The Enhanced Monocyte Adhesiveness after UVB Exposure Requires ROS and NF-κB Signaling in Human Keratinocyte

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Introduction

The skin is the primary organ that functions as a physical barrier as well as an active immune organ. The human skin consists of various epidermal and dermal cells including fibroblasts, keratinocytes, melanocytes, Langerhans cells etc. UV radiation in particular UVB (290-320 nm) from sunlight is one of the major external stimuli that cause inflammatory skin disease, photoaging, cancer (Katiyar et al., 1999). UVB has been shown to exert both antiinflammatory effects and proinflammatory effects on human skin, depending on the context of stimuli or the duration of exposure (Norris et al., 1990; Krutmann and Grewe, 1995). Previous studies demonstrated that UVB inhibited tumor necrosis factor (TNF)-α- or interferon (IFN)-γ-induced up-regulation of intercellular adhesion molecule-1 (ICAM-1) expression by keratinocyte (Krutmann et al., 1990; Krutmann et al., 1992). UVB selectively upregulates ICAM-1 in human keratinocyte and human dermal microvascular endothelial cells (Norris et al., 1990; Cornelius et al., 1994), indicating a proinflammatory activity of UVB.

UVB-induced infiltration of leukocytes (macrophage/neutrophils) into the skin is an important step in the development of skin inflammatory diseases. The increased trafficking of leukocytes into the skin has been described in the epidermis as well as the dermis of normal human skin after UVB exposure (Katiyar et al., 1999; Di Nuzzo et al., 2000). Induction of ICAM-1 on the surface of keratinocytes contributes to infiltration of leukocytes into the skin. Increased expression of ICAM-1 on the surface of epidermal keratinocytes as well as dermal microvascular endothelial cells has been proposed as an important regulator in the immunologic skin reactions (Cornelius et al., 1994). The
and epidermal keratinocyte, the major cell type in the skin, can express ICAM-1 in response to cytokines such as TNF-α, IL-1β, and IFN-γ as well as UV radiation (Knutmann et al., 1990; Knutmann et al., 1992).

UVB irradiation has been shown to induce a transient increase in the intracellular level of reactive oxygen species (ROS) in keratinocyte as well as ex vivo human skin (Hanson et al., 2002; Baek et al., 1997). Increased levels of ROS within the epidermis and the dermis can induce cellular damage that leads to various skin diseases, including immunomodulation, photocarcinogenesis, and skin aging that leads to various skin diseases, including immunosuppression (Katiyar et al., 1990; Brenneisen et al., 2002).

While it has been reported that UVB radiation may generate ROS, the possible roles of ROS involved in UVB-induced ICAM-1 expression have not been studied. In the present study, we show that ROS generated by UVB radiation is involved in the induction of ICAM-1 expression and subsequent adhesion of monocytic cells to keratinocyte through the NF-κB-dependent pathway.

Materials and Methods

Cell culture. The immortalized human keratinocyte cell line, HaCaT, was kindly provided by Professor N. Fusenig (German Cancer Research Center). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin G (100 U/ml), streptomycin (100 µg/ml) at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. THP-1 human monocytic cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin G (100 U/ml), streptomycin (100 µg/ml), and L-glutamine (2 mM) as described previously (Park et al., 2003; Choi et al., 2004).

Reagents. Diphenyl iodonium (DPI), N-acetyl cysteine (NAC) were obtained from Sigma. MG-132 was purchased from Calbiochem. Dichlorodihydrofluorescein diacetate (DCF-DA) and calcein acetoxymethyl ester (calcein-AM) were purchased from Molecular Probes.

UVB irradiation. The HaCaT cells were washed with phosphate-buffered saline (PBS) and exposed to a UVB irradiation system for culture, BIO-SUN with a UV peak at 312 nm in a thin layer of buffered saline (PBS) and exposed to a UVB irradiation system for varying time periods (0-60 min). To examine the effect of NAC and DPI on UVB-induced ROS production, HaCaT cells were incubated in the absence or presence of these inhibitors for 1 h, and then exposed to UVB irradiation. Cells were washed twice with Hank's balanced salt solution (HBSS) and incubated with DCF-DA (2.5 mM) for 10 min. The cellular fluorescent images were obtained using a Zeiss Axiosvert S100 microscope with a confocal laser-scanning system (Bio-Rad MRC-1024ES) by using laser excitation at 494 nm as previously described (Park et al., 2004).

Western blot analysis. Cell lysates were prepared from HaCaT cells as previously described (Kim et al., 2004), and 15 µg of total protein was fractionated by electrophoresis on 10% SDS-polyacrylamide gel. The proteins were then transferred to nitrocellulose membranes and probed with polyclonal rabbit anti-human ICAM-1 (1 : 1000), or monoclonal rabbit anti-human actin (1 : 500). The membrane was then incubated with a goat anti-rabbit IgG antibody (1 : 750) conjugated to horseradish peroxidase. Enhanced chemiluminescence (ECL; Amersham) was used to detect bound antibody.

RT-PCR analysis. Total RNA was isolated from HaCaT cells using a TRIzol reagent kit (Gibco BRL) according to the manufacturer's instructions. Five micrograms of total RNA was reverse-transcribed with 10,000 U of reverse transcriptase and 0.5 g/L oligo(dT)15 primer (Bioneer). One microliter of the cDNA synthesis was subjected to the standard PCR for 38 cycles by using primers as: human ICAM-1, 5'-GGTGGACGCTGATGGGGGTCC-3' (sense) and 5'-GTGGTCATGTTGGGGCTATAGACTC-3' (antisense) (907 bp); and human β-actin, 5'-GCTCTCCCATGAGAAGATC-3' (sense) and 5'-CATCCACATCTGCTGGGA-3' (antisense) (228 bp) (Choi et al., 2004). PCR products were resolved on a 1% agarose gel and visualized with UV light after ethidium bromide.

Measurement of intracellular ROS levels. For analysis of intracellular ROS levels, the oxidation-sensitive fluorescent probe, DCF-DA was used as described previously (Gottlieb et al., 2000; Park et al., 2004). To measure the levels of ROS production, HaCaT cells were exposed to UVB irradiation and incubated for varying time periods (0-60 min). To examine the effect of NAC and DPI on UVB-induced ROS production, HaCaT cells were incubated in the absence or presence of these inhibitors for 1 h, and then exposed to UVB irradiation. Cells were washed twice with Hank's balanced salt solution (HBSS) and incubated with DCF-DA (2.5 mM) for 10 min. The cellular fluorescent images were obtained using a Zeiss Axiosvert S100 microscope with a confocal laser-scanning system (Bio-Rad MRC-1024ES) by using laser excitation at 494 nm as previously described (Park et al., 2004).

NF-κB protein localization. The NF-κB p65 nuclear localization was detected by indirect immunofluorescence assay using confocal microscopy as described previously (Kim et al., 2003). HaCaT cells grown on a glass slide in a six-well plate were exposed to UVB and incubated for the indicated time periods. HaCaT cells were washed with PBS and then fixed with 4% ice-cold formaldehyde for 30 min. Polyclonal antibody to human NF-κB p65 (1 : 30) was applied for 1 h followed by 1 h incubation with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1 : 200). The cells were then incubated for 1 h with a goat anti-human ICAM-1 antibody (1 : 500). The cells were then incubated in a chamber on the stage of a Zeiss Axiosvert S100 microscope and observed with a confocal laser-scanning system (Bio-Rad MRC-1024ES). The fluorescent images (excitation 494 nm, emission 518 nm) of the cells were recorded every 0.25 s (x640).

Cell adhesion assay. Adherence of THP-1 cells to HaCaT cells was assayed using a cell-cell adhesion assay as described elsewhere (Choi et al., 2004). Briefly, HaCaT cells were cultured at a density of 6.0 × 10⁶ cells on a 4-well glass chamber slide containing 25 mmol/L HEPES-buffered M199 with 10% FBS, HaCaT cells were pretreated with inhibitors for the indicated time periods. After pretreatment, HaCaT cells were exposed to UVB irradiation. HaCaT cells were washed three times with PBS before cell-cell adhesion assay. THP-1 cells were labeled for 30 min with 5 µmol/L calcein-AM (Molecular Probes, Inc.). The labeled THP-1 (5.0 × 10⁶ cells × 30 min) were incubated with the HaCaT cells for 5 min. Cell-cell adhesion was then assessed by confocal laser scanning microscopy.
10^5) was cocultured with HaCaT cells for 1 h. Cocultured cells were washed three times with PBS. The fluorescence images were obtained at 485 nm excitation and 538 nm emission using a SPOT II digital camera–attached fluorescence microscope with Spot II data acquisition software (Diagnostic Instrument). For the adhesion quantification, the calcein-AM fluorescent intensity was measured at 485 nm excitation and 538 nm emission by a Fluoroskan ELISA plate reader (Labsystem Oy).

Results

Induction of ICAM-1 expression by UVB in human keratinocyte cells. It was previously demonstrated that UVB induces expression of ICAM-1 by human keratinocyte (Krutmann and Grewe, 1995). To understand the molecular mechanisms occurring in UVB-induced ICAM-1 expression, we examined the effects of UVB irradiation upon ICAM-1 expression in the human keratinocyte cell line HaCaT. HaCaT cells were exposed to varying doses of UVB (0-100 mJ/cm^2) and analyzed for expression of ICAM-1 protein after 20 h later. Maximal induction of ICAM-1 was observed at the dose of 40 mJ/cm^2 (Fig. 1A). Analysis of the kinetics of protein expression indicated that 20 h incubation after UVB exposure was optimal (Fig. 1B).

Next, we analyzed the induction of ICAM-1 mRNA expression in HaCaT cells after treatment with UVB (Fig. 1C). Cells were exposed with different UVB doses (0-100 mJ/cm^2) and at 10 h later ICAM-1 mRNA was measured by RT-PCR. Optimal induction of ICAM-1 mRNA was observed at the dose of 40 mJ/cm^2 (Fig. 1C). As shown in Fig. 1D, a time-dependent increase in levels of ICAM-1 mRNA was observed in HaCaT cells after UVB (40 mJ/cm^2) irradiation.

NF-κB is responsible for induction of ICAM-1 expression by UVB in human keratinocyte cells. Since NF-κB is a pivotal transcription factor of gene expression involved in photobiology (Legrand-Poels et al., 1998) and ROS has been known to be a key regulator in NF-κB activation (Sen and Packer, 1996; Legrand-Poels et al., 1998), we examined whether UVB can induce activation of NF-κB in HaCaT cells. HaCaT cells were exposed to UVB (40 mJ/cm^2) for various time periods, the translocation of NF-κB was analyzed by indirect immunofluorescence. Nuclear accumulation of NF-κB p65 in HaCaT cells were strongly induced 60 min after exposure to UVB (40 mJ/cm^2) (Fig. 2). Next, we investigated whether UVB-induced translocation of NF-κB is necessary for inducing ICAM-1 expression in HaCaT cells. To address this, we used various pharmacological NF-κB inhibitors, MG-132, TLCK, and Bay 11-7082. HaCaT cells were incubated in the absence or presence of these inhibitors for 1 h, exposed to UVB (40 mJ/cm^2), and then expression of ICAM-1 was assessed by RT-PCR and Western blot analysis. As shown in Fig. 3, pretreatment with MG-132, TLCK, or Bay 11-7082 suppressed UVB-induced expression of ICAM-1 mRNA (Fig. 3A) and protein (Fig. 3B). These results indicate that NF-κB activation is required for UVB-induced expression of ICAM-1 by HaCaT cells.

Generation of ROS by UVB in keratinocyte cells. Since ROS were generated in keratinocyte as well as ex vivo human skin after UVB irradiation (Hanson and Clegg, 2002; Baek et al., 2004), ROS may be involved in UVB-induced increase of ICAM-1 expression by human keratinocyte cells. We examined whether ROS can be generated after UVB irradiation in HaCaT cells. After exposure to UVB (40 mJ/cm^2) for various time periods, the time-dependent increase in levels of ROS was observed in HaCaT cells after UVB (40 mJ/cm^2) irradiation.

Fig. 1. UVB induces expression of ICAM-1 by human keratinocytes. A: HaCaT cells were exposed to varying dose of UVB (0-100 mJ/cm^2), and then analyzed for ICAM-1 expression by Western blot analysis. B: Cells were exposed to UVB (40 mJ/cm^2) for varying times, and then were examined for ICAM-1 expression. C: HaCaT cells were exposed to varying dose of UVB (0-100 mJ/cm^2), and then analyzed for ICAM-1 mRNA expression by RT-PCR. D: Cells were exposed to UVB (40 mJ/cm^2) for varying times, and then were examined for ICAM-1 mRNA expression.
periods (0-60 min), cells were stained with DCF-DA, which has been used as a general marker of intracellular ROS production. As shown in Fig. 4, ROS levels increased in a time-dependent manner in HaCaT cells as early as 5 min after UVB exposure and were maintained up to 1 h after treatment. To further characterize ROS production by UVB irradiation, we utilized an antioxidant, NAC, and a flavoprotein inhibitor, DPI. DPI has been used widely to inhibit NADPH oxidase activity in

Fig. 2. NF-κB nuclear translocation in HaCaT exposed to UVB. The NF-κB localization in HaCaT cells exposed to UVB (40 mJ/cm²) was visualized with a confocal microscopy by indirect immunofluorescence using anti-NF-κB p65 antibody. TNF-α (20 ng/ml for 20 min) was used as positive control. To analyze the effect of NF-κB inhibitor on UVB-induced NF-κB localization, HaCaT cells pre-treated with TLCK for 1 h were exposed to UVB and then the NF-κB p65 localization was examined.

Fig. 3. NF-κB inhibitors suppress UVB-induced ICAM-1 expression. HaCaT cells were incubated with MG-132, Bay 11-7082 and TLCK for 1 h, exposed to UVB (40 mJ/cm²), and then ICAM-1 expression were measured by RT-PCR (A) and Western blot assay (B). (Park et al., 2004). As shown in Fig. 4, ROS levels increased in a time-dependent manner in HaCaT cells as early as 5 min after UVB exposure and were maintained up to 1 h after treatment. To further characterize ROS production by UVB irradiation, we utilized an antioxidant, NAC, and a flavoprotein inhibitor, DPI. DPI has been used widely to inhibit NADPH oxidase activity in

Fig. 4. Analysis of ROS production in the HaCaT cells exposed to UVB. HaCaT cells exposed to UVB (40 mJ/cm²) were incubated for various time periods (0-1 h), and intracellular ROS levels were measured after staining with dichlorodihydrofluorescein diacetate (DCF-DA). Visualization of the ROS of the treated cells was measured by confocal microscopy (original magnification, X 200). Cells treated with H₂O₂ (100 µM) for 15 min were analyzed for intracellular ROS levels as positive controls. To analyze the effect of diphenyl iodonium (DPI) and N-acetyl cysteine (NAC) on UVB-induced ROS production, HaCaT cells were incubated in the absence or presence of NAC (20 mM) or DPI (10 µM) for 1 h, exposed to UVB (40 mJ/cm²), and incubated for an additional 1 h and then intracellular ROS were measured.
non-phagocytic cells (Cross and Jones, 1986; Doussiere and Vignais, 1992). Cells were incubated in the absence or presence of these inhibitors for 1 h, exposed to UVB (40 mJ/cm²) for 1 h, and then intracellular ROS was measured after staining with DCF-DA. As shown in Fig. 4, pretreatment with DPI and NAC suppressed UVB-induced increase of intracellular ROS levels in HaCaT cells. These results indicate that UVB irradiation induces production of ROS in a time-dependent manner in HaCaT cells.

ROS are responsible for UVB-induced activation of NF-κB and ICAM-1 expression. Next, we used DPI and NAC to investigate the role of ROS on UVB-induced translocation of NF-κB. HaCaT cells were incubated in the absence or presence of these inhibitors for 1 h, exposed to UVB (40 mJ/cm²), and 1 h later the translocation of NF-κB was analyzed by fluorescence microscopy. As shown in Fig. 5A, pretreatment with DPI suppressed UVB-induced translocation of NF-κB. UVB-induced translocation of NF-κB was also inhibited by pretreatment with NAC. We further used these inhibitors to investigate the role of ROS on UVB-induced increase of ICAM-1 expression in keratinocytes. Cells were incubated in the absence or presence of DPI or NAC for 1 h, exposed to UVB (40 mJ/cm²), and then ICAM-1 expression were analyzed by Western blot analysis. Pretreatment with DPI or NAC suppressed significantly ICAM-1 mRNA (Fig. 5B) and protein (Fig. 5C) expression.

ROS are responsible for UVB-induced monocyte adhesion to keratinocytes. Next, we examined the effect of DPI and NAC on monocyte adhesion to keratinocytes. HaCaT cells were incubated in the absence or presence of DPI or NAC for 1 h, exposed to UVB (40 mJ/cm²), and then analyzed for monocyte adhesion to keratinocytes. As shown in Fig. 6, significant adhesion of monocytes was observed following UVB exposure of HaCaT cells, as compared with untreated conditions. Adhesion between THP-1 and HaCaT cells was markedly attenuated in UVB-exposed cells pre-treated with NAC or DPI (Fig. 6B).

Discussion
One of characteristic features of skin inflammatory disease is the infiltration of leukocytes in the epidermis as well as dermis of the skin. In UVB-induced skin inflammation, adhesion of activated leukocytes to dermal microvascular endothelial cells is considered to be a prerequisite for the development of immune response in the skin. Once within the skin, these leukocytes may activate keratinocyte and fibroblast directly and/or via the production of proinflammatory cytokines, such as TNF and gamma interferon (Mitra et al., 1993). Therefore, expression of adhesion molecules such as ICAM-1 by skin cells including keratinocyte may be an important step in the induction of inflammatory skin diseases. The molecular mechanism by which UVB induces ICAM-1 expression of keratinocyte has not been fully understood. We demonstrate that ROS generated by UVB mediates activation of NF-κB, up-regulation of ICAM-1 and subsequent increased monocyte adhesion in human keratinocyte, HaCaT.
Our results are consistent with previous reports that UVB exerts pro-inflammatory effects on human skin (Krutmann and Grewe, 1995; Norris et al., 1999). Previous results have shown that UVB can exert both anti-inflammatory effects and pro-inflammatory effects depending on the duration of irradiation (Norris et al., 1999). They have demonstrated that UVB (100 mJ/cm²) significantly inhibited enhanced expression of ICAM-1 on the surface of cultured human keratinocytes by gamma interferon 24 h after irradiation. However, ICAM-1 expression was greatly induced at 48, 72, and 96 h after UVB irradiation (10 to 100 mJ/cm²) alone. They suggested that the late induction of ICAM-1 by UVB might be an important step in the induction of UVB-induced skin inflammation. Up-regulation of ICAM-1 mRNA was detected 6 h after UVB irradiation, and increase of ICAM-1 protein expression in the irradiated HaCaT cells was observed at 12 h after irradiation with a peak at 20 h. This early induction pattern of ICAM-1 protein expression after UVB irradiation is different from the results obtained from cultured human keratinocytes. This might be due to the different properties between the cell line and the primary cells.

ICAM-1 is constitutively expressed at low levels in keratinocyte. However, its expression is greatly enhanced by a variety of stimuli such as cytokines (interferon-γ, TNF-α) and UV radiation (Mitra et al., 1993; Norris et al., 1999). Enhanced expression of adhesion molecules such as ICAM-1 on the surface of keratinocyte is required for keratinocyte-leukocyte interaction. The increased infiltration of leukocytes into the skin has been observed in the epidermis of normal human skin after UVB exposure (Katiyar et al., 1999). Our results showed that UVB-induced increase in ICAM-1 expression is regulated at the transcriptional level in an NF-κB-dependent pathway. Although ICAM-1 expression is regulated in the NF-κB-dependent manner in response to UVB, signaling pathways involved in expression of this gene remain unknown. Our results showed that increase of ICAM-1 expression on the keratinocyte exposed to UV is mediated by ROS. UVB-induced generation of ROS was inhibited by DPI, suggesting that a NADPH oxidase is involved in the generation of ROS by UVB radiation. This result is consistent with previous studies demonstrating that the plasma membrane-bound NADPH oxidase is a major source of ROS production induced by UVB in keratinocytes (Beak et al., 2004; Wang and Kochevar, 2005). However, DPI is not specific for NADPH oxidase. DPI can inhibit a variety of flavoprotein oxidoreductases such as NADPH oxidase, nitric oxide synthase, xanthine oxidase, P-450 reductase, and mitochondrial respiratory chain complex I NADPH-ubiquinone oxidoreductase (Cross and Jones, 1986; Stuehr et al., 1991; Doussiere and Vignais, 1992; Li and Trush, 1998). Even though our results suggest that a NADPH oxidase may play a role in UVB-induced ROS production, further study is required to define the exact source.
of ROS. Since keratinocytes express NOX1, a homologue of gp91phox which is one of NADPH oxidase components (Chattopadhyay et al., 2004), it would be helpful to use a specific inhibitor of NADPH oxidase such as tetraethylpyridine which has been shown to inhibit p47(phox) association with gp91(phox) in NADPH oxidase (Rey et al., 2001).

UVB-induced NF-κB activation was significantly inhibited in cells exposed to UVB in the presence of NAC or DPI. In addition, both NAC and DPI inhibited ROS-mediated enhancement of expression of ICAM-1 and subsequent increased adhesion of monocyte to keratinocyte. These results indicate that ROS is involved in the signal transduction pathway leading to nuclear translocation of NF-κB and to NF-κB-activated ICAM-1 expression in HaCaT cells. Elucidation of the central role of ROS in the inducible expression of ICAM-1 and the subsequent increased adhesion of monocyte to keratinocyte may lead to the development of effective therapeutic agents for skin inflammation induced by UVB. Currently, available agents, such as corticosteroids, antihistamines, or immuno-suppressants, have been considered to have a beneficial effect against skin inflammatory diseases (Hengge et al., 2006, Musser and Lichtenstein, 1990, Tomi and Luger, 2003). However, the adverse effects of these agents resulted in limitation to treat skin inflammation. The observed inhibition of ICAM expression and subsequent increased adhesion of monocyte to HaCaT by the antioxidants suggested that the natural compounds with antioxidant activity may be useful as therapeutic agents for UVB-induced ROS-associated inflammatory skin diseases.

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References


UVB-induced ICAM-1 Expression in Keratinocyte


