Prevention of the Ultraviolet B-Mediated Skin Photoaging by a Nuclear Factor κB Inhibitor, Parthenolide

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ABSTRACT

The skin photoaging is characterized by keratinocyte hyperproliferation and degradation of collagen fibers, causing skin wrinkling and laxity and melanocyte proliferation that leads to pigmentation. UV is considered to be a major cause of such skin changes. It is well established that nuclear factor κB (NF-κB) is activated upon UV irradiation and induces various genes including interleukin-1 (IL-1), tumor necrosis factor α (TNFα), and matrix metalloprotease-1 (MMP-1). It is also known that basic fibroblast growth factor (bFGF) production is induced by UV and promotes the proliferation of skin keratinocytes and melanocytes. We found that UVB, IL-1, and TNFα induced NF-κB activation and then produced MMP-1 and bFGF in HaCaT keratinocytes and skin fibroblasts. In this experiment, we examined if parthenolide, an NF-κB inhibitor, could block the UVB-mediated skin changes. We found that parthenolide could effectively inhibit the gene expression mediated by NF-κB and the production of bFGF and MMP-1 from cells overexpressing p65, a major subunit of NF-κB. We also found that parthenolide could inhibit the UVB-induced proliferation of keratinocytes and melanocytes in the mouse skin. These findings suggest that NF-κB inhibitors should be useful for the prevention of skin photoaging.

Skin aging is a complex process that involves intrinsic and exogenous causes. Although intrinsic skin aging is associated with other physiological processes and is inevitable, exogenous aging is caused by extrinsic harmful environments and can be prevented. UV is one of the most noxious factors among the harmful environments (Ulrich et al., 2004). UV irradiation induces inflammatory processes in the skin, and the irradiated skin becomes metabolically hyperactive associated with epidermal hyperproliferation and accelerated collagen fiber breakdown. In contrast, physiologically aged skin is usually characterized by a slow decline in many of these processes (Kligman, 1989). The UV-irradiated skin is characterized by fine and coarse wrinkling, roughness, dryness, laxity, and pigmentation (Chung, 2003). Microscopically, these changes can be explained by keratinocyte hyperproliferation and degradation of collagen fibers (Brenneisen et al., 2002), causing skin wrinkling and laxity, and melanocyte proliferation that leads to pigmentary characterized by dysregulation of melanocyte homeostasis and increase in the melanocyte density (Hirobe et al., 2003). The UV-induced production of proinflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor α (TNFα), has been considered attributable to these changes (Corsini et al., 1997; Yarosh et al., 2000). Similarly, induction of matrix metalloprotease-1 (MMP-1) is responsible for the degradation of collagen fibers (Wlaschek et al., 1994; Barchowsky et al., 2000). In addition, UV irradiation is known to stimulate both keratinocytes and fibroblasts to induce basic fibroblast growth factor (bFGF) that is responsible for the proliferations of melanocytes and keratinocytes.

It is well established that a transcription factor, nuclear factor κB (NF-κB), is activated upon UV irradiation and induces various genes including IL-1 and TNFα, which subsequently stimulate the signal transduction pathway to activate NF-κB, thus conferring a vicious cycle (Okamoto et al., 1997; Saliou et al., 1999). In fact, NF-κB is known to increase MMP-1 in dermis (Bond et al., 1999; Sun et al., 2002; Chung, 2003). It is also reported that UV irradiation induces bFGF production (Sabourin et al., 1993), presumably through NF-κB activation (Wakisaka et al., 2002). Thus, inhibition of the NF-κB activation pathway would block the vicious cycle elicited by UV irradiation and effectively prevent the UVB-mediated cutaneous alterations.

NF-κB is sequestrated in the cytoplasm as an inactive

ABBREVIATIONS: IL-1, interleukin-1; TNFα, tumor necrosis factor α; MMP-1, matrix metalloprotease-1; bFGF, basic fibroblast growth factor; NF-κB, nuclear factor κB; IκB, inhibitor κB.
complex with a class of inhibitory molecules known as inhibitor \( \kappa B \) (I\( \kappa B \)). Treatment of cells with a variety of inducers such as IL-1 and TNF-\( \alpha \) results in phosphorylation, ubiquitination, and degradation of the I\( \kappa B \) proteins (Hayden and Ghosh, 2004). The phosphorylation of I\( \kappa B \) is catalyzed by I\( \kappa B \) kinase complex. The phosphorylated I\( \kappa B \) is subjected to ubiquitination and proteolytic degradation by proteasome. The degradation of I\( \kappa B \) exposes the nuclear localization sequence in the remaining NF-\( \kappa B \) dimers, followed by the rapid translocation of NF-\( \kappa B \) to the nucleus, where it activates the target genes by binding to the DNA regulatory element.

Parthenolide is a sesquiterpene lactone compound and an active substance in medical herb Feverfew (Tanacetum parthenium) traditionally used in the treatment of inflammation in Mexico (Heinrich et al., 1998). It was shown that parthenolide blocked the NF-\( \kappa B \) activation pathway at multiple levels such as inhibiting I\( \kappa B \) kinase activity (Kwok et al., 2001) and DNA binding of NF-\( \kappa B \) (Garcia-Pineros et al., 2001). In this study, we examined the effect of parthenolide in blocking the processes of UVB-mediated cutaneous alterations using cultured cell and animal models.

**Materials and Methods**

**Reagents and Plasmids.** Parthenolide, recombinant human TNF\( \alpha \), and IL-1 were purchased from Wako Pure Chemicals (Osaka, Japan). Antibodies to bFGF and epidermal growth factor were purchased from R&D Systems (Minneapolis, MN). The reporter plasmid expressing firefly luciferase under the control of NF-\( \kappa B \) (pGL3-4xBwt-Luc) was constructed by inserting four tandem copies of the \( \kappa B \) sequence (GGGACTTCCC) from HIV-1 enhancer into pGL3-promoter vector (Promega, Madison, WI) as reported previously (Sato et al., 1998; Tetsuka et al., 2000). Construction of the mutant NF-\( \kappa B \) reporter plasmid, pGL3-4xBm-luc, containing mutated NF-\( \kappa B \) binding sites, was described previously (Tetsuka et al., 2000). Control luciferase reporter plasmids under controls of CRB, pCRE-luc, and AP1, pAP-1-luc, were purchased from Stratagene (La Jolla, CA). The p65-expressing plasmid, pCMV-p65, was described previously (Tetsuka et al., 2000).

**Cell Culture.** The HaCaT human keratinocyte cell line (Boukamp et al., 1988) was generously provided by N. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany). HaCaT cells were grown at 37°C in RPMI 1640 medium supplemented with 1% fetal bovine serum, 100 units/ml penicillin, and 100 \( \mu \)g/ml streptomycin. Human normal epidermal melanocytes (Toyobo Engineering, Osaka, Japan) were grown at 37°C in melanocyte basic medium (Toyobo Engineering) supplemented with 1% fetal bovine serum, 100 units/ml penicillin, and 100 \( \mu \)g/ml streptomycin. Human normal fibroblasts (KURABO, Osaka, Japan) were grown at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 1% fetal bovine serum, 100 units/ml penicillin, and 100 \( \mu \)g/ml streptomycin. Human embryonic kidney 293 cells (Riken, Tsukuba, Japan) were grown at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1 mM glutamate, 100 units/ml penicillin, and 100 \( \mu \)g/ml streptomycin. Human embryonic kidney 293 cells (Riken, Tsukuba, Japan) were grown at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1 mM glutamate, 100 units/ml penicillin, and 100 \( \mu \)g/ml streptomycin. For UVB irradiation, cell culture medium was changed to phosphate-buffered saline and UVB of 280- to 320-nm wavelength was irradiated at a dose of 0.5 mJ/cm\(^2\) using an FL20 S-E sunlamp every other day for 12 days. For the group of parthenolide treatment, 250 \( \mu \)g/kg parthenolide in saline was injected i.p. every day during the period of UVB irradiation. The same amount of saline was injected to UV treatment and control groups. After 12 days, ears were excised from all subjects. One of the ear specimens of each animal was stored in \(-80^\circ C\) for the microscopic observations of melanocytes, and the other ear specimen was paraffin-embedded for the immunohistochemical analysis of MMP-1 and the determination of skin thickness by hematoxylin and eosin staining (H&E staining).

**Transfection and Luciferase Assay.** Cells were transfected with various plasmids using Fugene-6 transfection reagent (Roche Diagnostics, Basel, Switzerland). Briefly, cells were cultured in 12-well plates, and transfections were performed with 1.5 \( \mu \)l of Fugene-6 transfection reagent/ml culture medium and a total of 0.5 \( \mu \)g of plasmid DNA as previously described (Tetsuka et al., 2000; Uranishi et al., 2001). Control plasmid pUC19 was used to equalize the amount of DNA for each transfection. Fugene-6 DNA complexes were allowed to form for 15 min at room temperature in serum-free medium before being added to the cells. After 24 h of transfection, cells were incubated for additional 24 h and then harvested. The luciferase activity was measured by the luciferase assay system (Promega). The relative light units were determined with a TD-20/20 Luminometer (Promega). Transfection efficiency was monitored by Renilla luciferase activity with pRL-TK plasmid containing TK promoter as an internal control. All luciferase activities shown in transient transfection assays were corrected by the internal control activity of Renilla luciferase by pRL-TK. The assays were performed in triplicates. The results were presented as the fold increases in luciferase activities (means ± S.D.) relative to the control in three independent transfections.

**Melanocyte Growth Assay.** To assess the activity promoting the growth of melanocyte in the supernatant of HaCaT cells, pCMV-p65 was transfected into HaCaT cultured in 12-well plates, cultured for 48 h, and the supernatant was obtained. Melanocytes were cultured in 24-well plates, and the supernatant of HaCaT was added to the melanocyte culture medium, the HaCaT culture supernatant was incubated with the anti-bFGF antibody premixed with 5% Sepharose A beads (Amersham Biosciences AB, Uppsala, Sweden) at 4°C for 4 h.

**Quantitative Determination of bFGF and MMP-1.** The commercial ELISA kits were used to determine the concentrations of bFGF (Cytimmune, College Park, MD) and MMP-1 (Amersham Biosciences AB) according to the suppliers’ protocol. All the measurements were performed in triplicates and repeated at least twice.

**Mouse Model for the UVB-Irradiated Skin.** Twenty male DBA/2 mice 6 weeks of age were subjected to this study. All mice were randomly allocated to the following four groups: UV + parthenolide treatment, UV treatment, parthenolide treatment, and control. For the groups of UV + parthenolide and UV treatments, the heads of mice were locally exposed to UVB of 280 to 320 nm wavelength at a dose of 180 mJ/cm\(^2\) using an FL20 S-E sunlamp every other day for 12 days. For the group of parthenolide treatment, 250 \( \mu \)g/kg parthenolide in saline was injected i.p. every day during the period of UVB irradiation. The same amount of saline was injected to UV treatment and control groups. After 12 days, ears were excised from all subjects. One of the ear specimens of each animal was stored in \(-80^\circ C\) for the microscopic observations of melanocytes, and the other ear specimen was paraffin-embedded for the immunohistochemical analysis of MMP-1 and the determination of skin thickness by hematoxylin and eosin staining (H&E staining). The thickness of epidermis was measured using software for image analysis (Win ROOF; Mitani, Fukui, Japan). These animal experiments were performed according to the institutional regulation and were approved by the institutional review board.

**Melanocyte Counting.** The melanocyte count in skin tissues was determined microscopically according to the method of Hiramoto et al. (2003). The cartilages were manually removed from the excised mouse ear specimen, and the skin tissues were soaked in 2 N NaBr solution at 37°C for 2 h. The epidermal and basal layers were exfoliated from the skin by this procedure, and melanocytes were stained by immersing in 0.1 M phosphate-buffered saline (pH 7.2) containing 0.14% L-DOPA at room temperature for 3 h and counted under a microscope.

**Statistical Analyses.** The data were collected from at least three independent experiments. Animal experiments were performed with at least five animals per each treatment group. Quantitative data were expressed as the mean ± S.D. Statistical significance was examined by the analysis of variance and the paired Student’s t test. Differences were considered statistically significant if \( p < 0.05 \). The levels of statistical significance were indicated as the following: *, \( p < 0.05 \); **, \( p < 0.01 \); and n.s., not significant.
Results

Induction of NF-κB and the Inhibitory Effects of Parthenolide in Keratinocytes and Fibroblasts. We examined the effects of UVB, IL-1, TNFα, and p65 overexpression on NF-κB dependent gene expression using transient luciferase assay. Since keratinocytes and fibroblasts are major cellular components of the skin, we used human keratinocyte cell line HaCaT as reported previously (Tebbe et al., 2001; Ahn et al., 2003) and primary human fibroblasts. Although UVB cannot penetrate the keratinocyte layer completely, approximately 10% of UVB is known to reach the upper layer of dermis consisting of fibroblasts (Fujisawa et al., 1997). These cells were transfected with luciferase reporter plasmids containing either wild-type κB sites or mutated κB sites, and extents of gene expression were compared in the presence or the absence of these stimuli (Fig. 1).

In HaCaT cells, UVB, IL-1, TNFα, and p65 stimulated gene expression from the reporter plasmid containing NF-κB binding sites by 2.5-, 2.4-, 5.8-, and 6.9-fold, respectively, whereas no significant stimulation was observed when the reporter plasmid containing mutant NF-κB sites was used. Even higher NF-κB-dependent activation was observed in primary fibroblasts. UVB, IL-1, TNFα, and p65 stimulated the luciferase gene expression by 3.3-, 6.7-, 8.9-, and 19.2-fold, respectively. Similar observations were made in 293 cells, a fibroblast cell line derived from human kidney (data not shown). We then examined the effect of parthenolide on the TNFα-mediated NF-κB-dependent gene expression. As shown in Fig. 2, when parthenolide was added to the 293-cell culture, the NF-κB-mediated gene expression was inhibited in a dose-dependent manner for the concentration of parthenolide. In contrast, no such effect of parthenolide was observed on AP-1- or CREB-dependent gene expression. These effects were observed under noncytotoxic concentrations of parthenolide (data not shown).

Induction of bFGF and MMP-1 by NF-κB and the Effects of Parthenolide. The UV-induced cutaneous alterations are known to be mediated by bFGF and MMP-1 (Pittelkow and Shipley, 1989; Chung, 2003). Since it was previously shown that production of bFGF and MMP-1 was induced by UVB (Sabourin et al., 1993; Brenneisen et al., 2002) and that UVB irradiation induced production of IL-1 and TNFα in keratinocytes and fibroblasts (Corsini et al., 1997; Fujisawa et al., 1997), we examined if p65 overexpression, mimicking NF-κB activation, induced production of bFGF and MMP-1. As shown in Fig. 3A, when p65 was overexpressed in keratinocytes and fibroblasts, bFGF production into the culture supernatant was significantly augmented although the transfection efficiency was approximately 3.8 and 9.2% for HaCaT cells and fibroblasts, respectively. Similar effects were observed in the MMP-1 production (Fig. 3B). The amounts of bFGF and MMP-1 production were significantly reduced, almost to the basal level, luciferase activity. As a positive control (denoted as p65), pCMV-p65, expressing the p65 subunit of NF-κB, was cotransfected. As an internal control, pRL-TK, expressing Renilla luciferase under the control of TK promoter, was cotransfected. All luciferase activities were corrected by the internal control activity of Renilla luciferase. Values (-fold activation) represent the mean ± S.D. of three independent transfections. Similar results were achieved repeatedly. * p < 0.05; ** p < 0.01; n.s., not significant.
by the treatment with parthenolide. When mice were treated with parthenolide i.p., significant reduction of the MMP production upon UVB irradiation was observed (data not shown), consistent with the results with cultured cells.

Effects of Parthenolide on Epidermal Hyperproliferation and Melanocyte Growth. It is well known that the UV-induced epidermal hyperplasia, consisting of the hyperproliferative keratinocytes and melanocytes (Brenneisen et al., 2002; Chung, 2003; Hirobe et al., 2003) is considered to be due to the action of bFGF induced by UVB (Pittelkow and Shipley, 1989; Bielenberg et al., 1998). In Fig. 4, effects of parthenolide on the UVB-induced epidermal hyperproliferation were shown. UVB (180 mJ/cm²) was irradiated at the head of mice. Although UVB induced epidermal hyperproliferation by 2.9-fold compared with the control untreated skin, it was significantly reduced by the treatment with parthenolide to 1.6-fold.

We then examined the effects of parthenolide on the melanocyte growth (Fig. 5). The epidermal and basal layers were exfoliated from the skin tissue and melanocytes were stained by L-DOPA. As demonstrated in Fig. 5B, although UVB induced melanocyte proliferation by 3.1-fold in the number of melanocytes compared with the control untreated skin, it was significantly reduced by the treatment with parthenolide to 2.1-fold compared with the control.
Promotion of the Melanocyte Growth by Keratinocytes and Involvement of bFGF. In Fig. 6, we examined if bFGF stimulated the growth of melanocyte and if keratinocytes, upon NF-κB activation, produced bFGF. After 48 h of the bFGF treatment, a dose-dependent stimulation of melanocyte growth was observed (Fig. 6A). Since keratinocytes are known to produce bFGF (Bielenberg et al., 1998), which acts as a growth factor for melanocytes (Krasagakis et al., 1995), and NF-κB has been shown to activate production of bFGF (Wakisaka et al., 2002), we examined the effect of the culture supernatant of keratinocytes in which NF-κB/p65 was activated on the melanocyte growth. In Fig. 6B, p65 was overexpressed in HaCaT keratinocytes (HaCaT-p65), and the culture supernatant was examined for the growth-promoting effect on melanocytes. As shown here, a significant increase in the melanocyte growth was observed after incubation with the culture supernatant of HaCaT-p65 (Fig. 6B). In fact, bFGF was detected in the HaCaT-p65 culture supernatant (14.8 ± 1.53 ng/ml), whereas the level of bFGF in parental HaCaT cells was less than 6 ng/ml. When the HaCaT-p65 supernatant was preincubated with the neutralizing antibody to bFGF, the melanocyte growth-promoting effect was completely abolished (Fig. 6B), whereas the anti-EGF antibody did not show such effect. From these observations, it was suggested that keratinocytes were responsible for the melanocyte proliferation and that this effect was mediated by bFGF.

Discussion

UV is one of the most harmful environmental factors for skin (Kligman, 1989; Ulrich et al., 2004; Wulf et al., 2004) and is responsible for the skin aging. Most characteristic changes of the skin induced by UVB have been ascribed to the production of proinflammatory cytokines, including IL-1 and TNFα (Wlaschek et al., 1994; Fisher et al., 1996; Corsini
et al., 1997; Yarosh et al., 2000) and effector molecules, such as MMP-1 and bFGF (Wlaschek et al., 1994; Barchowsky et al., 2000). It is well established that expressions of IL-1, TNFα, MMP-1, and bFGF are controlled by NF-κB, which is activated by UV irradiation (Barchowsky et al., 2000; Wakisaka et al., 2002). NF-κB is a crucial factor for the immunoinflammatory responses and is also implicated in various skin diseases including allergic dermatitis, psoriasis vulgaris, and skin cancer (Bell et al., 2003). Hence, although NF-κB is involved in maintaining the skin homeostasis (Pasparakis et al., 2002; Takao et al., 2003), excessive activation is pathogenic. Thus, inhibition of NF-κB is considered to prevent the pathogenetic changes induced by UVB. In this study, we have examined the causal association of NF-κB with the UVB-induced changes in the skin and the efficacy of one such inhibitor, parthenolide.

Although parthenolide is known to have inhibitory action on NF-κB (Heinrich et al., 1998; Hehner et al., 1999), its specificity has not been clearly demonstrated. We found that parthenolide specifically inhibited the NF-κB-dependent gene expression and did not affect the actions of other transcription factors such as AP-1 and CREB. Although Won et al. (2004) recently reported the inhibitory action of parthenolide on AP-1 and claimed its chemopreventive activity against UVB-induced skin cancer, we did not observe such inhibitory effect on AP1, which might be due to the differences of cell lineages used or the experimental procedures applied. Thus, the effect of parthenolide seems to be specific. Since parthenolide was effective in blocking the induction of MMP-1 and bFGF even in cells overexpressing p65, bypassing the activation signaling of NF-κB, it is possible that parthenolide can inhibit NF-κB DNA binding or transactivation process following the DNA binding such as the recruitment of basal transcription factors or coactivators (Uranishi et al., 2001; Jiang et al., 2003). However, further analysis of parthenolide action is needed to clarify its mode of action.

The possible involvement of NF-κB in the UV-mediated cutaneous alteration, or skin photoaging, through induction of 2001) and IκB kinase (Hehner et al., 1999), its specificity has not been clearly demonstrated. We found that parthenolide specifically inhibited the NF-κB-dependent gene expression and did not affect the actions of other transcription factors such as AP-1 and CREB. Although Won et al. (2004) recently reported the inhibitory action of parthenolide on AP-1 and claimed its chemopreventive activity against UVB-induced skin cancer, we did not observe such inhibitory effect on AP1, which might be due to the differences of cell lineages used or the experimental procedures applied.

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MMP-1 and bFGF is involved (Bond et al., 1999; Abeyama et al., 2000; Barchowsky et al., 2000; Wakisaka et al., 2002). We have confirmed these findings in this study using cultured cells and short-term irradiation experiments using mice. In addition, we found that NF-κB activation is involved in the melanocyte proliferation, which was consistent with the previous observations by others as the involvement of bFGF in melanocyte proliferation (Pittelkow and Shively, 1989). Interestingly, impaired bFGF production is reported in the vitiliginous patient skin where melanin deposition in the skin is partially affected (Moretti et al., 2002). In addition, kojic acid and ascorbic acid, identified as active whitening ingredients for the skin, have been shown to inhibit the NF-κB-mediated gene expression (Ahn et al., 2003).

These findings support an idea that NF-κB is involved in various steps in the UVB-mediated skin change both directly and indirectly. Thus, inhibition of NF-κB can be effective in preventing the process of UVB-mediated cutaneous alterations and eventually photoaging (schematically demonstrated in Fig. 7). Although we did not observe any side effect in experimental parthenolide therapy of mice as long as we observed (12 days), longer treatment might cause unexpected side effects such as immunosuppression and deteriorated host defense considering NF-κB being a major determinant for immunological and inflammatory responses. Further studies are needed regarding the chronic effects and the possible toxicity of parthenolide.

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References


