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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. Ultraviolet (UV) is considered to be a major cause of such skin changes. It is well established that nuclear factor κ B (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.

Introduction

Skin aging is a complex process that involves intrinsic and exogenous causes. Whereas intrinsic skin aging is associated with other physiological processes and is inevitable, exogenous aging is caused by extrinsic harmful environments and can be prevented. Ultraviolet (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 pigmentation 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 conforming a vicious cycle (Okamoto et al., 1997). In fact, NF-κB is known to increase MMP-1 in dermis (Chung, 2003; Sun et al, 2002; Bond, 1999). 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 complex with a class of inhibitory molecules known as inhibitor κB (IκB). Treatment of cells with a variety of inducers such as IL-1 and TNF-α results in phosphorylation, ubiquitination, and degradation of the IκB proteins (Hayden and Ghosh, 2004). The phosphorylation of IκB is catalyzed by IκB kinase (IKK) complex. The phosphorylated IκB is subjected to ubiquitination and proteolytic degradation by proteasome. The degradation of IκB exposes the nuclear localization sequence in the remaining NF-κB dimers, followed by the rapid translocation of NF-κB 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-κB activation pathway at multiple levels such as inhibiting IKK activity (Kwok et al., 2001) and DNA binding

of NF-κB (Garcia 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.

Methods

Reagents and plasmids. Parthenolide, recombinant human TNFα and IL-1 were purchased from Wako (Osaka, Japan). Antibodies to bFGF and epidermal growth factor were purchased from R&D system (Minneapolis, Minnesota). The reporter plasmid expressing firefly luciferase under the control of NF-κB (pGL3-4κBwt-Luc) was constructed by inserting four tandem copies of the κB sequence (GGGACTTTCC) 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-κB reporter plasmid, pGL3-4κBm-luc, containing mutated NF-κB 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 (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, 100units/ml penicillin and 100μg/ml streptomycin. Human normal epidermal melanocytes (TOYOBO, Tokyo, Japan) were grown at 37°C in melanocyte basic medium (TOYOBO) supplemented with 1% fetal bovine serum, 100units/ml penicillin and 100μg/ml streptomycin. Human normal fibroblasts (KURABO, Osaka, Japan) were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 100units/ml

penicillin and 100μg/ml streptomycin. Human embryonic kidney 293 cells (Riken, Tsukuba, Japan) were grown at 37°C in DMEM supplemented with 10% fetal bovine serum, 1mM glutamate, 100units/ml penicillin and 100μg/ml streptomycin. For UVB irradiation, cell culture medium was changed to PBS and UVB of 280-320 nm wavelength was irradiated at a dose of 0.5 mJ/cm^2 using a FL20 S-E sunlamp (Toshiba, Tokyo, Japan). After the UVB irradiation, the fresh medium was supplied for further cultures.

Transfection and luciferase assay. Cells were transfected with various plasmids using Fugene-6 transfection reagent (Roche, Basal, Switzerland). Briefly, cells were cultured in 12-well plates, and transfections were performed with 1.5μl Fugene-6 transfection reagent per ml culture medium and a total of 0.5μg 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 15min 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, Madison, WI). The relative light units were determined with an 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. In order 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 at 2:1, continued for additional 48 h, and the numbers of melanocytes were counted using WST-1 (Roche, Basal, Switzerland). In order to remove bFGF from the HaCaT culture medium, the HaCaT culture supernatant was incubated with **t**he anti-bFGF antibody premixed with 5% Sepharose A beads (Amersham Biosciences, Uppsala, Sweden) at 4°C for 4h.

Quantitative determination of bFGF and MMP-1. The commercial EIA kits were used to determine the concentrations of bFGF (Cytimmune, College Park, MD) and MMP-1 (Amersham Biosciences, Uppsala, Sweden) 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 of six-weeks 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 head of mice was locally exposed to UVB of 280-320 nm wavelength at a dose of 180 mJ/cm^2 using a FL20 S-E sunlamp every other day for

12 days. For the group of parthenolide treatment, 250 μg /kg of parthenolide in saline was injected intraperitoneally 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°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 a 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 (Hiramoto et al., 2003). The cartilages were manually removed from the excised mouse ear specimen and the skin tissues were soaked in 2N NaBr solution at 37°C for 2 h. The epidermal and basal layers were exfoliated from rest of the skin tissue by this procedure and melanocytes were stained by immersing in 0.1M PBS (pH7.2) containing 0.14% L-DOPA at room temperature for 3h, and counted under a microscope.

Statistical analyses. The data were collected from at least three independent experiments. Animal experiments were performed with at least 5 animals per each treatment group. Quantitative data were expressed as the mean +/- S.D. Statistical significance was examined

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by the ANOVA 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: *, $p<0.05$; **, $p<0.01$; 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 utilized human keratinocyte cell line HaCaT as reported previously (Tebbe et al., 2001; Ahn and Moon, 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, 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 folds, 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 folds, 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 dependent or CREB-dependent gene expression. These effects were observed under non-cytotoxic 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 (Chung, 2003; Pittelkow and Shipley, 1989). Since it was previously shown that production of bFGF and MMP-1 was induced by UVB (Brenneisen et al., 2002; Sabourin et al., 1993) and that UVB irradiation induced production of IL-1 and TNFα in keratinocytes and fibroblasts (Corsini et al., 1997; Fujisawa, 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 on the MMP-1 production (Fig. 3B). The amounts of bFGF and MMP-1 production were significantly reduced, almost to the basal level, by the treatment with parthenolide. When mice were treated with parthenolide intraperitoneally, significant reduction of the MMP production upon UVB irradiation was observed (data not shown), consistently 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 (Chung, 2003; Brenneisen et al., 2002; Hirobe et al., 2003) is considered to be due to the action of bFGF induced by UVB (Pittelkow, 1989; Bielenberg, 1998). In Fig. 4, effects of parthenolide on the UVB-induced epidermal hyperproliferation were shown. UVB (180 mJ/cm^2) was irradiated at the head of mice. Whereas UVB induced epidermal hyperproliferation by 2.9 fold as compared with the control untreated skin, treatment with parthenolide significantly reduced the epidermal hyperproliferation 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 melanocyte as compared with the control untreated skin, it was significantly reduced by the treatment with parthenolide to 2.1 fold as 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 hr of the bFGF treatment, a dose-dependent stimulation of melanocyte growth was observed (Fig. 6A). Since keratinocytes are known to produce bFGF (Bielenberg, 1998), which acts as a growth factor for melanocytes (Krasagakis, 1995), and NF-κB has been shown to activate production of bFGF (Wakisaka, 2002), we examined the effect of the culture supernatant of keratinocytes in which NF-κB 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 (Ulrich et al., 2004; Kligman, 1989; Wulf, 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 (Corsini et al., 1997; Yarosh et al., 2000; Wlaschek et al., 1994; Fisher, 1996), 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 that is activated by UV irradiation (Barchowsky et al., 2000; Wakisaka, 2002). NF-κB is a crucial factor for the immuno-inflammatory 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, 2002; Takao, 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 of such inhibitors, parthenolide.

Although parthenolide is known to have inhibitory action on NF-κB (Heinrich et al., 1998; Hehner et al., 1999), such as inhibitions of NF-κB DNA binding (Garcia et al., 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.

Thus, the effect of parthenolide appears 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 MMP-1 and bFGF has been implicated (Barchowsky et al, 2000; Bond et al., 1999; Wakisaka, 2002, Abeyama, 2000). 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 such as the involvement of bFGF in melanocyte proliferation (Pittelkow and Shipley, 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 activation should be effective in preventing the process of UVB-mediated cutaneous alterations and eventually photoaging (schematically demonstrated in e Figure 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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Footnotes

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Legends for Figures

Figure 1. Induction of the NF-κB-dependent gene expression in cultured human skin cells. *A*, Diagram of reporter gene plasmids. κBwt-luc, a luciferase reporter plasmid pGL3 containing four tandem copies of the wild type NF-κB binding site (GGGACTTTCC); κBm-luc, containing four tandem copies of the mutant NF-κB binding site (CTCACTTTCC). *B*, NF-κB-dependent gene expression in HaCaT human keratinocyte cell line. *C*, NF-κB-dependent gene expression in human skin fibroblasts. Experiments were carried out essentially identical to those with HaCaT cells (*B*). Cells were transfected with κBwt-luc or κBm-luc reporter plasmid, stimulated with UVB (0.5 mJ/cm^2), IL-1 (1.0 ng/ml), or TNFα (1.0 ng/ml). These stimuli were given 24h after the transfection and the cell lysates were prepared after additional 24 h incubation for determination of the 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.

Figure 2. Inhibition of NF-κB-dependent gene expression by parthenolide. *A*, Effects of parthenolide on NF-κB-dependent gene expression. Experiments were similarly performed as

in Fig. 1 except that cells were pretreated with parthenolide at 2.5, 5 or 10 μM of final concentration 2h prior to the TNFα (1.0 ng/ml) treatment. *B and C*, Effects of parthenolide on gene expression dependent on AP-1 (B) and CREB (C). Parthenolide, at final concentrations of 2.5, 5 or 10 μM, was added 2h prior to the TNFα or forskolin treatment. Values represent the luciferase activity means +/- S.D. of three independent transfections. **, $p<0.01$; n.s., not significant.

Figure 3. Effect of NF-κB on production of bFGF and MMP-1 from cultured skin cells and its inhibition by parthenolide. *A*, Effect of NF-κB on production of bFGF. HaCaT or skin fibroblasts were transiently transfected with pCMV-p65 and the culture supernatants were collected after 72h of transfection for determination of bFGF. Parthenolide of 10μM was added to each cell culture 24h after the transfection. The amount of bFGF produced from 10^4 cells was indicated. *B*, Effect of NF-κB on production of MMP-1. HaCaT or skin fibroblasts were transiently transfected with pCMV-p65, incubated for 72 h, and the MMP-1 production in the culture supernatant per 10^4 cells was determined. The inhibitory effect of parthenolide was similarly evaluated. Experiments were performed in triplicates and the values represent the mean +/- S.D. of three independent experiments. Similar results were achieved repeatedly. *, $p<0.05$; **, $p<0.01$.

Figure 4. Epidermal hyperproliferation by UV and inhibition by parthenolide. *A.* Epidermal hyperproliferation by UVB. Parthenolide of 250μg/kg or physiological saline as

control was administered to DBA/2 mice (n=10 for each group) by repeated intraperitoneally injections every day for 12 days. Half of the mice in each group were exposed to UVB irradiation at a 180 mJ/cm^2 every other day. Ear samples were prepared from the anesthetized mice, paraffin-embedded, and cut with a sliding microtome to 5μm thickness. Tissue sections were subjected to histological examination (H&E staining). Representative pictures are shown. Arrowheads indicate the width of epidermis. *B.* Measurement of the epidermal hyperproliferation induced by UVB and effect of parthenolide. All specimens (n=5 per group) were examined microscopically for the measurement of epidermal thickness. Data shown are the mean +/- S.D. (n=5) of the average epidermal thickness measured using software for image analysis (Win ROOF). Fold increase in the epidermal thickness as compared with the control is indicated. **, $p<0.01$; Scale bar = 20μm.

Figure 5. Induction of melanocyte proliferation by UVB and its inhibition by parthenolide. *A.* L-DOPA staining of skin melanocytes. DBA/2 mice were treated with parthenolide (250μg/kg) and exposed to UVB (180 mJ/cm^2) as in Figure 4. Parthenolide or physiological saline was repeatedly administered to DBA/2 mice by intraperitoneally injection once a day for 12 days. Half of the mice in each group were exposed to UVB irradiation of 180 mJ/cm^2 every other day. To stain melanocyte, the ears obtained from each mouse were soaked in 2N NaBr solution for exfoliating epidermis, and immersed in 0.14% L-DOPA solution for 3 h at room temperature. *B,* Melanocyte counting. All specimens (n=5 per group) were examined microscopically for counting the DOPA-positive melanocytes in the epidermis.

Data shown are the mean +/- S.D. of the number of melanocytes per mm^2. Fold increase in the melanocyte numbers as compared with the control is indicated. **, $p<0.01$; scale bar = 80μm.

Figure 6. Involvement of bFGF in the melanocyte proliferation and its production from keratinocytes. *A,* Induction of melanocyte proliferation by bFGF. Primary human melanocytes were cultured in melanocyte basic medium and the effects of bFGF of 1, 10, and 100 ng/ml were examined. The numbers of melanocytes were determined by WST method after 48 h of culture. *B,* Involvement of bFGF in the keratinocyte-mediated melanocyte proliferation. HaCaT cells were transfected with pCMV-p65 as in Figure 3, and the culture supernatant was collected after 48 h of transfection. As a control, pUC19 plasmid was transfected into HaCaT. The anti-bFGF or anti-EGF antibody was incubated with the supernatants of these transfected HaCaT cells and bFGF or EGF was removed. These supernatant samples were then added to the melanocyte and incubated for additional 72 h. The numbers of melanocyte were counted by WST method. Data shown are the mean +/- S.D. (n=5) of the number of melanocytes. *, $p<0.05$; **, $p<0.01$.

Figure 7. Diagrammatic representation of the action of NF-κB in the process of UVB-mediated cutaneous alterations (photoaging). Environmental stimuli, such as UV irradiation and inflammatory signaling, induce the NF-κB activation that leads to production of MMP-1 and bFGF in epidermal keratinocytes. The upregulation of bFGF promotes proliferation of keratinocytes and melanocytes as a protection mechanism to these

environmental insults. In dermis, skin fibroblasts are stimulated by UV and proinflammatory cytokines, such as IL-1 and TNFα, produced by keratinocytes, and then NF-κB is activated, leading to the MMP-1 production. Thus, NF-κB inhibitors are considered to be effective in preventing the UVB-mediated cutaneous alterations and eventually the skin photoaging process.

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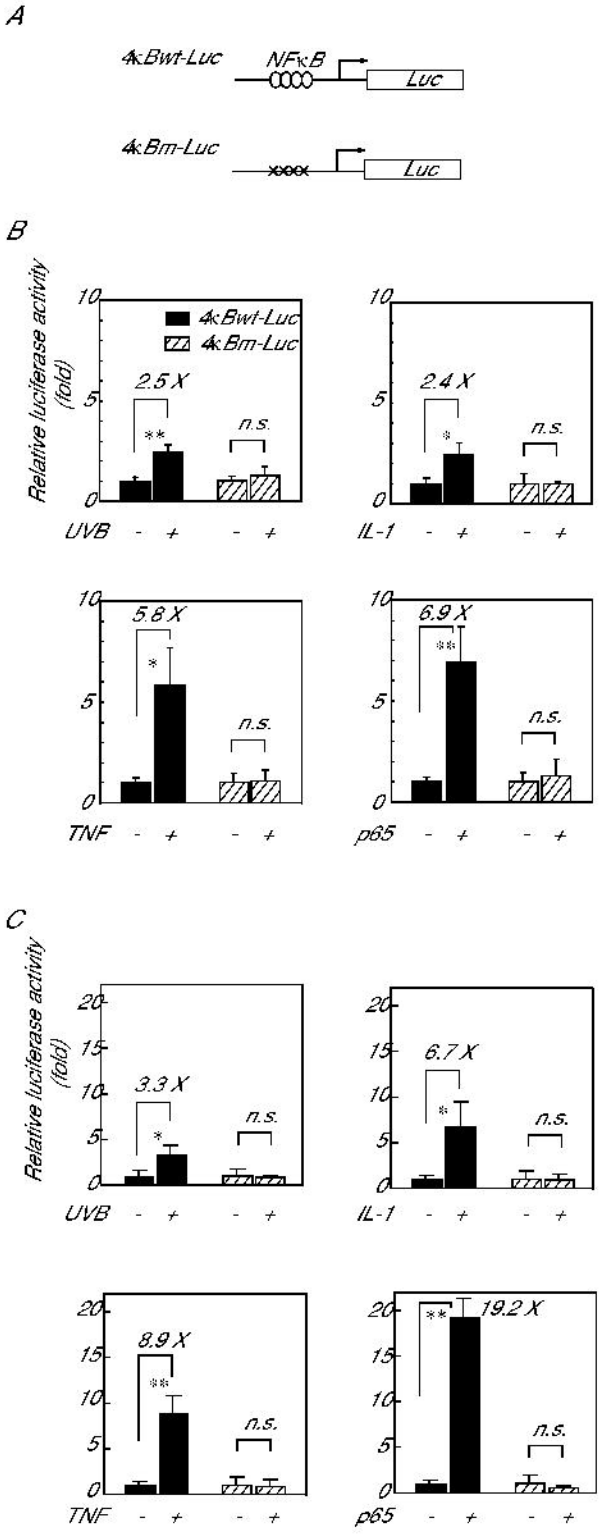

Fig. 1

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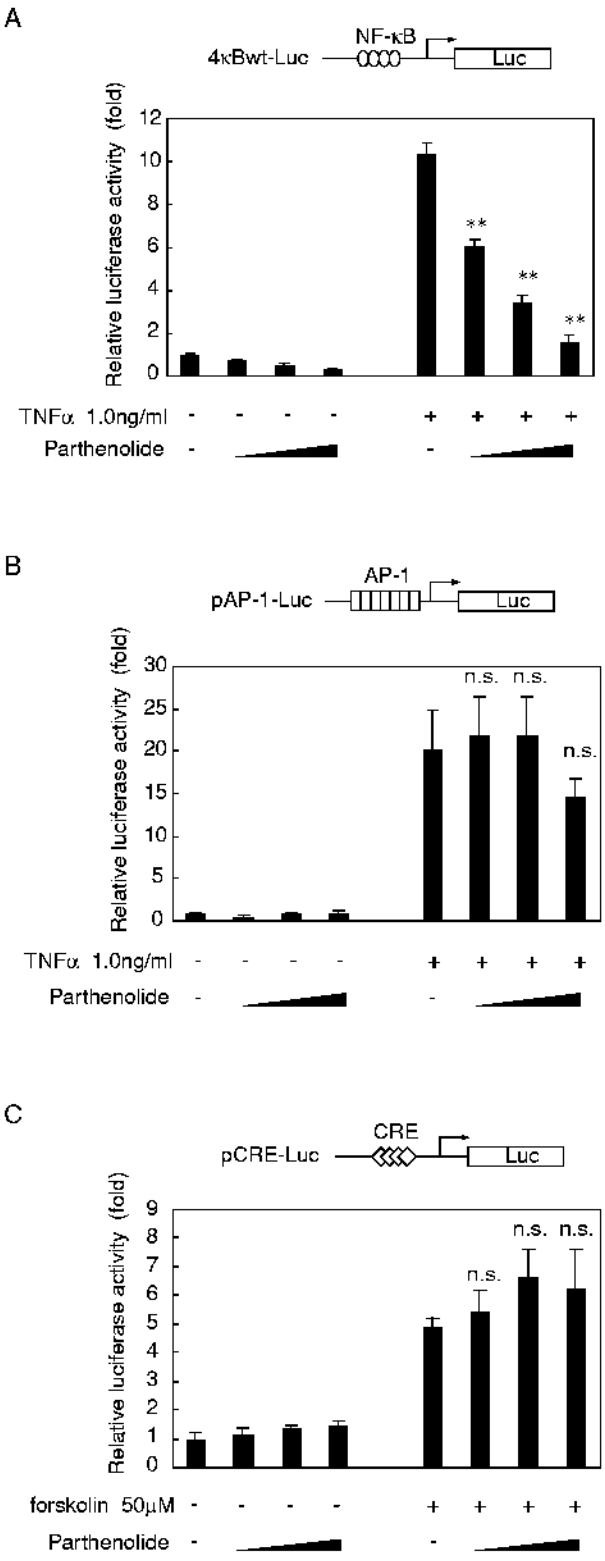

Fig. 2

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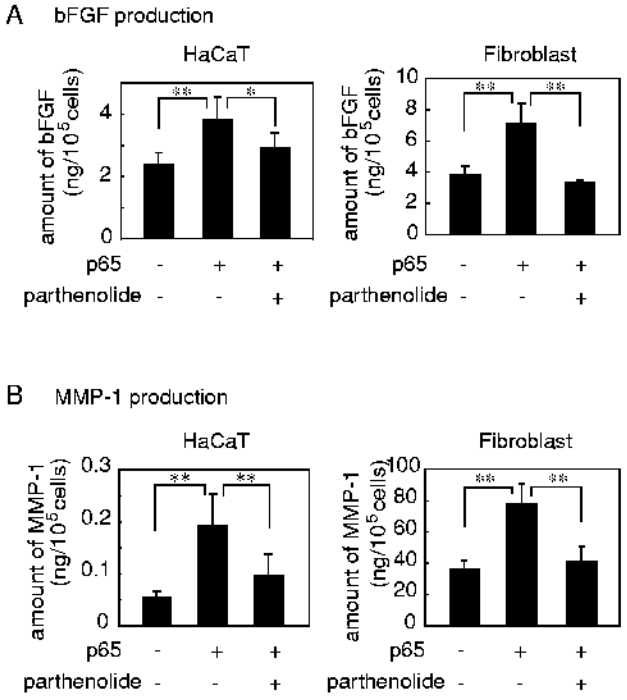

Fig. 3

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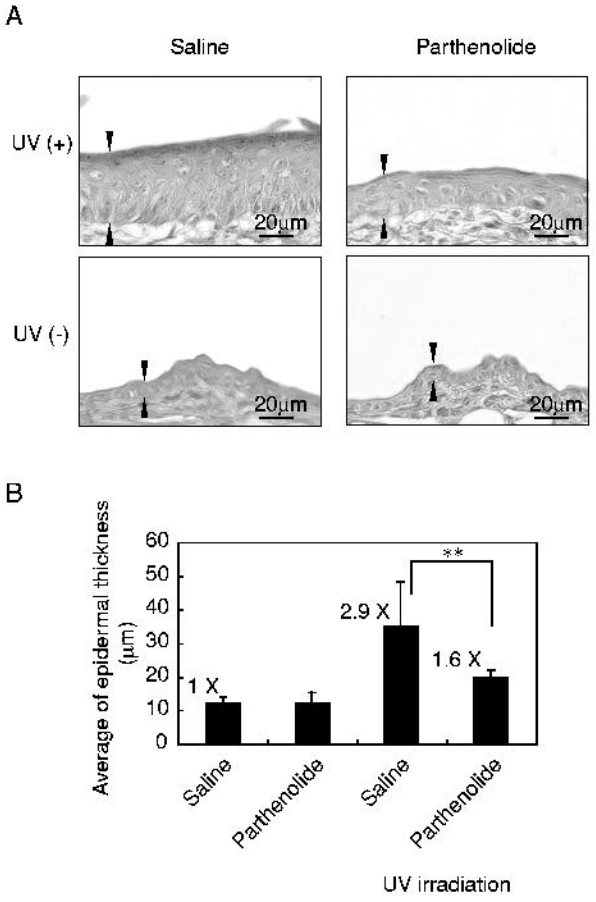

Fig. 4

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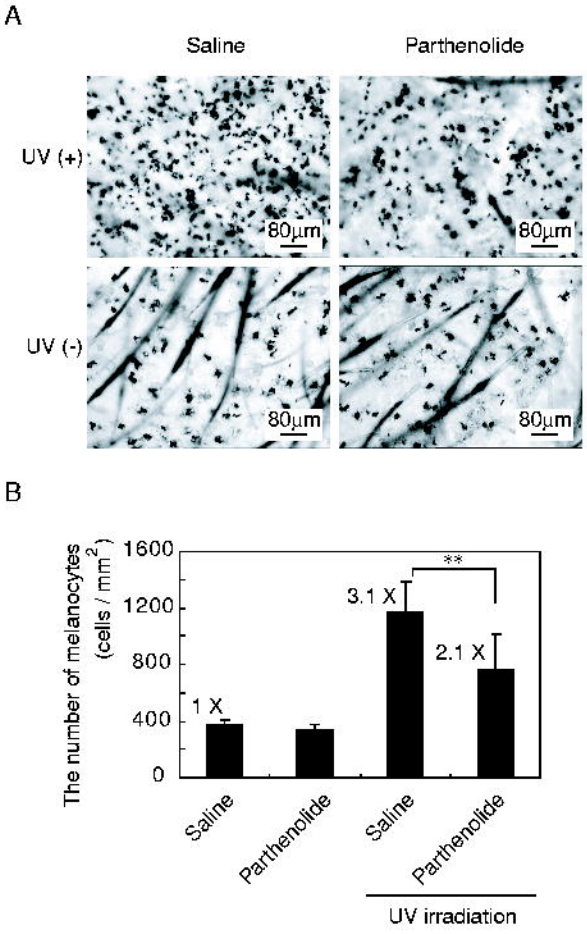

Fig. 5

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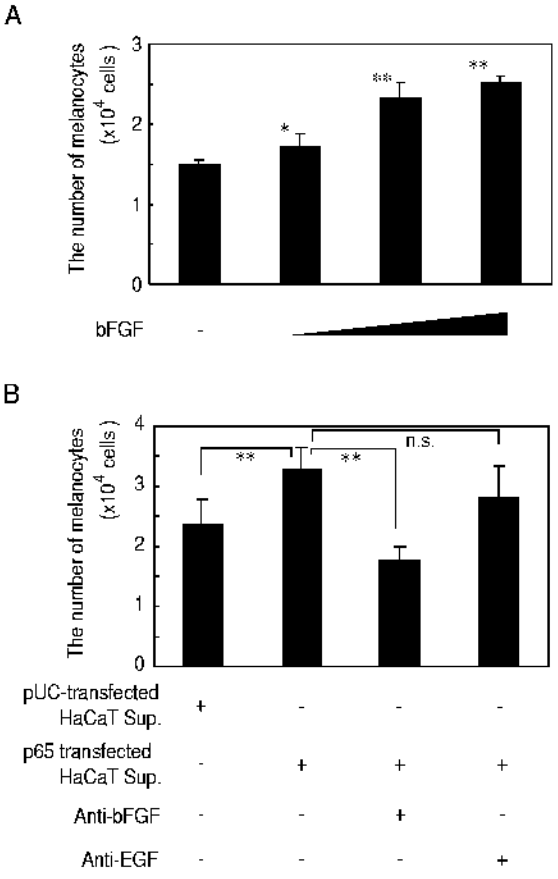

Fig. 6

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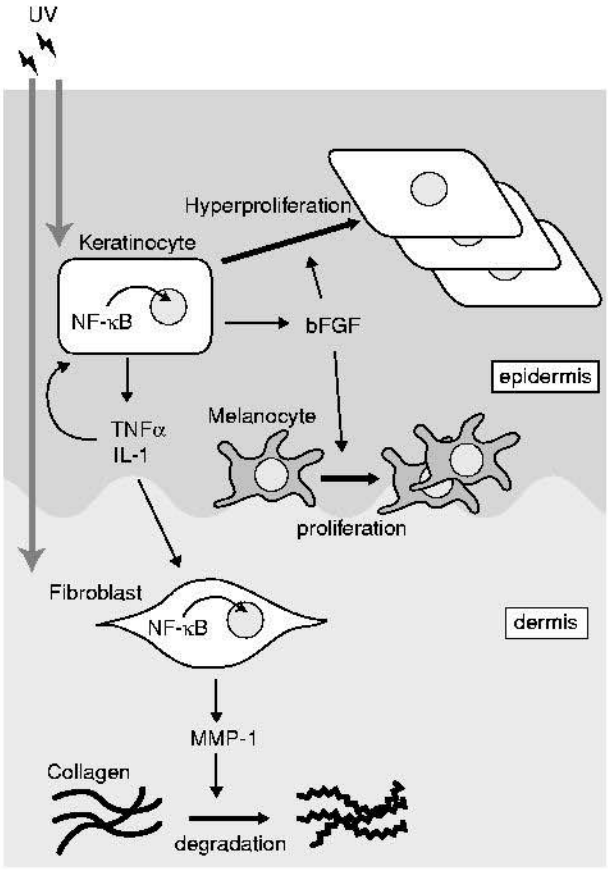

Fig. 7