



# Pre-elafin is Involved in Ultraviolet-induced Keratinocyte Apoptosis via Pro-caspase-3 Activation Associated with Cystatin-A Down-regulation

Hyangmi KIM<sup>1</sup>, Minsoo NOH<sup>2</sup>, Ok-Nam BAE<sup>3</sup>, Chang-Hoon LEE<sup>4</sup> and Ai-Young LEE<sup>1</sup>

<sup>1</sup>Department of Dermatology, Dongguk University Ilsan Hospital, Gyeonggi-do, <sup>2</sup>College of Pharmacy, Natural products Research Institute, Seoul National University, Seoul, <sup>3</sup>College of Pharmacy, Institute of Pharmaceutical Science and Technology, Hanyang University, Ansan, and <sup>4</sup>College of Pharmacy, Dongguk University, Seoul, South Korea

**Pre-elafin controls keratinocyte integrity via cornified envelope formation and inhibition of desquamation, but its role in ultraviolet (UV)-induced keratinocyte apoptosis is unknown. This study examined the role of pre-elafin in volunteer skin samples and primary cultured normal human keratinocytes irradiated with phototoxic doses of UVA/narrow-band UVB, and in keratinocytes with pre-elafin overexpression/knockdown, under conditions of low and high calcium. Phototoxic doses of UV increased pre-elafin mRNA and protein expression in inverse proportion to keratinocyte survival. Pre-elafin overexpression under conditions of low calcium, which, in contrast to conditions of high calcium, was localized to the cytoplasm, increased keratinocyte apoptosis, whereas knockdown inhibited UV-induced apoptosis. Pre-elafin was co-localized with, but not bound to, cleaved caspase-3. Pre-elafin reduced cystatin-A expression, which was bound to pro-caspase-3. In conclusion, UV phototoxicity-induced pre-elafin inside keratinocytes prior to cornified envelope formation could be involved in UV-induced keratinocyte apoptosis via cystatin-A downregulation resulting in pro-caspase-3 activation.**

**Key words:** UV; keratinocyte apoptosis; pre-elafin; cellular location; cystatin-A; pro-caspase-3.

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**Corr:** Ai-Young Lee, Department of Dermatology, Dongguk University Ilsan Hospital, 814 Siksa-dong, Ilsandong-gu, Goyang-si, Gyeonggi-do 410-773, South Korea. E-mail: lay5604@naver.com

Overexposure to ultraviolet radiation (UVR) induces acute and long-term phototoxicities on human skin. UVR-induced DNA damage is important in the development of these harmful effects. If DNA damage is not completely removed, growth arrest or apoptosis occur to protect the cells from carcinogenesis (1). The sunburn cell, which is characterized by pyknotic nuclei and eosinophilic cytoplasm, is an apoptotic cell with positive staining on terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay (2, 3). Considering that these cells are keratinocytes, sunburn cells could be a representative example of UV-induced keratinocyte apoptosis. Although molecular pathways have been mentioned as the mechanism of UVR-induced

keratinocyte apoptosis (4, 5), the detailed picture of UV-induced apoptosis remains to be clarified.

Apoptosis pathways converge to activate pro-caspases. However, there is increasing evidence that proteases other than caspases, such as cathepsins and endogenous cathepsin inhibitor cystatin-A, are also involved in apoptosis of keratinocytes (6–9). The role of serine protease and inhibitors in apoptosis has been suggested, despite differing results (10–12). In a microarray analysis of human skin samples, which were irradiated by a single phototoxic dose of UVA and narrow-band UVB (NB-UVB), the serine protease inhibitor pre-elafin was found to be an upregulated differentially expressed gene (DEG) common to both UVA and NB-UVB-induced phototoxicities. Pre-elafin, also known as skin-derived anti-leukoproteinase or skin-derived peptidase inhibitor 3 (PI3), is a secretory protein derived from the precursor pro-pre-elafin. Pre-elafin is formed by cleavage of a signal sequence from the precursor and contains a putative substrate domain for epidermal transglutaminase (TG) at the N-terminus and a proteinase-inhibiting domain at the C-terminus (13). Elafin is released from pre-elafin by proteolytic cleavage (14), although the term elafin has often been used regardless of precursor form (pre-elafin) and cleaved form (real elafin).

Pre-elafin in human skin has previously been shown to play at least 2 different roles in the control of epithelial integrity, depending on keratinocyte differentiation: cornified envelope (CE) formation in terminally differentiated keratinocytes and desquamation inhibition in keratinocytes before terminal differentiation (15, 16). In this study, another role of pre-elafin in the apoptosis of keratinocytes damaged by UV phototoxicity was examined in primary cultured human keratinocytes and in skin samples from volunteers irradiated with phototoxic doses of UVA and NB-UVB, as well as in cultured keratinocytes subject to pre-elafin overexpression or knockdown. Calcium is an important regulator of keratinocyte differentiation (17). For CE formation in terminal differentiation, constituting proteins of keratinocytes should be cross-linked by TG. TG activity is also regulated by calcium. Therefore, the role of pre-elafin in cultured keratinocytes under low or high calcium concentrations was examined in order to identify whether cellular location of pre-elafin involved in the apoptosis was different from that involved in CE formation or in inhibition of desquamation.

## MATERIALS AND METHODS (for complete details see APPENDIX S1<sup>1</sup>)

### *Skin samples from volunteers*

UVA-irradiated, NB-UVB-irradiated, and non-irradiated abdominal skin specimens were obtained from 8 healthy volunteers for real-time PCR and immunofluorescence analysis. For irradiation, 3 minimal erythema doses (MEDs) of UVA and NB-UVB were used. All volunteers provided written informed consent prior to the study. The Institutional Review Board of Dongguk University Ilsan Hospital approved this study, which was conducted according to the principles of the Declaration of Helsinki.

### *Primary cultured normal human keratinocytes*

Adult skin specimens were obtained from previous Caesarean section scars and used to establish cell cultures. Primary cultured normal human keratinocytes were irradiated with single doses of UVA or NB-UVB, producing 70–80%, 50%, and 20–30% cell survival, or were transfected with a pCMV plasmid containing the skin-derived *PI3* gene or small interfering (si) RNA against human pre-elafin in the absence or presence of extra calcium (low or high concentrations of calcium, respectively). Cells were harvested 48 h after treatment, and were subjected to a cell viability assay, a cytotoxicity test, fluorescence-activated cell sorter (FACS) analysis, real-time PCR, Western blot analysis, immunoprecipitation, and confocal microscopy.

### *Statistical analysis*

Statistical analysis was performed using Student's *t*-test. The results are expressed as the mean  $\pm$  standard deviation (SD). A *p*-value  $< 0.05$  was considered significant.

## RESULTS

### *UVR-induced phototoxicity increases pre-elafin expression in human skin and primary cultured normal human keratinocytes*

Previous experiments indicate that Fas expression, which is important and dose-dependent in UV-induced apoptosis, does not occur at doses below 3 MEDs UVA (18). In this study, irradiation with 3 MEDs UVA and NB-UVB also increased the number of sunburn cells compared with non-irradiated control ( $p < 0.05$ ; Fig. 1a). Hence, 3 MEDs UVA and NB-UVB were applied in 8 abdominal skin samples from volunteers. Pre-elafin mRNA expression was increased in UVA-irradiated and NB-UVB-irradiated compared with non-irradiated skin ( $p < 0.05$ ; Fig. 1b). Immunohistochemistry using anti-pre-elafin antibody in paired (non-irradiated, UVA-irradiated, NB-UVB-irradiated) skin specimens from the same volunteers showed stronger staining intensities with irradiation of either UVA or NB-UVB. Without UV irradiation, pre-elafin expression was hardly detectable (Fig. 1c). Irradiation with different doses of UVA (4, 10 and 12 J/cm<sup>2</sup>) or NB-UVB (500, 1,000 and 1,200 mJ/cm<sup>2</sup>), which showed 70–80% cell survival, 50%

cell survival, and 20–30% cell survival, respectively, in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, increased pre-elafin mRNA and protein expression in a dose-dependent manner in primary cultured human keratinocytes ( $p < 0.05$ , Fig. 1d,e, respectively).

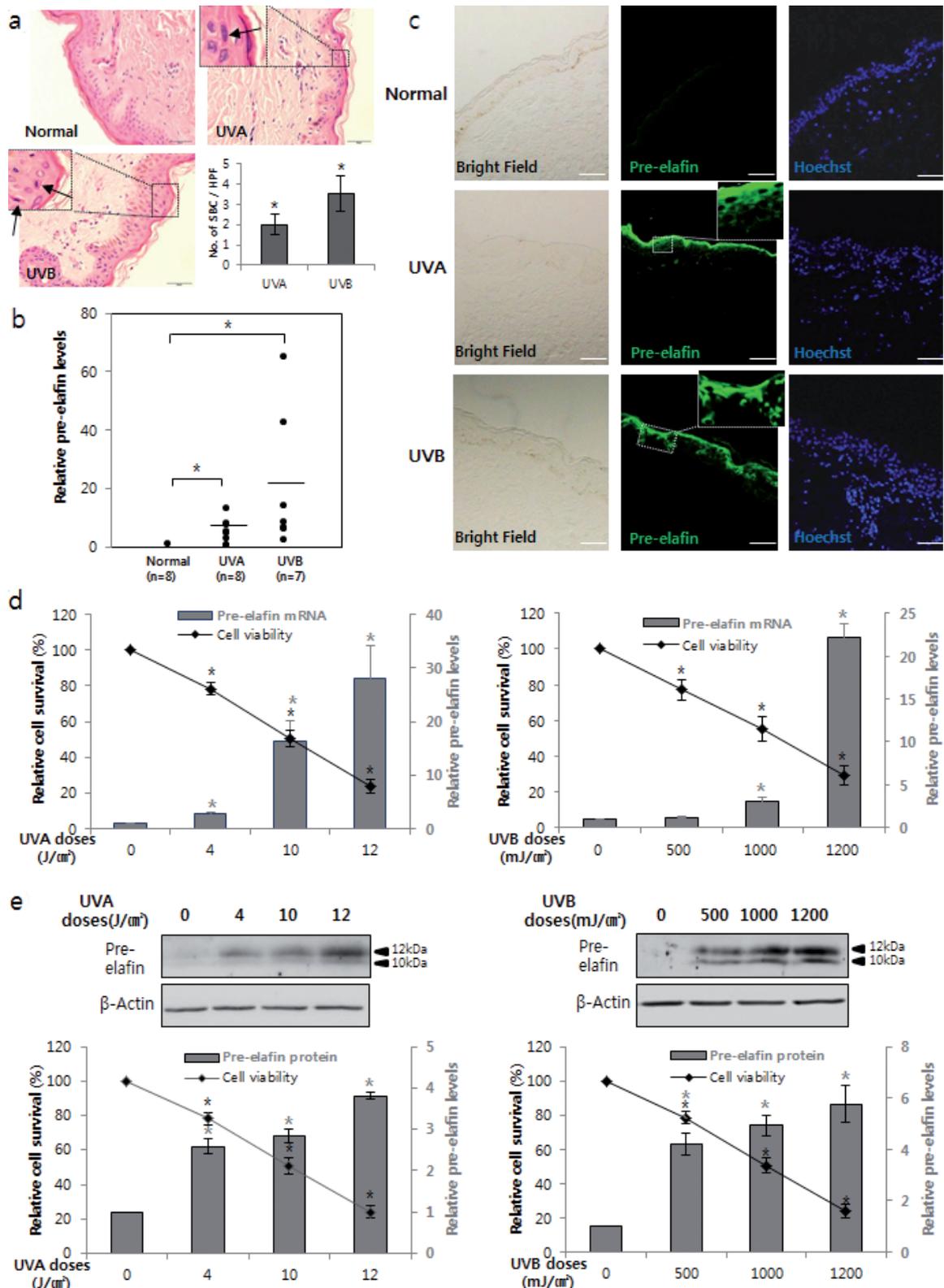
### *Pre-elafin stimulates keratinocyte apoptosis*

Based on the observation that increases in pre-elafin mRNA and protein expression are inversely proportional to the survival of UV-irradiated keratinocytes (Fig. 1d, e), the effect of pre-elafin on cell survival/death was examined in primary cultured normal human keratinocytes. A preliminary time-course analysis of Western blots for apoptosis and cell survival-related molecules from day 1 to day 6 (data not shown), suggested that overexpression and knockdown experiments of pre-elafin should be carried out for 48 h. Because levels of pre-elafin expression were too low in keratinocytes without UV irradiation (Fig. 2a), UV irradiation was used to induce pre-elafin expression levels in keratinocytes for knockdown study. Overexpression, which increased the expression of pre-elafin protein in the form of 2 bands (12 kDa and 10 kDa (Fig. 2a)) similar to keratinocytes irradiated with phototoxic doses of UVR, reduced the number of viable keratinocytes by MTT assay ( $p < 0.05$ , Fig. 2b) and increased the cytotoxicity by lactate dehydrogenase (LDH) release ( $p < 0.05$ , Fig. 2c). FACS analysis showed that the percentage of annexin V-positive/propidium iodide (PI)-negative (early apoptotic) and double-positive (late apoptotic) cells (19) was increased by the overexpression ( $p < 0.05$ , Fig. 2d). Pre-elafin overexpression increased the activation of pro-caspase-9 and pro-caspase-3, but decreased the phosphorylation of PI3K, AKT, ERK and NF- $\kappa$ B ( $p < 0.05$ ; Fig. 2e). On the other hand, pre-elafin knockdown reduced the changes induced by phototoxic doses of UVA and NB-UVB in cleavage of pro-caspase-9 and pro-caspase-3 and phosphorylation of PI3K, AKT, ERK and NF- $\kappa$ B ( $p < 0.05$ ; Fig. 2f).

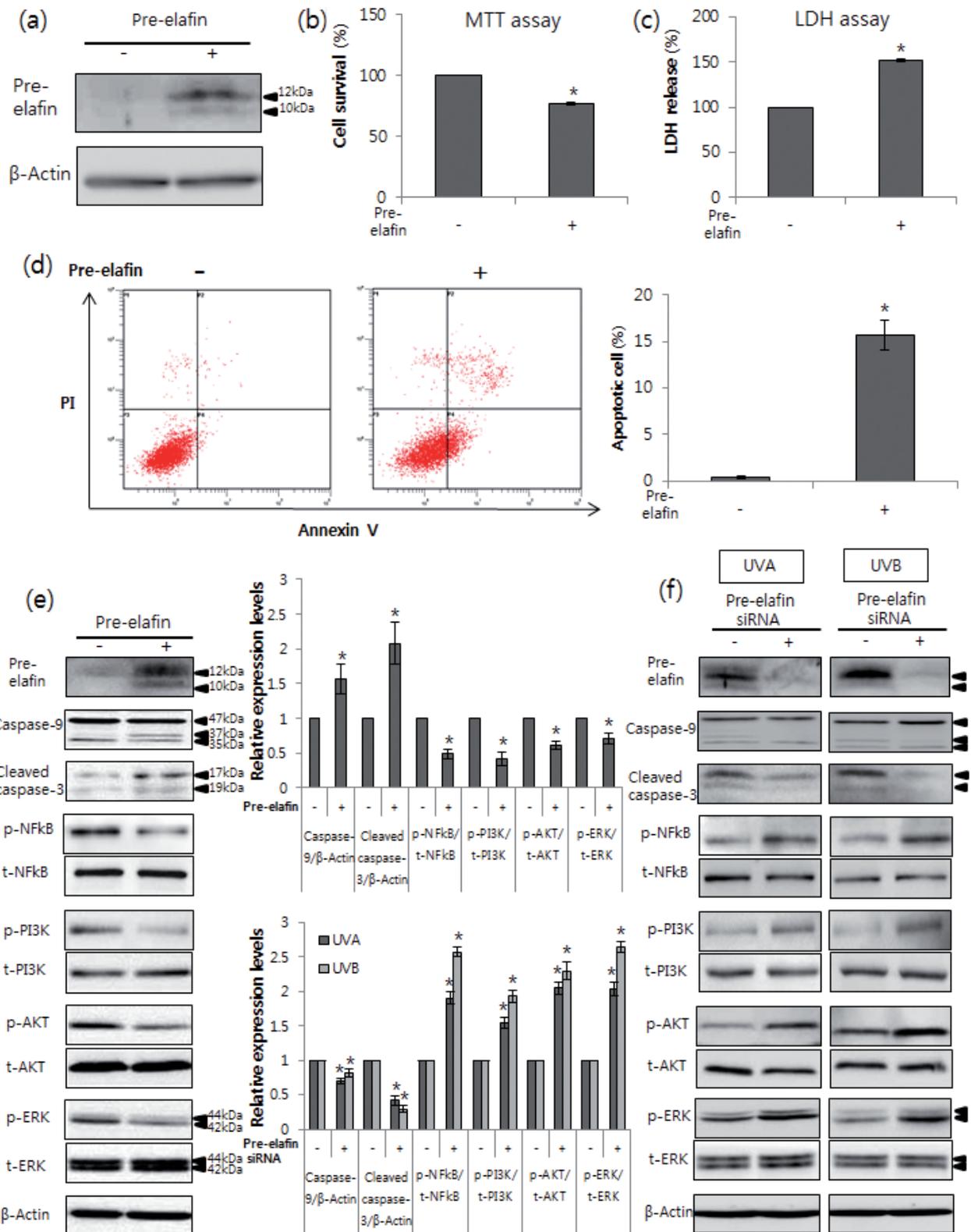
### *Pre-elafin increases pro-caspase-3 activation via reducing pro-caspase-3-bound cystatin-A expression*

Pre-elafin overexpression stimulated keratinocyte apoptosis along with an increase in cleaved caspase-3 expression, whereas knockdown inhibited UV phototoxicity-induced keratinocyte apoptosis along with a decrease in cleaved caspase-3 (Fig. 2e, f, respectively). We examined whether pre-elafin was directly involved in pro-caspase-3 activation in keratinocytes irradiated with phototoxic doses of UV. If so, whether there was a connection between pre-elafin and cystatin-A based on the known role of cystatin-A in apoptosis of keratinocytes (9). Confocal microscopy after double staining with anti-pre-elafin and cleaved caspase-3 antibodies, showed that phototoxic doses of UV increased the number and

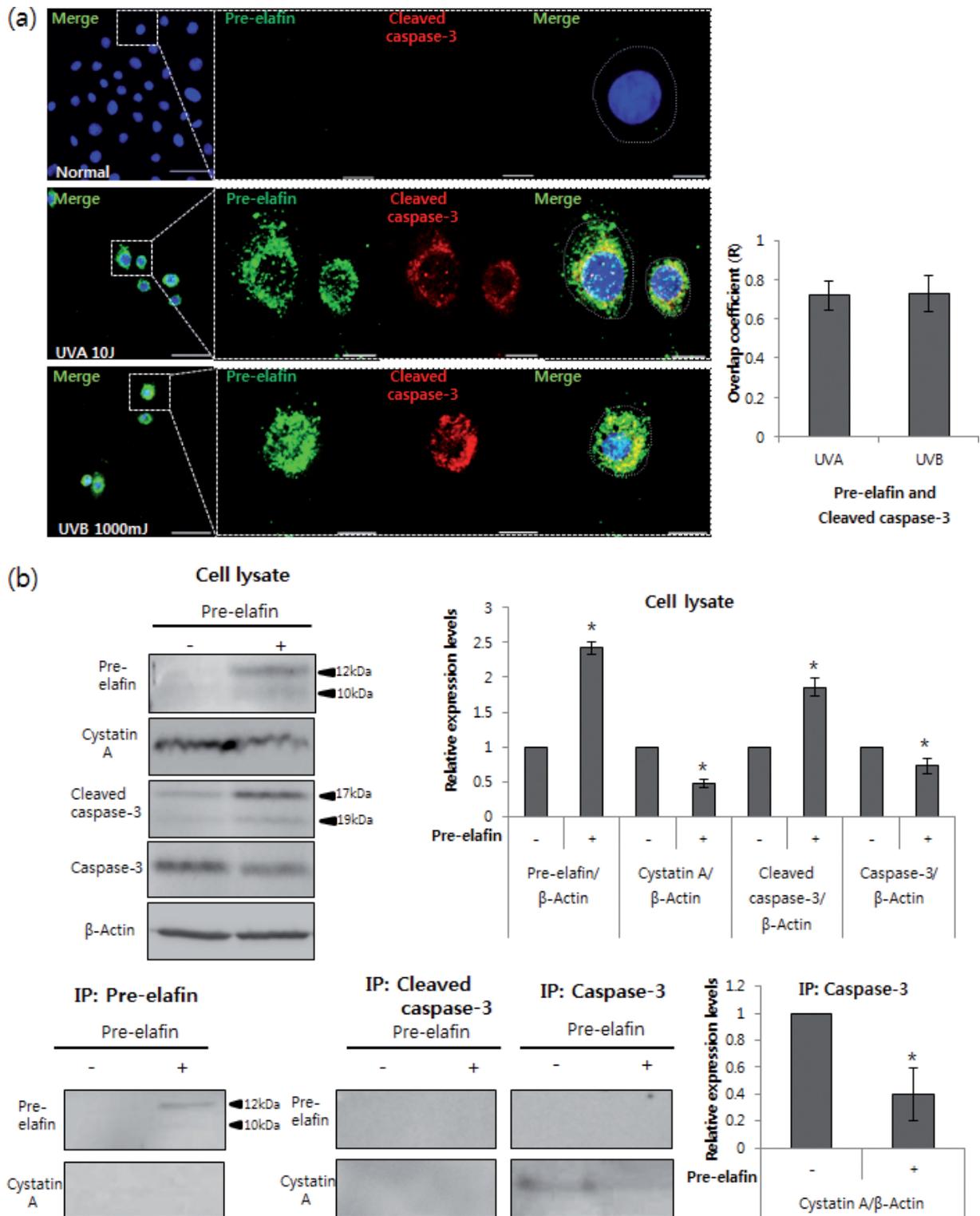
<sup>1</sup><https://doi.org/10.2340/00015555-2621>



**Fig. 1. Ultraviolet radiation (UVR)-induced phototoxicity increased pre-elafin expression in human skin and primary cultured normal human keratinocytes.** (a) Representative microscopic findings stained with haematoxylin and eosin. (b) Real-time PCR of relative pre-elafin mRNA expression. (c) Representative immunofluorescence staining with anti-pre-elafin antibody, whose nuclei were counterstained with Hoechst 33258, in abdominal skin specimens from 8 healthy volunteers at 2 days after irradiation with the 3 minimal erythema doses (MEDs) of ultraviolet A (UVA) and narrow band-UVB (NB-UVB) (*bar* = 100  $\mu$ m). *Arrow* indicates sunburn cell (SBC). (d) Real-time PCR. (e) Western blot analysis to compare relative pre-elafin mRNA and protein expression levels in cell lysates of cultured keratinocytes at 48 h after irradiation with the 3 different cytotoxicity doses of UVA (4, 10 and 12 J/cm<sup>2</sup>) and NB-UVB (500, 1,000 and 1,200 mJ/cm<sup>2</sup>) along with corresponding rates of cell survival (70–80%, 50% and 20–30%).  $\beta$ -actin was used as a loading control. Data in the graph represents mean  $\pm$  standard deviations (SD) of relative values compared with non-irradiated control from 3 independent experiments (\**p* < 0.05 vs. non-irradiated control, HPF: high-power field).



**Fig. 2. Pre-elafin stimulated keratinocyte apoptosis.** (a) Pre-elafin overexpression increased expression of pre-elafin protein in cell lysates. β-actin was used as a loading control. (b) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay results showing cell viability. (c) Lactate dehydrogenase (LDH) release for cytotoxicity. (d) Fluorescence-activated cell sorter (FACS) analysis for apoptosis proportion in cultured keratinocytes with or without overexpression of pre-elafin for 48 h. Data in the graph represents mean ± standard deviations (SD) of relative values, compared with keratinocytes with overexpressing control RNA from 3 independent experiments (\**p* < 0.05 vs. control). (e, f) Western blotting using corresponding antibodies in cultured keratinocytes with or without pre-elafin overexpression for 48 h (e) and in phototoxic dose of UV-irradiated keratinocytes with or without pre-elafin knockdown for 48 h (f). β-actin was used as a loading control. Data in the graph represents mean ± SD of relative values compared with keratinocytes with overexpressing control RNA (e) and keratinocytes irradiated with phototoxic dose of UVR with control siRNA knockdown (f) from 3 independent experiments (\**p* < 0.05).



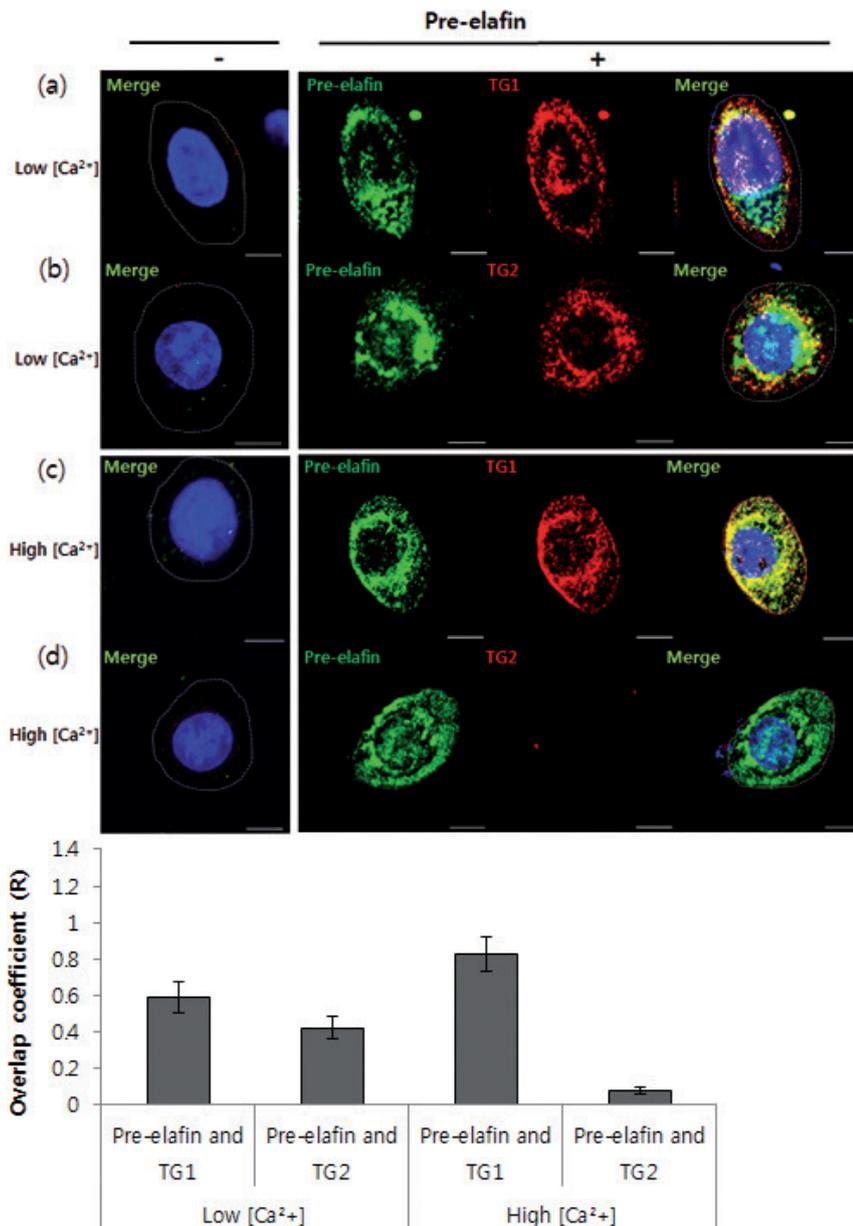
**Fig. 3. Pre-elafin increased pro-caspase-3 activation via reducing pro-caspase-3-bound cystatin-A expression.** (a) Representative confocal microscopy analysed after staining of keratinocytes 48 h after irradiation with phototoxic dose of ultraviolet A (UVA) and narrow band-UVB (NB-UVB) using anti-pre-elafin and cleaved caspase-3 antibodies (*bar* = 50  $\mu$ m) with magnification of the representative cell under each condition (*bar* = 10  $\mu$ m). Overlap coefficient (R) to demonstrate co-localization efficiency was calculated using Wright Cell Imaging Facility (WCIF) ImageJ software, Toronto, Canada. Dotted line indicates cellular boundary. (b) Immunoprecipitation using anti-pre-elafin, caspase-3 or cleaved caspase-3 in keratinocytes with/without pre-elafin overexpression for 48 h.  $\beta$ -actin was used as a loading control. Data in the graph represents mean  $\pm$  standard deviations (SD) of relative values compared with keratinocytes without overexpression from 3 independent experiments (\**p* < 0.05).

staining intensity of double-positive cells. In addition, considerable levels of pre-elafin, which was detected in the cytoplasm, but not in the cell membrane of irradiated keratinocytes, was co-localized with cleaved caspase-3 (Fig. 3a). Without UV irradiation, none of the cells were stained by pre-elafin or activated caspase-3 (Fig. 3a). However, immunoprecipitation did not show binding between pre-elafin and activated caspase-3 (Fig. 3b). On the other hand, pre-elafin reduced cystatin-A expression without directly binding to cystatin-A ( $p < 0.05$ ; Fig.

3b). Cystatin-A was bound to pro-caspase-3, but not to activated caspase-3 ( $p < 0.05$ ; Fig. 3b).

*Pre-elafin is increased in the cytoplasm, but not in the cell membrane of keratinocytes under conditions of low calcium*

Increased pre-elafin involved in keratinocyte apoptosis was located in the cytoplasm beneath the cell membrane of cells cultured in low-calcium media (Fig. 3a). Because the 2 different roles of pre-elafin under normal physiological conditions, CE formation and desquamation inhibition, depend on keratinocyte differentiation (15, 16), cellular location of the upregulated pre-elafin was compared under different calcium conditions. TG1 is known to be bound to the plasma membrane from the cytoplasmic side, whereas TG2 is a cytoplasmic protein (15, 16). In addition, TG1 forms CE in terminally differentiating keratinocytes. Therefore, co-localization between pre-elafin and TG1 or TG2 was examined in pre-elafin-overexpressing keratinocytes cultured in low-calcium and high-calcium media for 48 h. Confocal microscopy showed, that under low-calcium conditions, pre-elafin increased the TG1 and TG2 expression levels, and it was co-localized with some of these levels in the cytoplasm of pre-elafin-overexpressing keratinocytes compared with control keratinocytes (Fig. 4a, b, respectively). On the other hand, under high-calcium conditions, pre-elafin increased TG1 expression levels and it was mostly co-localized with TG1 (Fig. 4c). TG2 expression was not detectable in high-calcium media (Fig. 4d).



**Fig. 4. Pre-elafin was increased in the cytoplasm, but not in the cell membrane of keratinocytes under conditions of low calcium.** Representative confocal microscopy analysed in pre-elafin-overexpressing keratinocytes cultured in low-calcium media for 48 h by double-staining with anti-pre-elafin and (a) TG1 or (b) TG2 antibodies. Representative confocal microscopy analysed in pre-elafin-overexpressing keratinocytes cultured in high-calcium media for 48 h by double-staining with anti-pre-elafin and (c) TG1 or (d) TG2 antibodies (bar = 10  $\mu$ m). Overlap coefficient (R) indicating co-localization efficiency was calculated using WCIF ImageJ software. Dotted line indicates cellular boundary.

## DISCUSSION

Increased expression of pre-elafin has been identified in a number of inflammatory and proliferative skin diseases, particularly in psoriasis (20, 21). In relation to UVR, a protective role of pre-elafin derived from UV-irradiated dermal fibroblasts in elastic fibre degeneration was investigated (22), although the main source of pre-elafin generation is keratinocytes (23). In this study, the results of real-

time PCR and immunofluorescence showing increased expression of pre-elafin, in both UVA and NB-UVB-irradiated skin from 8 healthy volunteers (Fig. 1b, c, respectively), indicated a potential role of pre-elafin in UVR-induced phototoxicity on epidermal keratinocytes. Dose-dependent increases in pre-elafin mRNA and protein expression in the cell lysates of primary cultured human keratinocytes irradiated with UVA and NB-UVB, reaching 70–80%, 50%, and 20–30% of cell survival (Fig. 1d, e), supported that UV phototoxicity generated pre-elafin. Although foetal calf serum induces pre-elafin (23), keratinocytes in this study were cultured in a serum-free medium. Therefore, UVR-induced skin phototoxicity could be another example of pre-elafin induction in keratinocytes.

The role of pre-elafin in UV-induced cytotoxicity on keratinocytes has not hitherto been examined. The reported serine protease inhibitor involved in UVB-induced keratinocyte apoptosis is hurpin (10). In this study, the decrease in the number of viable keratinocytes, increase in LDH release, and increased proportion of annexin V-positive/PI-negative and double-positive cells by pre-elafin overexpression, and increase/decrease in cleaved caspase-9 and caspase-3 by pre-elafin overexpression/knockdown (Fig. 2b–f), suggest that pre-elafin could be involved in keratinocyte apoptosis. The UV-irradiated samples contained very few cells compared with the control sample (Fig. 3a), indicating that the apoptotic cells were lost. Although protein localization and interaction were examined in the surviving cell population rather than the dying cell population, increased expression and co-localization between pre-elafin and cleaved caspase-3 with phototoxic doses of UVR (Fig. 3a) provided more evidence for the role of pre-elafin in keratinocyte apoptosis. However, immunoprecipitation suggested no direct binding between cleaved caspase-3 and pre-elafin (Fig. 3b). With regards to UVB-induced keratinocyte apoptosis, a protective role of cystatin-A has been presented (10). Cystatin-A is a cysteine protease inhibitor, whereas pre-elafin is a serine protease inhibitor. No direct binding was observed between pre-elafin and cystatin-A (Fig. 3b), as reported (24). Instead, pre-elafin reduced cystatin-A expression (Fig. 3b). Because cystatin-A was bound to pro-caspase-3, but not to cleaved caspase-3 (Fig. 3b), pre-elafin-induced cystatin-A downregulation could lead to more cleavage of unbound pro-caspase-3.

The cellular location of pre-elafin involved in apoptosis was in the cytoplasm beneath the cell membrane of keratinocytes (Fig. 3a), which was different from extracellular pre-elafin involved in desquamation regulation (14). Although the inside location of pre-elafin involved in apoptosis was similar to that of pre-elafin in CE formation, which occurs in terminally differentiated keratinocytes (15, 16), apoptosis was observed in keratinocytes cultured in monolayers in low-calcium media (Figs 2b–f and 3b), which are not thought to be

terminally differentiated (25). In addition, TG1 plays a role in CE formation, and co-localization of pre-elafin with TG1 under conditions of high calcium (Fig. 4c) supported the role of pre-elafin under conditions of high calcium in CE formation. Although most of TG1 in terminally differentiating keratinocytes is attached to cell membranes, some of TG1 is present as cytosolic protein (26), as in this study (Fig. 4c). No detectable TG2 in high-calcium media (Fig. 4d) may result from degradation of TG2 by calcium overload (27). On the other hand, pre-elafin under conditions of low calcium was co-localized with some of the TG1 and TG2 (Fig. 4a, b). The result indicated that pre-elafin under conditions of low calcium may not be involved in CE formation, because the location of TG1 under conditions of low calcium was different from that under conditions of high calcium, in the cytoplasm (Fig. 4a, c), and TG2 was not detected in the cell boundary (Fig. 4d). In fact, TG1 has also been detected in the basal layer with potentiation in the granular layer (28, 29). Considering the location of sunburn cells in the epidermis (Fig. 1a), the finding that pre-elafin in keratinocytes before CE formation was involved in apoptosis is plausible.

In conclusion, pre-elafin in the cytoplasm beneath the cell membrane of the keratinocytes before CE formation, could play a protective role in UVA and NB-UVB-induced harmful effects by inducing keratinocyte apoptosis, through downregulating cystatin-A, which, in turn, activates pro-caspase-3.

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*The authors declare no conflicts of interest.*

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