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ULTRAVIOLET (UV)-AND UV-RELATED CYTOKINE-MEDIATED TRANSCRIPTIONAL MECHANISMS OF TYPE VII COLLAGEN GENE (COL7A1) EXPRESSION IN THE SKIN, WITH SPECIAL REFERENCE TO PHOTOAGED SKIN AND ANTI-AGING

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Abstract Chronic ultraviolet (UV) radiation results in photoaged skin characterized by deep wrinkle formation. Recent studies suggest that diminishment of anchoring fibrils (AF), stabilizing the association of the basement membrane to the underlying dermis, are involved in photoaged skin. Expression of COL7A1, encoding type VII collagen that is a major component of AF, is also decreased in photoaged skin. In this study, we have investigated COL7A1 transcription by UV and UV-related cytokines involved in photoaged skin. Nuclear run-on assay, luciferase assay and gel shift assay revealed that UV and UV-related cytokines (TNF-α, IL-1β) tissue specifically downregulated COL7A1 transcription in epidermal keratinocytes, whereas COL7A1 transcription was upregulated by each modulator in dermal fibroblasts. The responsive element of each modulator was located between nucleotide -524 and -22 of COL7A1 promoter and AP-1 and NF-κB families band to this region. Taken together, these data strongly suggest that the downregulation of COL7A1 transcription in epidermal keratinocytes, main cells expressing COL7A1 in the skin, and subsequent diminishment of AF are involved in photoaged skin. We also have discussed relation between these results and pathophysiology of wrinkle formation, characteristic feature of photoaged skin.

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Key words: type VII collagen; COL7A1, anchoring fibril; ultraviolet radiation; photo-aging; anti-aging

Introduction

Chronic exposure to ultraviolet (UV) radiation results in cutaneous photoaging, characterized by the formation of deep skin wrinkles[1,2]. Various histopathological changes occur in both the epidermis and the dermis of photoaged skin[3-7]. The epidermis becomes atrophic, and shows keratinocyte atypia and dyspolarity, together with increased melanocyte numbers. In the dermis, collagen fibers are damaged and reduced by ultraviolet A radiation (UVA)- and B radiation (UVB)-induced metalloproteinases, which degrade extracellular matrix proteins including collagen. Excessive deposition of abnormal elastic fibers (solar elastosis), induced by UVB radiation, is also observed in the dermis. Furthermore, recent studies have shown that alterations in the structure of the cutaneous basement membrane, including a flattened dermal-epidermal interface, and cleavage and reduplication of the sub-lamina densa, are also involved in the pathophysiology of photoaging[4].

Anchoring fibrils are structures which play a critical role in stabilizing the attachment of the cutaneous basement membrane zone to the underlying papillary dermis[8,9]. Type VII collagen is the predominant, if not the exclusive, component of anchoring fibrils. Therefore, mutations in the type VII collagen gene (COL7A1) can manifest clinically as dystrophic epidermolysis bullosa, a mechano-bullous skin disease characterized by extreme cutaneous fragility and a tendency to develop blisters in

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the sub-lamina densa[10-14]. Recent studies have demonstrated that both the number of anchoring fibrils in the basement membrane zone and COL7A1 expression in epidermal keratinocytes are reduced in photoaged skin[15]. UV and UV-inducible cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β also decrease COL7A1 expression in cultured epidermal keratinocytes[16,17]. Taken together, these data strongly suggest that downregulation of COL7A1 expression and the resultant reduction in anchoring fibril formation are involved in photoaging of the skin.

The present study was designed to investigate the effects of UV and UV-inducible cytokines (TNF-α and IL-1β) on COL7A1 transcription in cultured epidermal keratinocytes. Our results indicate that all of these factors downregulate COL7A1 transcription in epidermal keratinocytes, but enhance COL7A1 transcription in dermal fibroblasts. Their effects are therefore tissue-specific. Furthermore, we demonstrated that the UV- and cytokine-responsive sequence of COL7A1 is located between nucleotides -524 and -22 of its promoter region. We also discuss the potential relevance of our data to wrinkle formation, a feature of photoaged skin.

Materials and methods

Materials

Human recombinant TNF-α and IL-1β were purchased from Roche Diagnostics (Tokyo, Japan). All other reagents were of analytical grade and obtained from commercial sources.

Cell culture

Human epidermal keratinocyte cell line, HaCaT[18], human dermal fibroblasts and NIH 3T3 fibroblast cells were maintained in Minimal Essential Medium supplemented with 10% fetal bovine serum (10% MEM). Human dermal fibroblasts from passages 3-6 were utilized in the experiments.

In vitro nuclear run-on assay

The nuclei were isolated from the cultured epidermal keratinocytes and in vitro synthesis of radiolabeled nascent RNA was performed as described elsewhere[19]. The RNA transcripts were hybridized with COL7A1 cDNA bound to nylon membranes (Zeta probe; BioRad, Hercules, CA). The hybridization and washing conditions were as described before[19]. The radioactive signals from the cDNA-mRNA hybrids were visualized by exposure to X-ray film and quantified by densitometry.

Plasmid constructs

To study human COL7A1 transcription in epidermal keratinocytes, the cells were transfected with plasmid constructs containing sequential 5'-end deletions of the human COL7A1 promoter region. -1804 COL7A1/Luc consisted of a fragment spanning nucleotides -1804 to +92 of the promoter (GenBank/EMBL No. L23982), relative to the transcription start site, cloned into a promoterless luciferase vector (pGL3-basic; Promega, Madison, WI). This fragment was generated by polymerase chain reaction (PCR) amplification of template DNA isolated from human peripheral blood, following a standard protocol[20]. Two additional deletion fragments (-524/+92 and -22/+92) were synthesized by the similar PCR strategy, described previously[21]. -1804 COL7A1/GFP was constructed by subcloning the -1804/+92 fragment into a promoterless green fluorescent protein (GFP) expression vector (pEGFP-1; BD Biosciences Clontech, Palo Alto, CA).

Transient cell transfection and luciferase assay

Transient transfection of epidermal keratinocytes and dermal fibroblasts were performed using the polybren-DMSO method, as described elsewhere[22]. After 6 h of incubation, both types of cells were exposed to either UV, TNF-α or IL-1β for 24 h. They were then washed, harvested and lysed in PicaGene^{TM}

cell culture lysis reagent LCβ (Toyo Ink MFG, Tokyo, Japan). Luciferase activity was determined using the PicaGene^{TM} LT luminescence kit (Toyo Ink MFG) according to the manufacturer's protocol.

Stable cell transfection

HaCaT keratinocytes and NIH 3T3 fibroblasts were stably transfected with the various constructs (-1804, -524, -22 COL7A1/Luc and -1804 COL7A1/ GFP). The COL7A1/Luc constructs were co-transfected with the pCI-neo vector (Promega). Two days after transfection, 1.5 and 1.0 mg/ml of Geneticin (Invitrogen) were added to the HaCaT keratinocytes and NIH 3T3 fibroblasts, respectively, to select the transfected cells. After 28 days, the Geneticin-resistant colonies were pooled and GFP expression in each cell was analyzed by fluorescence microscopy.

Results

UV and UV-inducible cytokines (TNF-αand IL-1β) downregulate COL7A1 transcription in epidermal keratinocytes

Previous studies have demonstrated that UV downregulates COL7A1 expression in cultured epidermal keratinocytes[16]. We have also demonstrated that UV-inducible cytokines inhibit COL7A1 expression in both human epidermal keratinocytes and HaCaT keratinocytes in culture[17]. On the other hand, both UV and UV-inducible cytokines (TNF-α and IL-1β) upregulate COL7A1 expression in dermal fibroblasts[16,24-26]. Thus, UV- and cytokine-mediated COL7A1 expression is tissue-specific. In order to determine whether UV, TNF-α and IL-1β transcriptionally downregulate COL7A1 expression in cultured epidermal keratinocytes, in vitro nuclear run-on assays were performed. The number of COL7A1 RNA transcripts in the nuclei of cultured epidermal keratinocytes was reduced by treatment with either UVA (30 J per cm^2), UVB (15 mJ per cm^2), TNF-α (30 ng/ml) or IL-1β (100 U/ml) (data not shown). These data suggest that UV, TNF-α and IL-1β all inhibit COL7A1 expression at the transcriptional level.

UV and UV-inducible cytokines (TNF-α and IL-1β) exert inhibitory effects on the COL7A1 promoter region.

To analyze the inhibitory effects of UV, TNF-α and IL-1β on COL7A1 transcription in detail, cultured epidermal keratinocytes were transiently transfected with -1804 COL7A1/Luc, a plasmid construct containing the promoter region (nucleotides -1804/+92) of COL7A1. As shown in Figure 1, exposure to UVA decreased

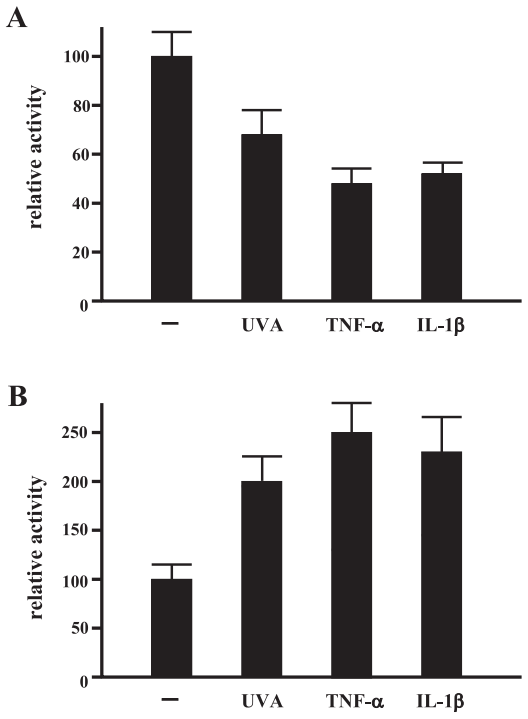

Figure 1 Inhibitory effects of UV and cytokines (TNF-α and IL-1β) on COL7A1 promoter activity in epidermal keratinocytes. Cultured epidermal keratinocytes (A) and dermal fibroblasts (B) were transfected with the -1804 COL7A1/Luc construct as described in Materials and Methods. Subsequently, the cells were exposed to either UVA radiation (30 J per cm^2), TNF-α (30 ng/ml) or IL-1β (100 U/ml) for 24 h. Cell extracts were then assayed for luciferase activity. The results are expressed as mean± SD values for five separate experiments.

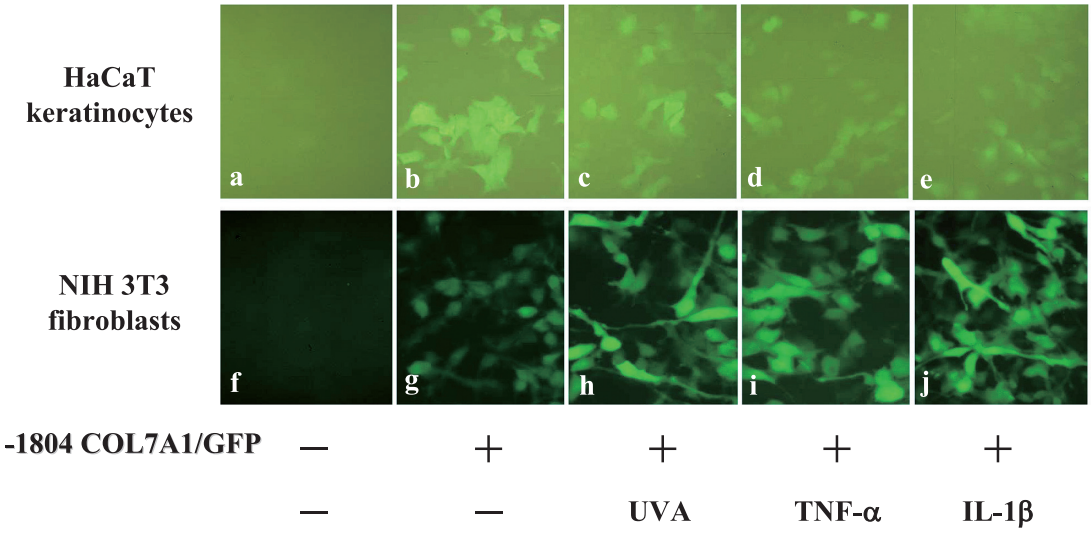

Figure 2 Inhibition of GFP production in epidermal keratinocytes transfected with the COL7A1/GFP construct after exposure to UV and cytokines. HaCaT keratinocytes (a-e) and NIH 3T3 fibroblasts (f-j) were transfected with either a mock vector (pEGFP-1) (a, f) or the -1804 COL7A1/GFP construct (b-e, g-j). Geneticin was subsequently added to select transfected cells, which were exposed to UVA radiation (30 J per cm^2), TNF-α (30 ng/ml) or IL-1β (100 U/ml) for 24 h. GFP expression in the transfected cells was then analyzed by fluorescence microscopy.

COL7A1 promoter activity by 32%. Similar inhibitory effects were observed when epidermal keratinocytes were treated with TNF-α and IL-1β which decreased COL7A1 promoter activity by 52% and 49%, respectively. These results indicate that both UVA and these cytokines downregulate COL7A1 expression by acting on its promoter region, located between nucleotides -1804 and +92.

Expression of GFP protein in HaCaT keratinocytes transfected with a COL7A1/GFP construct is reduced by UV and UV-inducible cytokines (TNF-α and IL-1β)

In order to confirm the inhibitory effects of UV, TNF-α and IL-1β on COL7A1 transcription, stable transfection experiments were perfomed with the -1804 COL7A1/GFP construct in both HaCaT keratinocytes and NIH 3T3 fibroblasts. After selection by Geneticin, the -1804 COL7A1/GFP-transfected cells were pooled and GFP expression was analyzed by fluorescence microscopy. As shown in Figure 2, GFP-associated fluorescence was observed in all areas of both HaCaT keratinocytes and NIH 3T3 fibroblasts. However, within each cell, the level of fluorescence was higher in the nucleus than in the cytoplasm. After exposure to UV, GFP-associated fluorescence in HaCaT cells was decreased. Similar results were observed in cells treated with either TNF-α or IL-1β. In contrast, the expression of GFP in NIH 3T3 fibroblasts was enhanced by UVA and cytokine treatment.

Identification of the UV- and UV-inducible cytokine-responsive regions of the COL7A1 promoter

To characterize the regions of the COL7A1 promoter that respond to UVA, TNF-α and IL-1β, cultured epidermal keratinocytes were transiently transfected with various COL7A1/Luc constructs containing sequential 5'-end deletions of the promoter region. The cells were then irradiated with UV or stimulated with either TNF-α or IL-1β as described in Materials and Methods. As shown in Figure 3, UVA (30 J per cm^2) and UVB (15 mJ per

cm^2) (data not shown) reduced the activities of the -1804 COL7A1/Luc and -524 COL7A1/Luc constructs by 26% and 30%, respectively. However, further deletion to position -22 of the promoter abolished the inhibitory effect of UVA. Similarly, TNF-α and IL-1β both had inhibitory effects on COL7A1 promoter activity. TNF-α and IL-1β reduced the COL7A1 promoter activity of -524 COL7A1/Luc by 29% and 51%, respectively. On the contrary, COL7A1 promoter activity in dermal fibroblasts was increased by UV and both cytokines, as reported previously[16, 24-26].

To verify this responsiveness to UV and UV-induced cytokines, COL7A1/Luc constructs (-1804, -525 and -22 COL7A1/Luc) were stably transfected into HaCaT keratinocytes and NIH 3T3 fibroblasts. As shown in Figure 4, UV, TNF-α and IL-1β decreased the promoter activities of -1804 and -524 COL7A1/Luc, whereas further deletion to position -22 produced no effect. These results are similar to those obtained during the transient transfection experiments (see Figure 3), and indicate that the region of the COL7A1 promoter that responds to both UV and UV-inducible cytokines (TNF-α and IL-1β) is located between nucleotides -524 and -22.

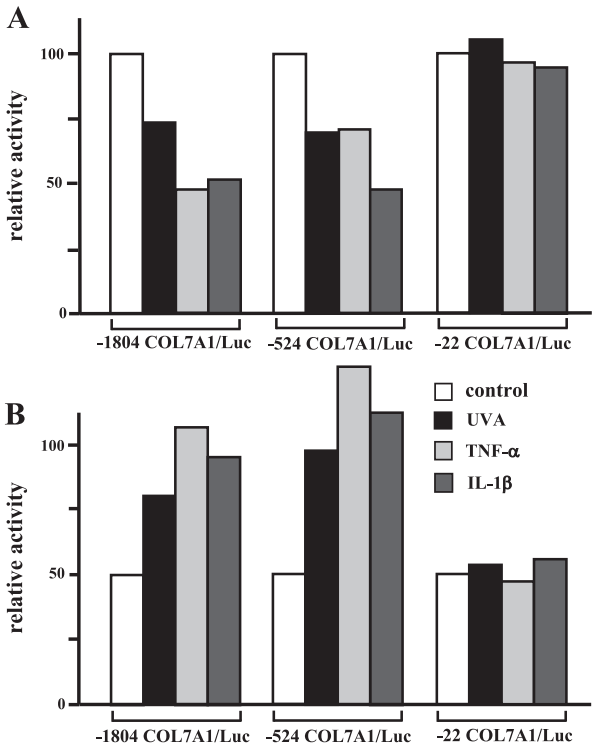

Figure 3 Inhibitory effects of UV and cytokines on the activity of 5'-end deletions of the COL7A1/Luc promoter during transient cell transfection experiments. Cultured epidermal keratinocytes (A) and dermal fibroblasts (B) were were transfected with either -1804, -524 or -22 COL7A1/Luc constructs. After subsequent exposure to UVA radiation (30 J per cm^2), TNF-α (30 ng/ml) or IL-1β (100 U/ml), cell extracts were assayed for luciferase activity as described in Figure 1. The results are expressed as mean±SD values for five separate experiments.

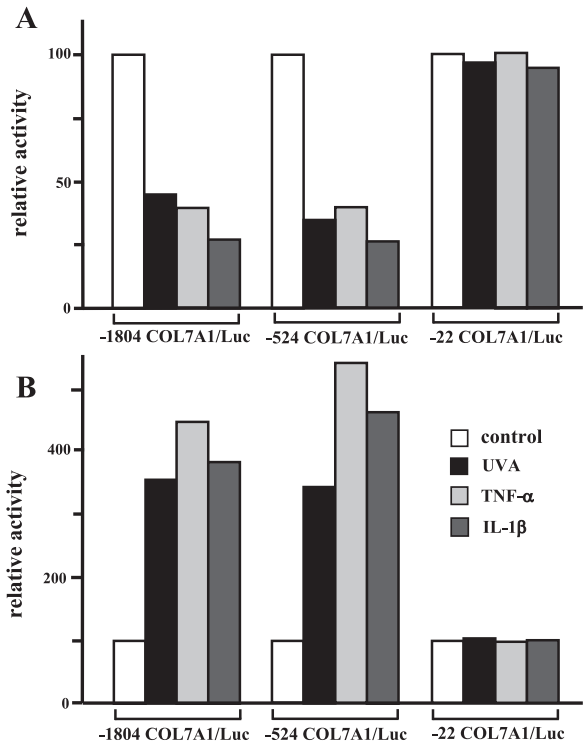

Figure 4 Effects of UV and cytokines on the activity of 5'-end deletions of the COL7A1/Luc promoter during stable cell transfection experiments. HaCaT keratinocytes (A) and NIH 3T3 fibroblasts (B) were stably co-transfected with various COL7A1 constructs (-1804, -524, -22 COL7A1/Luc or -1804 COL7A1/ GFP) and the pCI-neo vector. Transfected cells were selected using Geneticin and exposed to UVA radiation (30 J per cm^2), TNF-α (30 ng/ml) or IL-1β (100 U/ml). Cell extracts were then assayed for luciferase activity. The results are expressed as mean±SD values for nine separate experiments.

Discussion

Chronic exposure to solar ultraviolet radiation results in cutaneous photoaging, which is characterized by wrinkle formation[1,2]. Previous studies have demonstrated that the number of anchoring fibrils in the basement membrane zone is lower in sun-exposed skin than in sun-protected skin, and that COL7A1 expression in epidermal keratinocytes is reduced[15]. Furthermore, we and other investigators have demonstrated that both UV and the cytokines TNF-α and IL-1β downregulate COL7A1 expression in cultured epidermal keratinocytes[16,17]. These results strongly suggest that downregulation of COL7A1 expression in epidermal keratinocytes (the main VII collagen-synthesizing cells in the skin), and the subsequent reduction in anchoring fibril formation, are involved in the pathophysiology of photoaging of the skin. Based on these observations, in the present study we investigated the impact of UV, and UV-inducible cytokines, TNF-α and IL-1β on COL7A1 transcription, and demonstrated that all of these factors downregulate COL7A1 transcription in cultured epidermal keratinocytes (Figures 1A and 2A). Conversely, but in agreement with previous reports[16,24-26], they transcriptionally upregulated COL7A1 expression in dermal fibroblasts (Figures 1B and 2B). Thus, the effects of UV, TNF-α and IL-1β on COL7A1 transcription are tissue-specific.

Deletion analyses revealed that the region of the COL7A1 promoter that responds to both UV and UV-inducible cytokines is located between nucleotides -524 and -22 (Figs 3 and 4). UV is one of the most potent inducers of cytokine release, and the UV-inducible cytokines TNF-α and IL-1β regulate the expression of target genes through transcription factors, such as AP-1 and NF-κB[27,28]. Putative AP-1 and NF-κB binding sites have been identified within the COL7A1 promoter region, and are located upstream of the transcription initiation site, at nucleotide positions -408 and 237, respectively[29]. Hence, TNF-α and IL-1β may mediate UV-induced expression of COL7A1 in epidermal keratinocytes. Recently, we demonstrated that NF-κB binds to a specific site located at nucleotide position -237, and that this binding triggers TNF-α-mediated upregulation of COL7A1 transcription in cultured dermal fibroblasts[26]. Taken together, these results support the hypothesis that the AP-1 and NF-κB binding sites of the COL7A1 promoter may be involved in the downregulation of COL7A1 transcription in epidermal keratinocytes.

As described in the introduction, several studies have reported damage of the basement membrane zone in photoaged skin. The changes include a flattened dermal-epidermal interface, cleavage and reduplication of the sublamina densa[4], and diminished anchoring fibril formation[15], and there is strong support for the involvement of these injuries in photoaging of the skin. In the present study, we have demonstrated that the effects of UV and UV-inducible cytokines on COL7A1 transcription are tissue-specific; transcription is downregulated in epidermal keratinocytes but upregulated in dermal fibroblasts. However, paradoxically, anchoring fibril formation is reduced rather than increased in photoaged skin[15]. It is still unclear exactly which cells express COL7A1, synthesize type VII collagen and finally form anchoring fibrils. However, there are more keratinocytes in the epidermis than fibroblasts in the dermis. Furthermore, the expression of type VII collagen in epidermal keratinocytes is higher than in dermal fibroblasts at both the mRNA and protein levels[30]. Considered together, these data suggest that a decrease in the overall expression of COL7A1 and type VII collagen protein may cause a corresponding decrease in anchoring fibrils formation in the skin, resulting in skin fragility. Additional and repeated external forces together with UV effects on the face and

posterior neck may then accelerate the formation of deep wrinkles, a clinical feature of photoaged skin.

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