Epithelial–mesenchymal interaction during UVB-induced up-regulation of neutral endopeptidase

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We recently reported that overexpression of the elastase NEP (neutral endopeptidase) by fibroblasts plays a pivotal role in the mechanism of UVB-induced skin wrinkling by degrading dermal elastic fibres. Since UVB does not penetrate to the dermis, we hypothesized that factors secreted by UVB-exposed keratinocytes in the epidermis trigger fibroblasts in the dermis to increase their expression of NEP which then degrades the elastic fibres. In the present study, we characterized the epithelial–mesenchymal interaction between keratinocytes and fibroblasts which leads to increased expression of NEP. Human fibroblasts co-cultured with UVB-exposed human keratinocytes in cell inserts significantly increased their expression of NEP at the transcriptional, translational and enzymatic levels. Neutralizing antibodies to IL-1α or GM-CSF (granulocyte/macrophage colony-stimulating factor) significantly abolished the increased expression of NEP at the enzymatic levels in human fibroblasts co-cultured with UVB-exposed human keratinocytes, whereas neutralizing antibodies to IL-6, IL-8 or TNFα (tumour necrosis factor α) had no such effect. The addition of IL-1αr or GM-CSF, but not TNFαr, IL-6 or IL-8, at concentrations ranging from 1 to 10 nM, significantly stimulated the expression of NEP in human fibroblasts at the transcriptional and translational levels. These findings suggest that IL-1α and GM-CSF are intrinsic cytokines secreted by UVB-exposed keratinocytes that stimulate expression of NEP by fibroblasts.

Key words: cytokine, elastase, fibroblast, neutral endopeptidase (NEP), skin wrinkling, UV light.

INTRODUCTION

Repetitive exposure to sunlight has been thought to be the most plausible factor that causes skin wrinkles since they frequently appear on the face. However, little is known about the precise physicochemical and biological mechanism(s) which lead to the formation of wrinkles due to sunlight exposure. In clinical studies, the formation of facial wrinkles has been associated with the loss of elastic properties of the skin [1–3]. Repetitive exposure of the skin to UVB radiation at suberythemal doses significantly reduces its elastic properties, which in turn leads to the formation of wrinkles [4]. Repetitive UVB irradiation of the skin also elicits a marked alteration in the three-dimensional structure of elastic fibres, which is closely linked to a subsequent reduction in elastic properties [5,6]. The alteration of elastic fibres has been substantiated by a marked continuous up-regulation of the elastin-degrading enzyme elastase in wrinkled skin after repetitive UVB irradiation [7,8] as well as after ovariotomy [9].

A study to determine which matrix protease(s) is/are linked to the loss of skin elasticity in the dermis of UVB-irradiated mouse skin reported a significant increase in the activity of elastase in UVB-irradiated skin compared with non-irradiated skin [9]. The increase in dermal elastase activity appeared 2 weeks after the onset of UV exposure and continued until 18 weeks later [8]. In contrast, there was a slight, but not significant, increase in the activity of collagenase I in the dermis at week 14 in UVB-irradiated skin compared with non-irradiated skin [9]. On the other hand, there was a decrease in the activity of collagenase IV in UVB-irradiated skin at the same time compared with non-irradiated skin [9]. Thus we hypothesized that enhanced elastase activity in the dermis of UVB-irradiated skin plays an important role in the degeneration of elastic fibres, which eventually results in the loss of skin elasticity. This elastase-triggered alteration of the three-dimensional structure of elastic fibres was validated by our previous UVB studies [10,11], which used a synthetic inhibitor specific for skin fibroblast elastases to demonstrate a close interrelationship between wrinkle formation, elastic properties and elastic fibre linearity, a marker for the alteration of elastic fibres. The fact that elastase inhibitors can serve as anti-wrinkling agents was also substantiated by another study [12] in which we showed that the topical application of a Zingiber officinale (L.) Rose extract that inhibits fibroblast elastase activity significantly abolished the UVB-induced wrinkle formation, reduced the degradation of elastic fibres and prevented the reduction of elastic properties. Furthermore, the clinical effect of the Z. officinale (L.) Rose extract on facial wrinkles and skin elasticity was proven during a one year clinical study of human volunteers [13]. The sum of this evidence strongly suggests that enhanced elastase activity produced by dermal fibroblasts plays a pivotal role in the mechanism of UVB-induced wrinkling of the skin.

Skin fibroblast-derived elastase is a 94 kDa membrane-bound type metalloprotease with a neutral optimum pH [14–21]. Although there were several candidate enzymes for skin fibroblast-derived elastase, such as 92, 72 kDa type IV collagenase (gelatinase) [22,23], neutrophil elastase [24], cathepsin G [25] and protease 3 [26], none of those had properties which matched the characteristics of skin fibroblast-derived elastase. Since there are similarities between skin fibroblast-derived elastase and NEP (neutral endopeptidase) [27] in terms of their size (97 000 Da), their membrane-bound nature and their inhibitory profiles, we used immunoprecipitation and Western blotting with an anti-NEP antibody to show that skin fibroblast-derived elastase is identical to NEP [28]. The identification of skin fibroblast-derived elastase as NEP served as a plausible factor that causes skin wrinkles since they frequently appear on the face.
elastase as NEP enabled us to clarify epithelial–mesenchymal interactions, especially at the gene and protein levels, whereby UVB irradiation, which penetrates only the epidermis, causes fibroblasts in the dermis to stimulate their expression of NEP. Thus we hypothesized that soluble cytokines secreted by UVB-exposed keratinocytes pass through the basement membrane into the dermis and cause dermal fibroblasts to increase their expression of NEP, which then disrupts the three-dimensional networks of elastic fibres. This results in the loss of the elastic properties of skin, an essential parameter that leads to wrinkle formation.

In the present study, we characterized the epithelial–mesenchymal interaction between UVB-exposed epidermal keratinocytes and dermal fibroblasts that leads to wrinkle formation in the skin. We determined the mechanism involved using a co-culture system in which the two cell populations are co-cultivated in different compartments that are physically separated, but can communicate via paracrine signalling through the pores of a membrane. We report that IL (interleukin)-1α and GM-CSF (granulocyte/macrophage colony-stimulating factor) are intrinsic paracrine cytokines secreted by UVB-exposed human keratinocytes that stimulate the expression of NEP by human fibroblasts.

EXPERIMENTAL

Materials

Human primary keratinocytes, human fibroblasts, serum-free keratinocyte growth medium (Medium 154S) containing low calcium (0.2 mM), BPE (bovine pituitory extract), and EGF (epidermal growth factor), were obtained from Kurabo. The human HaCaT keratinocyte cell line was supplied by Dr M. Furue (Department of Dermatology, School of Medicine, Kyushu University, Fukuoka, Japan). The synthetic substrate for elastase, STANA (N-succinyl-tri-alanyl-p-nitroaniline) was purchased from the Peptide Institute. Anti-human NEP [CD10(SN5c); sc-19586] was purchased from Santa Cruz. Neutralizing antibodies such as anti-(human IL-6) monoclonal mouse antibody (MAB206), anti-[human CXCL8 (CXC chemokine ligand 8)/IL-8] monoclonal mouse antibody (MAB208), anti-[human TNF-α (tumour necrosis factor α)] monoclonal mouse antibody (MAB2101) and anti-(human GM-CSF) monoclonal mouse antibody (MAB215) were purchased from R&D Systems. The neutralizing mouse antibody against human IL-1α (L122M) was obtained from EXALPHA Biologicals. Recombinant human IL-6 (206-IL-010), recombinant human CXCL8/IL-8 (618-010) and recombinant human GM-CSF (215-GM-010) were purchased from R&D Systems. Recombinant human IL-1α (200-01A) and recombinant human TNF-α (300-01A) were obtained from PeproTech. ELISA kits for IL-1α, IL-6, IL-8, GM-CSF and TNFα were obtained from Endogen (Thermo Fisher Scientific). Horse radish peroxidase-conjugated goat polyclonal anti-mouse IgG was obtained from Transduction Laboratories. Other chemicals of reagent grade were purchased from Sigma–Aldrich.

Cell cultures

Human fibroblasts derived from human foreskins were cultivated in DMEM (Dulbecco’s modified Eagle’s medium) with 10% FBS (fetal bovine serum), 100 mg/ml penicillin, 100 mg/ml streptomycin and 250 ng/ml amphotericin B at 37°C in a 95% air, 5% CO₂ atmosphere. Human primary keratinocytes were maintained in serum-free keratinocyte medium (Medium 154S) (Kurabo) supplemented with 5 ng/ml EGF and 50 μg/ml BPE at 37°C with 5% CO₂. HaCaT cells were maintained in DMEM with 10% FBS, 100 μg/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B at 37°C in a 95% air, 5% CO₂ atmosphere. In the experiments to measure cytokine levels using ELISA kits, HaCaT cells were cultivated in DMEM without FBS after UVB irradiation.

Co-cultures with cell culture inserts

Human primary keratinocytes or HaCaT cells were seeded in Medium 154S supplemented with 5 ng/ml EGF and 50 μg/ml BPE or in DMEM with 10% FBS respectively, at 37°C with 5% CO₂ at a density of 1.5×10⁵ or 1.0×10⁵ cells/well respectively, in six-well format cell culture inserts with translucent membranes and 1.0 μm pores (Becton Dickinson). The cell culture inserts were then inverted and placed in Multowell™ six-well plates (Becton Dickinson) where human fibroblasts had been seeded in DMEM with 10% FBS, 100 μg/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B at 37°C in a 95% air, 5% CO₂ atmosphere at a density of 2×10⁴ cells/well. After the co-culture units with the cell culture inserts had been maintained for 12 h in Medium 154S without EGF and BPE (for human keratinocytes) or in DMEM without FCS (for HaCaT cells), the cell culture inserts were exposed to UVB after exchanging the Medium 154S or DMEM with PBS. After UVB irradiation, the cell culture inserts were again inverted and placed in six-well plates and the co-culture units were cultured in Medium 154S without EGF and BPE or in DMEM without FCS for 48–72 h as indicated.

UVB irradiation

Human primary keratinocytes, HaCaT cells or human fibroblasts cultured in 10-cm-diameter dishes or in the six-well format cell culture inserts were washed twice with PBS and were then exposed to UVB. The UVB source was a SE fluorescent lamp (Clinical Supply) that emitted an energy spectrum with high fluency in the UVB region (280–320 nm) with a peak at 305 nm. The emitted dose was calculated using a UVB radiometer photodetector (Torex). The duration of UVB irradiation delivered to cells was altered by sliding a plastic lid covered with aluminum foil on to the flat-bottomed plate. After UVB irradiation, the cells or the co-culture units were maintained for 48–72 h in DMEM without FBS (for HaCaT cells) or in Medium 154S without EGF and BPE (for human primary keratinocytes) at 37°C in a 95% air, 5% CO₂ atmosphere. Control samples were mock-irradiated and were maintained under the same culture conditions as those used for the UVB-irradiated specimens. Preliminary testing determined no significant effect on cell viability of HaCaT cells, human primary keratinocytes or human fibroblasts by UVB irradiation with 80 mJ/cm² as measured by the MTT [3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyl-2H-tetrazolium bromide] assay [29].

Cytokine measurements

HaCaT cells were cultured at a density of 1.0×10⁵ cells/well in six-well format cell culture inserts, which were placed in Multowell™ six-well plates and exposed to UVB at a dose of 80 mJ/cm². The secretion levels of IL-1α, IL-6, IL-8, GM-CSF and TNFα in the conditioned medium were measured by ELISA kits at 5, 10, 20 and 40 h post-irradiation. The secretion levels are expressed as pg/ml.

Neutralizing antibody experiments

In experiments using the co-culture units with cell culture inserts, after the HaCaT cells or human primary keratinocytes were exposed to UVB and were replaced in the six-well plates with
Neutral endopeptidase-induction by keratinocyte-derived cytokines

Neutral endopeptidase (NEP), a metallopeptidase that can degrade several matrix metalloproteases, is considered as one of the enzymes responsible for the loss of skin elasticity in the UVB-exposed skin. The authors investigated the effects of UVB exposure on NEP protein and activity levels in human fibroblasts.

**RESULTS**

Effect of exposure of human fibroblasts to UVB on NEP protein and activity levels

The authors first asked whether direct UVB exposure of human fibroblasts stimulates their expression of NEP at the protein and enzymatic activity levels. Western blotting analysis for NEP protein revealed that when human fibroblasts were exposed to UVB at doses of 40 or 80 mJ/cm², NEP protein levels were significantly down-regulated at 4 h post-irradiation in a dose-dependent manner (Figure 1A). Consistently, enzymatic assays for NEP revealed that UVB irradiation at doses of 40 or 80 mJ/cm² significantly suppressed the enzymatic activity in human fibroblasts at 72 h post-irradiation (Figure 1B). Thus it is likely that direct UVB exposure of human fibroblasts is not linked to the up-regulated expression of NEP by which elastic fibres are degraded, leading to the loss of skin elasticity in the UVB-exposed and wrinkled skin.

**Figure 1** Effect of UVB exposure of human fibroblasts on NEP protein and activity levels

Effects of UVB exposure of human fibroblasts on NEP protein level (A) and on NEP enzymatic level (B). Human fibroblasts were exposed to UVB at doses of 0, 40 and 80 mJ/cm² and were then cultured for 72 h. Cell lysates were prepared at 72 h post-irradiation and were subjected to Western blotting analysis and enzymatic assay using STANA for NEP protein and activity levels respectively. Values are means ± S.D. derived from three independent experiments. Representative immunoblots from three independent experiments are shown and molecular masses in kDa are indicated. *P < 0.05; **P < 0.01.

**Statistical analysis**

All data are expressed as means ± S.D. (n = 3) unless noted otherwise. For pairwise comparisons, either the Student’s t test or Welch’s t test was applied. For multiple comparisons, data were tested by one-way ANOVA, and subsequently using the Tukey’s or Dunnet multiple comparison test. P values less than 0.05 were considered statistically significant.
Effects of UVB exposure of human keratinocytes on expression levels of NEP in human fibroblasts in co-culture

Effects of UVB exposure of human keratinocytes on NEP mRNA levels (A) and on NEP protein levels (B). (C) Effects on NEP enzymatic activity at 48 h post-irradiation. HaCaT cells cultured in cell culture inserts were washed twice with PBS and were then exposed to UVB at a dose of 80 mJ/cm². Human fibroblasts (NHF) in co-culture units with UVB-exposed HaCaT cells in cell culture inserts were cultured for 48–72 h to measure expression of NEP at the mRNA, protein and enzymatic activity levels. For (A), results are normalized to GAPDH. Values are means ± S.D. derived from three independent experiments. * P < 0.05 compared with 0 mJ/cm². For (B), representative immunoblots from three independent experiments are shown and molecular masses in kDa are indicated. In (C), the results are expressed as relative to elastase activity. Values are means ± S.D. derived from three independent experiments. * P < 0.05 and ** P < 0.01 compared with 0 mJ/cm².

Effects of UVB-exposure of human keratinocytes on the expression levels of NEP in human fibroblasts in co-culture

We asked whether UVB-exposure of human keratinocytes (in the cell inserts) stimulates the expression of NEP in human fibroblasts (in the well plates) at the gene, protein and/or enzymatic activity levels. Real-time RT–PCR for NEP mRNA revealed that UVB exposure of HaCaT cells at a dose of 80 mJ/cm² slightly stimulated the NEP mRNA level at 18 h post-irradiation and elicited a significant increase at 48 h post-irradiation (Figure 2A). Western blotting analysis for NEP protein demonstrated that UVB-exposure of HaCaT cells stimulated NEP protein levels at 48 and 72 h post-irradiation in a UVB dose-dependent manner (Figure 2B). Consistently, enzymatic assays using the substrate STANA revealed that UVB exposure of HaCaT cells stimulated the enzymatic activity of NEP at 48 h post-irradiation in a UVB dose-dependent manner with significant differences between 0 and 80 or 100 mJ/cm² (Figure 2C).

Cytokine secretion in UVB-exposed human keratinocytes

HaCaT cells were exposed to UVB at a dose of 80 mJ/cm² and levels of cytokines secreted into the conditioned medium were measured by ELISA kits at 5, 10, 20 and 40 h post-irradiation. The secretion of IL-6 and IL-8 began to significantly increase at 10 h post-irradiation and reached a plateau at 20 h post-irradiation (Figure 3A). On the other hand, the secretion of IL-1α, GM-CSF and TNFα began to significantly increase at 10 h post-irradiation and reached a plateau at 20 h post-irradiation (Figure 3B).
Neutral endopeptidase-induction by keratinocyte-derived cytokines

Figure 4  Abrogating effect of neutralizing antibodies on the stimulated NEP activity in the co-culture system with UVB-exposed human keratinocytes

Neutralizing antibodies against IL-1α (A), GM-CSF (B), IL-6 (C), IL-8 (D) and TNFα (E) were added at concentrations of 100 pg/ml, 1 ng/ml and 10 ng/ml to the co-culture system with UVB- or non-exposed HaCaT cells 0 (top panel) or 12 (bottom panel) h after UVB irradiation at a dose of 80 mJ/cm², after which the co-culture system was continued for 48 h. The lysates of human fibroblasts were then assessed for NEP activity using STANA at 48 h post-irradiation. Values are means ± S.D. from three independent experiments. *P < 0.05 and **P < 0.01.
Neutralizing antibodies against IL-1α (A) and GM-CSF (B) were added at concentrations of 100 pg/ml, 1 ng/ml and 10 ng/ml to the co-culture system with UVB- or non-exposed human primary keratinocytes at 0 h post-irradiation with a dose of 80 mJ/cm² after which the co-culture was continued for 48 h. The lysates of human fibroblasts were then assessed for NEP activity using STANA at 48 h post-irradiation. Values are means ± S.D. from three independent experiments. **P < 0.01.

Abrogation by cytokine antibodies of the stimulated NEP activity elicited by UVB-exposed human keratinocytes in the co-culture system

Since human fibroblasts co-cultured with UVB-exposed human keratinocytes significantly increased the expression of NEP at the transcriptional, translational and enzymatic levels, we then attempted to identify the cytokine(s) responsible for the stimulated expression of NEP by neutralizing experiments with antibodies against cytokines secreted in the conditioned medium. Thus co-culture units consisting of UVB- or non-exposed HaCaT cells (in the cell inserts) and human fibroblasts (in the well plates) were cultured for 48 h immediately after UVB irradiation and neutralizing antibodies to IL-1α, GM-CSF, IL-6, IL-8 and TNFα were added at 0 and at 12 h after UVB irradiation. The stimulation of NEP activity in human fibroblasts co-cultured in the 6 well plates was markedly abrogated at 48 h post-irradiation by the addition of the antibodies to IL-1α or GM-SCF at 0 h, but not at 12 h, post-irradiation (Figures 4A and 4B), whereas antibodies to IL-6, IL-8 or TNFα at 0 and at 12 h post-irradiation did not abolish the stimulation of NEP activity (Figures 4C–4E). When co-culture units consisting of UVB- or non-exposed human primary keratinocytes (in the cell inserts) and human fibroblasts (in the well plates) were cultured with neutralizing antibodies to IL-1α or GM-CSF at 0 h post-irradiation and were cultured for 48 h, the stimulation of NEP activity was markedly abrogated by both antibodies (Figure 5).

Effects of cytokines on NEP mRNA levels in human fibroblasts

To verify the results for the neutralizing experiments using the co-culture system, we examined the potential of the secreted cytokines to stimulate the NEP mRNA levels in human fibroblasts. When human fibroblasts were treated for 6–24 h with IL-1α, GM-CSF, IL-6, IL-8 or TNFα at 0, 1, 5 and 10 nM, the NEP mRNA levels were significantly stimulated over 24 h incubation by IL-1α, GM-CSF or TNFα, but not by IL-6 or IL-8 (Figure 6).

Effects of cytokines on NEP protein levels in human fibroblasts

We next examined the effects of various cytokines on NEP protein levels. When human fibroblasts were treated for 72 h with IL-1α, GM-CSF, IL-6, IL-8 or TNFα at 0, 1, 5 and 10 nM, the NEP protein levels were distinctly stimulated over 72 h incubation by IL-1α or GM-CSF, but not by IL-6, IL-8 or TNFα (Figure 7).

DISCUSSION

Although the enhanced activity of NEP in skin dermal fibroblasts is responsible for the UVB-induced cascade of biological events that leads to the degeneration of elastic fibres and reduced skin elasticity, which in turn leads to wrinkle formation [10], little is known about the mechanism(s) whereby NEP activity in dermal fibroblasts is stimulated by UVB irradiation. On the basis of the fact that UVB penetrates to the dermis to only a minor extent, but it down-regulates NEP expression in human keratinocytes in the dermis, the most plausible mechanism is that UVB elicits the production of factor(s) in the epidermis which then stimulates the expression of elastases by fibroblasts in the dermis. To test that hypothesis, we asked whether a co-culture with them stimulates the expression of matrix proteins or matrix metalloproteases, especially NEP, in human dermal fibroblasts. Real-time RT–PCR analysis revealed that a co-culture with UVB-exposed human keratinocytes stimulates NEP mRNA levels at 48 h post-irradiation. Western blotting and enzymatic assay of human fibroblasts co-cultured with the UVB-exposed human keratinocytes demonstrated that NEP protein and enzymatic levels in human fibroblasts are significantly increased at 48 h post-incubation. The sum of these findings indicates that paracrine factors are secreted by UVB-exposed human keratinocytes which penetrate into the dermis to trigger dermal fibroblasts to stimulate NEP activity. Comparison of the in vivo profiles for matrix proteins/metalloproteases in UVB-exposed and wrinkled skin and the in vitro cellular effects of conditioned medium from UVB-exposed human keratinocytes on gene expression patterns in human fibroblasts [11], revealed that the effects of the conditioned medium on fibroblasts mimics the in vivo situation in wrinkled skin despite the fact that the frequency of UVB exposure differs greatly between the in vitro and in vivo situations. This suggested that the enhanced activity of NEP or matrix metalloproteinase-1 in UVB-irradiated skin [7–9] is mediated by basement membrane-permeable soluble factors secreted by UVB-exposed keratinocytes. Taken together, the sum of available evidence supports our hypothesis for a mechanism of wrinkle formation by which cytokines are released by keratinocytes following UVB irradiation, triggering dermal fibroblasts to stimulate the expression and activity of NEP. The enhanced NEP activity then results in the deterioration of the three-dimensional architecture of elastic fibres, reducing skin elasticity and eventually leading to the formation of wrinkles.

To determine which cytokine(s) secreted from human keratinocytes following UVB irradiation are responsible for the increased expression of NEP, we previously compared the expression of NEP (measured by real-time RT–PCR) and the levels of cytokines released into the medium by UVB-exposed human primary keratinocytes at different UVB doses [11]. There were close correlations ($R^2 = 0.949$ and 0.947) between the secreted levels of IL-1α and GM-CSF respectively, and the expression of elastase mRNA, but no correlation with TNFα, IL-8 or endothelin-1. This suggested that both IL-1α and GM-CSF play roles in the stimulation of the expression of NEP [10,11], although additional studies using neutralizing antibodies was required to reach a final conclusion.
Figure 6  Effects of cytokines on NEP mRNA levels in human fibroblasts as revealed by real-time RT–PCR analysis

Effects of IL-1α (A), GM-CSF (B), IL-6 (C), IL-8 (D) and TNFα (E) on NEP mRNA levels. Cytokines were added at the indicated concentrations to the medium of human fibroblasts in culture, after which human fibroblasts were cultured for 24 h. NEP mRNA levels in human fibroblasts were measured by real-time RT–PCR at 6, 12 and 24 h post-incubation. Values are means ± S.D. derived from three independent experiments. *P < 0.05 compared with 0 nM.

Figure 7  Effects of cytokines on NEP protein levels in human fibroblasts as revealed by Western blotting analysis

Effects of IL-1α (A), GM-CSF (B), IL-6 (C), IL-8 (D) and TNFα (E) on NEP protein levels. Cytokines were added at the indicated concentrations to the medium of human fibroblasts in culture, after which human fibroblasts were cultured for 24 h. NEP protein levels in human fibroblasts were measured by Western blotting analysis at 72 h post-incubation. Representative immunoblots from three independent experiments are shown and molecular masses in kDa are indicated. Values in the histograms are means ± S.D. derived from three independent experiments. *P < 0.05 and **P < 0.01 compared with 0 nM.
To determine which cytokines secreted by UVB-exposed human keratinocytes are intrinsic soluble factors that cause dermal human fibroblasts to stimulate the activity of NEP, we used the co-culture system to evaluate the inhibitory effects of several neutralizing antibodies to cytokines or a chemokine secreted by UVB-exposed human keratinocytes on NEP expression in human fibroblasts. Those results revealed that among the neutralizing antibodies tested (anti-IL-1α, anti-GM-CSF, anti-IL-6, anti-IL-8 and anti-TNFα antibodies), the anti-IL-1α and anti-GM-CSF (but not anti-IL-6, anti-IL-8 or anti-TNFα) antibodies significantly abolished the enhanced activity of NEP in human fibroblasts after the co-culture with UVB-exposed human keratinocytes. The sum of these findings strongly suggests that IL-1α and GM-CSF are intrinsic keratinocyte-derived cytokines associated with the up-regulated expression of NEP. Those two antibodies also reduced the activity of NEP after the co-culture with non-exposed human keratinocytes. These findings indicate that the co-culture with non-exposed human keratinocytes also contains IL-1α and GM-CSF at concentrations sufficient, although to a lesser extent, to stimulate NEP activity in human skin fibroblasts. Of considerable interest in the co-culture experiments using neutralizing antibodies is the fact that the addition of anti-IL-1α or anti-GM-CSF antibodies at 12 h post-irradiation did not abolish the up-regulated activity of NEP, although their addition at 0 h post-irradiation elicited significant preventive effects on the enhanced activity of NEP. As the secretion of IL-1α and GM-CSF was significantly increased even at 10 h post-irradiation, the lack of a neutralizing effect by the later timing of addition suggests that the initial release of IL-1α and GM-CSF by UVB-exposed human keratinocytes had already proceeded at 12 h post-irradiation.

To ascertain whether the neutralizing effects of the antibodies against IL-1α or GM-CSF are associated with the potential to stimulate the expression of NEP in human fibroblasts, we assessed the effects of the corresponding cytokines on the expression of NEP in the gene and protein levels. Those results demonstrated that although IL-1α, GM-CSF and TNFα, but not IL-6 or IL-8, have the potential to significantly stimulate the expression of NEP mRNA in human fibroblasts, the addition of IL-1α or GM-CSF, but not IL-6, TNFα or IL-8, up-regulates the protein levels of NEP. Although TNFα has distinct effects at the gene and protein levels in an opposite way, probably due to TNFα-enhanced proteolysis, these findings indicate that IL-1α and GM-CSF exclusively have the potential to stimulate NEP expression by skin fibroblasts and antibodies against those two cytokines are specifically associated with neutralizing effects on the enhanced NEP activity in human fibroblasts co-cultured with UVB-exposed human keratinocytes.

Before speculating about the paracrine cytokine mechanism involved in the activation of NEP in vivo, it should be noted that the co-culture system used a single UVB exposure in contrast to the repeated UVB exposure under in vivo conditions required for wrinkle formation [6–8]. In spite of this reservation, our data clearly show that IL-1α and GM-CSF are the only cytokines whose antibodies have specific neutralizing effects on the stimulated activity of NEP in human fibroblasts co-cultured with UVB-exposed human keratinocytes. Consistent with this, they are the only cytokines capable of significantly stimulating the gene and protein expression of NEP in human fibroblasts. Although it remains to be clarified whether, similar to the interaction between IL-1α and IL-8 [30], IL-1α serves in an autocrine loop to trigger the secretion of GM-CSF [31], the same timing for the onset of the UVB-induced increase in the secretion of IL-1α and GM-CSF seems to indicate that UVB radiation directly causes keratinocytes to secrete GM-CSF, which then stimulates the expression of NEP in human fibroblasts.

Thus we propose a UVB-induced wrinkling mechanism as follows. As depicted in Figure 8, repetitive UVB exposure causes keratinocytes to secrete IL-1α and GM-CSF. The secreted IL-1α and GM-CSF penetrate the dermis to stimulate the expression of NEP by fibroblasts, which then cleaves elastic fibres surrounding the fibroblasts, leading to deterioration of the threedimensional configuration of elastic fibres. This results in a loss of skin elasticity and in turn leads to wrinkle formation.

**REFERENCES**


Imokawa, G. (2008) Recent advances in characterizing biological mechanisms underlying


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