to UV results in photoaging of the skin and is considered an initiating event of skin carcinogenesis (Chainiaux *et al*., 2002). UVB radiation not only causes inducing DNA damage and cell mutagenecity, but can also modulate the expression of several genes at dose levels to natural solar exposure (Shindo and Hashimoto, 1998). Some of these genes are regulated by an oxidative mechanism (Lee *et al*., 1998; Takeda *et al*., 2002). It has been reported that the effect of UVB radiation on the *in vitro* regulation of important enzymes that participate in inflammation and cancer progression (Kulms *et al*., 2002). The polyamines, (putrescine, spermidine, and spermine) have been shown to be essential for mammalian cell growth and function (Ahmad *et al*., 2001). Intracellular polyamine concentrations are highly regulated by the enzyme, ornithine decarboxylase (ODC), which catalyzes the conversion of ornithine to putrescine, the initial and often rate limiting step in polyamine biosynthesis (van Weelden *et al*., 1990; O'Brien *et al*., 1997). UVB light induces overexpression of ODC an enzyme that plays a critical role in photocarcinogenesis (Jansen *et al*., 2001). Therefore, the ODC activity served as a marker of the mutagenic and carcinogenic effects of UV light, because the activity of ODC in quiescent cells is extremely low and readily induced by UV light (Manzow *et al*., 2000). Furthermore, ODC is also activated by many other stimuli including tumor promoters and growth factors (Oguro and Yoshida, 2001), as well. Melatonin (N-acetyl-5-methoxytrptamine) is a hormone with multiple functions in human, produced by the pineal gland and stimulated by beta-adrenergic receptors (Ryoo *et al*., 2001). Areas of greater interest and potential importance include the antimitotic effects of melatonin on some types of tumor cells in culture and potent radical scavenging effects, but there has been little progress toward identifying the specific mechanisms

of its action. In the present study, we investigated whether the photoprotective actions of melatonin can attenuate the changes of polyamine levels following UVB induced cell damages in cultured skin fibroblasts.

Materials and Methods

Fibroblast culture

Primary cultures of dermal fibroblasts were established from children (n = 3, mean age = 5 yr) skin left over from cosmetic surgery and cultured on plastic culture dishes in DMEM supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 g/ml), and amphotericin B (1 g/ml). The cells were maintained in a humidified 5% CO_2, 95% air incubator at 37°C. Analyses of confluent fibroblast cultures were carried out at 3-6 passages of subcultivation, 1×10^5/ml. Cell viability was determined by trypan blue exclusion.

UVB irradiation

Immediately before irradiation, the medium was replaced by PBS. UVB was supplied by a closely spaced array of seven Westinghouse FS-40 sunlamps, which delivered uniform irradiation at a distance of 30 cm. The energy output of UVB (290-320 nm) was measured with a UVB photometer (IL 1350 photometer, International Light, Mass). The output of the FS-40 sunlamps was 0.1 mW/cm^2 at 38 cm. UVB irradiation doses were 0, 50, 70, 140, and 200 mJ/cm^2.

Melatonin treatment

Melatonin was purchased from Sigma (St Louis, MO) and dissolved in 95% ethanol. We treated cultured fibroblasts with 10-1,000 nM melatonin 3 min before UV irradiation and after UV irradiation for 24 h.

Propidium iodide staining

After 2 or 5 days of culture, the cells were fixed with 4% paraformaldehyde in 0.1% PBS (pH 7.4) for 10 min at room temperature, followed by 70% ethanol containing 1% HCl for 10 min at -20°C, and then stained with 1 g/ml propidium iodide containing 100 g/ml DNAase free RNase A for 30 min at 37°C to visualize the nuclei. The cells were examined with a confocal laser scanning microscopy. More than 100 cells in several microscopic fields were counted under the microscope to determine the percentage of apoptotic cells.

Polyamine extraction and HPLC analysis

The extraction procedure was carried out in ice-chilled conditions. Derivation and HPLC analysis of polyamines were based upon the methods of Spragg and Hutchings (1983) with some modification. Each cultured cells were lysed in 10 volumes of ice-chilled 0.4 M perchloric acid containing 2 mM EDTA and 40 M 1,8-diaminooctane as an internal standard. And centrifuged at 15,000 g for 10 min, at 4°C. 200 l of the supernatant was evaporated by a vacuum drier. The dried samples were dissolved in 100 l of 1 M sodium bicarbonate then derived with 300 l of 4-fluoro-3-nitrobenzo-trifluoride (FNBT) reagent (a mixture of 10 l of FNBT and 1 ml of DMSO at 60°C for 20 min. At the end of derivation, 40 l of 1 M histidine in 1 M sodium bicarbonate was added to the reaction mixture then the derivation continued for another 5 min to scavenge excess FNBT. After cooling the mixture in an ice basket, the N-2-nitro-4-trifluoromethylphenyl derivatives of polyamines were extracted twice with 2 ml of 2-methylbutane. After centrifugation at 3,000 g for 10 min, the organic phase was evaporated under nitrogen gas flow and the residue was reconstituted with 1 ml of HPLC grade methanol. The 20 l of the methanol solution was applied to the isocratic reversed phase HPLC system (Gilson Medical Electronics, Villiers-le-Bel, France), then the separation of NTP-polyamines was accomplished by elution of acetonitrile-water (85:15, v/v) mobile phase at the flow rate of 1.0 ml/min within 30 min. The eluent was monitored by UV/VIS detector set at 242 nm and a MicrosorbTM C18 column (5 M, 4.6 mm, 25 cm, Rainin instrument Co., Woburn, Mass) was used.

cDNA probe preparation

The following human-sequence-specific cDNAs were utilized in this study: a 3.2 kb cDNA for ODC; a 1.2 kb cDNA for GAPDH. The cDNAs were labeled with [^{32}P]-dCTP (NEG 036H, New England Nuclear, USA) by nick translation (Rigby *et al*., 1977) to a specific activity of approximately 1×10^8 cpm/ g.

Quantitation of mRNA levels by Northern blot analysis

Total RNA was isolated by the methods of Chomzynski and Sacchi (1987) from cultured normal skin fibroblasts. Total RNA (20 g) was fractionated by 1% agarose gel electrophoresis (85 V, 5 h) after denaturating the samples with formaldehyde and formamide (Wahl *et al*., 1979). RNA transcripts obtained were transferred to the charged nylon filter (Zeta-probe, BioRad, CA) in 20×SSC overnight at 4°C (Theiss *et al*., 2002). The filters was prehybridized for

12-18 h at 42°C with prehybridization mixture (50% formamide, 0.1% SDS, 3×SSC, 1×Denhart's solution, 50 g/ml ss-DNA) and hybridized with [^{32}P]-labeled cDNA by nick translation at 42°C for 24 to 36 h. Following hybridization, the filters were washed and autoradiography was performed.

Results

UVB induced cytotoxicity and melatonin defense

To examine the effect of UVB irradiation, cultured dermal fibroblasts were exposed to varying doses of UVB 50, 70, 140, and 200 mJ/cm^2 and checked their cell viability after 24 h later. Such treatment result in 80 ± 9.2%, 56 ± 6.9%, 43 ± 5.2%, 26 ± 5.8% of surviving cells, respectively (Figure 1). To clarify the effect of melatonin on dermal fibroblasts under UVB irradiation (140 mJ/cm^2), we treated with 10-1000 nM of melatonin. The protective effect was obtained from 1 M and 100 nM of melatonin, the cell viability in the pre-treated groups with 1 M and 100 nM of melatonin were 58% and 64.5%, respectively. The pre-treated groups with 100 nM of melatonin are significantly different from only UVB 140 mJ/cm^2 irradiated groups at $P < 0.01$ (Figure 2).

Apoptotic changes by UVB irradiation and melatonin defense

When cells were stained with propidium iodide and observed under confocal laser scanning microscopy, normal cells appear as homogenous fluorescent with oval nuclei. Using this staining method, we found that the UVB irradiated cells were irregular shaped with intensely fluorescent and contained fragmented round bodies characteristics of apoptotic cells after 24 h after treatment (Figure 3). The degree of apoptosis induced by UVB irradiation and the protective effects of melatonin were measured by counting the number of apoptotic cells among 10 high power field (×400). More apoptotic cells were observed in UVB irradiated group than melatonin treated group. The percentage of apoptotic cells in UVB treated group was 35.1 ± 8.21%, whereas those in melatonin protected group was 10.4 ± 5.95%. In control group, less than 2% of attached cells showed apoptotic features.

Effects of UVB and melatonin on polyamine level

The changes in polyamine level were examined 24 h after UVB and melatonin treatment by HPLC analysis. The level of putrescine of UVB irradiated

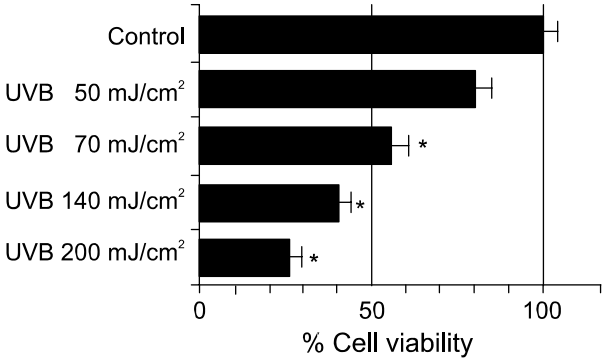

Figure 1. Viability of the dermal fibroblast following UVB treatment. The cells were treated with UVB (50, 70, 140 and 200 mJ/cm^2) and the number of viable cells was countered by trypan blue exclusion assay. Data shown are mean values ±standard errors determined from three independent experiments. *$P < 0.01$.

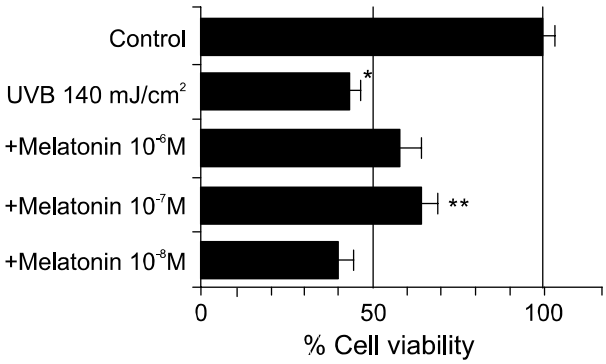

Figure 2. The effects of melatonin on cultured skin fibroblast viability under UVB irradiation (140 mJ/cm^2). Cell viability is represent as percent viable cells, where the untreated cells are regarded as 100%. *Significantly different from control and **significantly different from only UVB 140 mJ/cm^2 irradiated groups at $P < 0.01$.

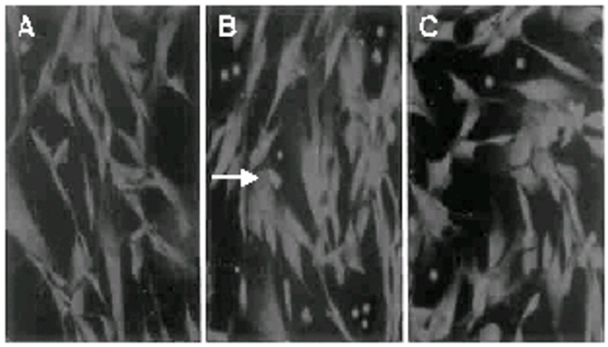

Figure 3. Detection of apoptotic cells by propidium iodide among adherent dermal fibroblasts treated with UVB and/or melatonin. A: untreated control, B: UVB (140 mJ/cm^2) irradiation, C: cells pretreated with melatonin before UVB irradiation and incubated for 24 h in DMEM containing melatonin (10^{-7} M). Apoptotic cells appear in B as condensed, brightly fluorescent, oval particles (arrow) that are easily distinguished from the controls (A) and these changes were moderately protected by melatonin (C).

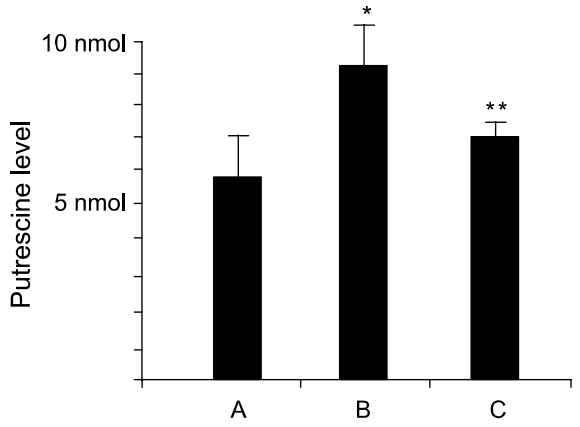

Figure 4. Quantitation of putrescine levels under UVB and melatonin by HPLC. A, control; B, UVB 140 mJ/cm^2; C, UVB 140 mJ/cm^2 + melatonin 10^{-7} M. Data are expressed as mean ± SD of three independent experiments. *Significantly different from control and **significantly different from only UVB 140 mJ/cm^2 irradiated groups at $P < 0.01$.

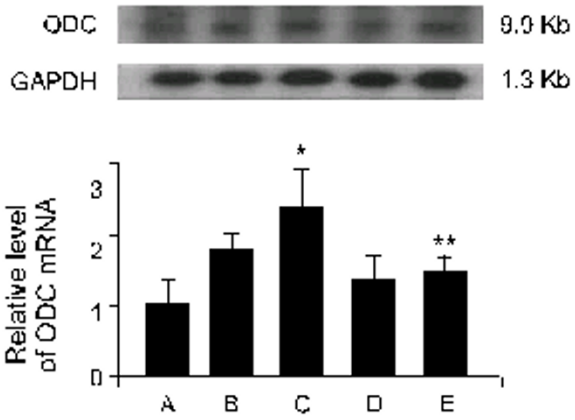

Figure 5. Quantitation of ODC gene expression by northern blot analyses, where the untreated cells are regarded as 100 densitometric absorbance unit (DAU). A, Control; B, C, UVB 70/140 mJ/cm^2; D, E, UVB 70/140 mJ/cm^2 + melatonin 10^{-7} M. Data are expressed as mean ± SD of three independent experiments. *Significantly different from control and **significantly different from only UVB 140 mJ/cm^2 irradiated groups at $P < 0.01$.

group was 9.0±0.79 nmol per ml which was increased compared to the untreated control (5.2±0.44 nmol). When pre-treated with melatonin before UVB irradiation, the level of putrescine was 6.9±0.55 nmol (Figure 4).

Expression of ODC gene

In northern blot analysis of cultured normal skin fibroblasts, [^{32}P]-labeled ODC and GAPDH cDNA probes specifically hybridized with each mRNA. ODC revealed 9.0 kb sized mRNA transcript, while GAPDH revealed 1.3 kb. There were no changes in size, indicating no alteration in quality. Northern analysis with cDNA probe for ODC after UVB (70, 140 mJ/cm^2) irradiation resulted in a signal markedly increased (by about 1.7±0.07, 2.2±0.79 folds respectively) compared to control, after correction of the signal by GAPDH mRNA levels. And these over-expressions were decreased up to 1.4±0.06, and 1.45 ±0.02 folds under melatonin pretreatment at concentration of 100 nM compared to control. This means melatonin pretreatment decreased UVB induced ODC mRNA levels up to 35% (Figure 5). Relative quantitation of ODC mRNA levels were measured, where the untreated cells are regarded as 100 densitometric absorbance units (Table 1).

Table 1. Steady-state levels of ODC mRNA.

Control	49 ± 9 (1)
UVB 70 mJ/cm^2	83 ± 8 (1.7)
UVB 140 mJ/cm^2	110 ± 11 (2.25)
UVB 70 mJ + Melatonin 10^{-7} M	68 ± 9 (1.4)
UVB 140 mJ + Melatonin 10^{-7} M	71 ± 6 (1.45)

The values are mean ± SD and expressed as densitometric absorbance unit which are the percentage of the value of GAPDH. Fold difference is shown in parethesis, *Significantly different from control and **significantly different from only UVB 140 mJ/cm^2 irradiated groups at $P < 0.01$

Discussion

Polyamines are present in living cells and play a pivotal role in cellular growth and developmental process (Theiss *et al*., 2002). The naturally occurring polyamines in mammamlian cells are putrescine, spermidine, and spermine (Megosh *et al*., 2002). ODC plays a critical role in the biosynthesis of those polyamines (Rebel *et al*., 2002). Actually, the level of ODC activity in quiescent cells is extremely low, but readily induced by a wide variety of growth-promoting agents or UV light (Nilsson *et al*., 2000; Tanaka *et al*., 2001). Also rapidly proliferating cells and neoplastic cells express elevated levels of ODC, therefore the level of ODC activity is served as a marker of the initial mutagenic and carcinogenic process. Ultraviolet radiation activates the expression of a wide variety of genes by pathways which differ between the short non-solar UVC wavelengths, which are strongly absorbed by nucleic acids, and the long solar UVA (320-380 nm) wavelengths, which generate active oxygen intermediates. Intermediate solar UV wavelengths in the UVB (290-320 nm) range also contain

an oxidative component, but also closely resemble UVC in their gene activating properties. Short wavelength UV, in common with other extracellular stimuli including growth factors, activates signal transduction events that involve both stress- and mitogen-activated protein kinase cascades (Reeve *et al*., 2000; Soriani *et al*., 2000; Tyrrell, 2000; Kim *et al*., 2001). This study describes the protective effects of melatonin in skin fibroblast against the UVB induced apoptotic changes. UVB induced cytotoxicity was examined 24 h after treatment by trypan blue exclusion test. A distinct dose response relationship was observed between the dose of UVB and the degree of survival rate. About 26% of cultured dermal fibroblasts were only survived at 200 mJ/cm^2. The UVB induced cytotoxicity is a rapid event, and melatonin prevents such damage when present at concentration of 1 nM (Fischer *et al*., 1999). In our study, the protective effect of melatonin was observed at concentration of 1 M and 100 nM. This discrepancy may result from different proctocol (*in vivo* vs *in vitro*). Moreover, melatonin treated cells showed marked suppression of UVB induced apoptotic changes. Like the other methods, this one also has some limitations in that it does not include all the floating cells. Therefore, these values are an underestimation of the actual degree of apoptosis. It was our interest to know whether the photoprotective actions of melatonin can attenuate the changes of polyamine levels and ODC activities following UVB irradiation in cultured skin fibroblasts. Putrescine is the first product from ornithine by the action of ODC and known as a marker of cell injury. The level of putrescine of UVB irradiated group was 1.7 times increased compared to the untreated control. When pre-treated with melatonin before UVB irradiation, the level of putrescine was decreased up to 75%. The expression of ODC gene after UVB irradiation resulted in markedly increased with dose dependent fashion. This result is supported by other previous reports (Bornman *et al*., 1999; Soriani *et al*., 1999). And these overexpressions were decreased up to 65% under melatonin pretreatment at concentration of 100 nM. The suppressive effect of melatonin to increased ODC mRNA can be resulted from increasing turn-over time of mRNA or decreasing transcription. Additionally, that effect of melatonin may be related with role of melatonin as antioxidant or not. Therefore, to elucidate the mechanisms of action of melatonin to polyamine and ODC gene, further experiments are needed. In conclusion, our results demonstrate that melatonin not only directly acts as a protector from cell damage induced by UVB irradiation only when present in relevant concentration at the site of action beginning but diminishes the UVB induced increase of polyamine synthesis and ODC gene expression. Collectively, ODC response to UVB induced changes are possibly involves melatonin sensitive regulatory pathway in normal human skin fibroblast.

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