**c-Jun–dependent inhibition of cutaneous procollagen transcription following ultraviolet irradiation is reversed by all-trans retinoic acid**

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The aged appearance of skin following repeated exposure to solar ultraviolet (UV) irradiation stems largely from damage to cutaneous connective tissue, which is composed primarily of type I and type III collagens. We report here that a single exposure to UV irradiation causes significant loss of procollagen synthesis in human skin. Expression of type I and type III procollagens is substantially reduced within 24 hours after a single UV exposure, even at UV doses that cause only minimal skin reddening. Daily UV exposures over 4 days result in sustained reductions of both type I and type III procollagen protein levels for at least 24 hours after the final UV exposure. UV inhibition of type I procollagen synthesis is mediated in part by c-Jun, which is induced by UV irradiation and interferes with procollagen transcription. Pretreatment of human skin in vivo with all-trans retinoic acid inhibits UV induction of c-Jun and protects skin against loss of procollagen synthesis. We have reported previously that UV irradiation induces matrix-degrading metalloproteinases in human skin and that pretreatment of skin with all-trans retinoic acid inhibits this induction. UV irradiation, therefore, damages human skin connective tissue by simultaneously inhibiting procollagen synthesis and stimulating collagen breakdown. All-trans retinoic acid protects against both of these deleterious effects and may thereby retard premature skin aging.

thesis and degradation can result in thickened, hardened skin due to collagen build up, or thin, fragile skin, due to collagen deficiency.

We have reported previously that low-dose UV irradiation induces collagenase and other matrix metalloproteinases that degrade mature collagen in human skin in vivo (19, 20). Since connective-tissue remodeling involves synthesis as well as degradation, we investigated the effect of UV irradiation on regulation of procollagen synthesis in human skin in vivo.

Methods

UV irradiation and tissue procurement. Healthy adult Caucasian subjects (45 subjects, approximately equal numbers of males and females), with light to mild skin pigmentation, were irradiated with four F36T12 ERE-VHO UVB tubes (UVB/UV A2 source), as described (20, 21). A Kodacel TA401/407 filter was mounted 4 cm in front of the tubes to remove wavelengths below 290 nm (UVC), since UVC does not reach Earth’s surface. Irradiation intensity was monitored using an IL1400A phototherapy radiometer and an SED240/UVB-1/W photodetector (International Light, Newburyport, Massachusetts, USA). Spectroradiometry was performed using an Optronics Laboratories (Orlando, Florida, USA) OL 754 system. For some studies, subjects were irradiated with a xenon arc solar simulator (Spex Instruments S.A. Inc., Edison, New Jersey, USA). Minimal erythema dose (MED) for each subject was determined 24 hours after irradiation. Irradiated and nonirradiated skin samples were obtained from each subject by punch biopsy, as described previously (22). All-trans retinoic acid (0.1%) and its vehicle (70% ethanol, 30% propylene/glycol, 0.05% butylated hydroxytoluene) were applied to skin under light-tight occlusion, 8 or 48 hours before UV treatment. For studies with multiple time points or treatments, tissue was obtained from each subject for each time point or treatment. n represents the number of subjects from whom tissue was taken for analysis. All procedures involving human subjects were approved by the University of Michigan Institutional Review Board, and all subjects provided written informed consent.

Western analysis. Type I(α1) procollagen and type III(α1) procollagen were measured in skin extracts by Western analysis (Ab’s from Research Diagnostics Inc., Flanders, New Jersey, USA) (23). C-Jun protein (Ab from Transduction Laboratories, Lexington, Kentucky, USA) was detected in nuclear extracts from cultured adult human skin fibroblasts using Western analysis (5). Immunoreactive proteins were visualized by enhanced chemiluminescence detection and quantified by laser densitometry or visualized by enhanced chemiluminescence detection and quantified by STORM PhosphorImager (Molecular Dynamics, Sunnyvale, California, USA).

Northern analysis. Frozen skin samples were ground with mortar and pestle under liquid nitrogen. Total RNA was isolated and hybridized against [32P]-labeled type I(α1), type I(α2), type III(α1) procollagen, and [32P]36B4 cDNA probes, as described previously (24). Hybridization signals were quantified by STORM PhosphorImager (Molecular Dynamics). Hybridization of 36B4 was used as an internal control to normalize all results.

In situ hybridization. Digoxigenin-labeled sense and antisense riboprobes for human type I(α1) and type III(α1) procollagen mRNA were synthesized using T3 and T7 ribonuclease acid polymerases. Frozen skin sections (5 μm) were mounted, fixed, treated, and hybridized, as described previously (24). Hybridization signals were detected immunohistochemically using alkaline phosphatase–conjugated antidigoxigenin Ab.

Transfection of fibroblasts and CAT assay. Primary cultured adult human skin fibroblasts (25) were transfected with 2 μg COL1A2 reporter-gene plasmid (−772 to +58) (26) alone or together with 100 ng expression vectors for either wild-type or dominant-negative mutant c-Jun (TAM-67) (27) using FuGene6 (Boehringer Mannheim, Indianapolis, Indiana, USA). All cells were cotransfected with pXJ40-LacZ plasmid (2 μg), containing the β-galactosidase gene. Twenty-four hours after transfection, cells were washed, covered with 0.5 mL PBS, and either irradiated with Kodacel-filtered UVB tubes, as described (30 mJ/cm²), or sham irradiated. Cells were harvested 8 hours after irradiation, lysates were normalized to β-galactosidase activity, and chloramphenicol acetyl transferase (CAT) activity measured as described (28).

Statistics. Endpoints involving single comparisons were analyzed with the paired t test. Endpoints involving multiple treatments or time intervals were analyzed with a repeated measures ANOVA. Specific comparisons were performed with either Tukey’s Studentized range test for all pairwise comparisons or Dunnett’s t test for treatment versus-control comparisons. Both tests control the experiment-wise type I error rate at the stated alpha level of 0.05. Summary data are represented as means plus or minus one SEM. The data were analyzed with SAS statistical software (SAS Institute Inc., Cary, North Carolina, USA).

Results

Acute UV transiently reduces type I and III procollagen mRNA and protein levels in human skin in vivo. Initially, we determined the effect of a single UV exposure (2 MED) on type I and type III procollagen mRNA expression in human skin in vivo. Figure 1, a–j, shows representative photomicrographs that display the epidermis and adjacent upper portion of skin connective tissue (dermis). Riboprobe in situ hybridization revealed that numerous cells in skin connective tissue expressed mRNA for type I(α1) (Figure 1a) and type III(α1) (Figure 1f) procollagen in sun-protected, nonirradiated human skin. Within 8 hours after a single UV exposure, cellular expression of both type I(α1) (Figure 1b) and type III(α1) procollagen mRNA (Figure 1g) were substantially reduced in the upper dermis. At 24 hours after UV exposure, neither type I(α1) (Figure 1c) nor type III(α1) procollagen mRNA (Figure 1h) was detectable in the
upper dermis. During the next 24 hours (i.e., 48 hours after UV exposure) cellular expression of type I(α1) (Figure 1d) and type III(α1) procollagen mRNA expression by digoxigenin-riboprobe in situ hybridization. (a–e) Type I(α1) procollagen and (f–j) type III(α1) procollagen mRNA expression in human skin. a and f: 0, b and g: 8, c and h: 24, d and i: 48, and e and j: 72 hours after irradiation. Data are representative of six subjects. Scale bar = 10 μm. In a–j, solid black lines demarcate border between epidermis and dermal connective tissue. Areas outlined in black are shown in 2.5-fold enlargements. (k) Northern analysis of type I(α1), type I(α2), and type III(α1) procollagen mRNA levels in human skin. Total mRNA (20 μg/lane) was analyzed from nonirradiated and UV-irradiated (2 MED) human skin, obtained 24 hours after UV irradiation (2 MED), and 36B4 mRNA was used as an internal control. Inset shows representative Northern blot. Procollagen mRNA levels were normalized to 36B4 mRNA levels. n = 6–7. *P < 0.01 vs. no UV exposure.

Figure 1
UV irradiation inhibits type I and type III procollagen gene expression in human skin in vivo. Nonirradiated and adjacent UV-irradiated (2 MED) human skin samples were obtained at the times indicated after UV irradiation. Skin was analyzed for type I(α1) and type III(α1) procollagen mRNA expression by digoxigenin-riboprobe in situ hybridization. (a–e) Type I(α1) procollagen and (f–j) type III(α1) procollagen mRNA expression in human skin. a and f: 0, b and g: 8, c and h: 24, d and i: 48, and e and j: 72 hours after irradiation. Data are representative of six subjects. Scale bar = 10 μm. In a–j, solid black lines demarcate border between epidermis and dermal connective tissue. Areas outlined in black are shown in 2.5-fold enlargements. (k) Northern analysis of type I(α1), type I(α2), and type III(α1) procollagen mRNA levels in human skin. Total mRNA (20 μg/lane) was analyzed from nonirradiated and UV-irradiated (2 MED) human skin, obtained 24 hours after UV irradiation (2 MED), and 36B4 mRNA was used as an internal control. Inset shows representative Northern blot. Procollagen mRNA levels were normalized to 36B4 mRNA levels. n = 6–7. *P < 0.01 vs. no UV exposure.
exposure and analyzed for type I and type III procollagen using Western blot analysis. As expected, a single UV exposure caused significant reduction of type I (Figure 3a) and type III (Figure 3b) procollagen and pN collagen protein levels in human skin. Inset shows representative Western blot. (a) Type I procollagen (filled bars) and type I pN collagen (open bars) protein levels in human skin. Inset shows representative Western blot. (b) Type III procollagen (filled bars) and type III pN collagen (open bars) protein levels in human skin. Inset shows representative Western blot. n = 7–9 subjects. *P < 0.05 vs. no UV exposure.

Figure 2
UV reduces type I and type III procollagen protein levels in human skin in vivo. Nonirradiated and adjacent UV-irradiated (2 MED) human skin samples were obtained at the times indicated after UV irradiation. Skin was analyzed for type I(α1) and type III(α1) procollagen protein levels using Western blot method. (a) Type I procollagen (filled bars) and type I pN collagen (open bars) protein levels in human skin. Inset shows representative Western blot. (b) Type III procollagen (filled bars) and type III pN collagen (open bars) protein levels in human skin. Inset shows representative Western blot.

UV dose dependence for reduction of type I procollagen protein levels in human skin in vivo. We next determined the UV dose dependence for reduction of type I procollagen protein levels in human skin. For these studies we used two different UV sources: a UVB/UVA2 source that emitted predominantly UVB (290–320 nm; used in the studies described above) and a solar simulator, which emitted predominantly UVA (320–400 nm) (Figure 4a). Human skin was exposed in vivo to UV doses between 0.1 MED and 2 MED from each source and analyzed for type I procollagen and pN collagen protein levels using Western blot analysis. A dose of 0.5 MED (one-half the dose required to cause slight skin reddening) from the UVB source caused significant reduction of type I procollagen and pN collagen (Figure 4b). Further reductions were observed at 1 and 2 MED. The UV dose dependence for loss of type III procollagen and pN collagen was similar to that for type I procollagen (data not shown). Solar-simulated UV also caused significant reduction of type I procollagen and pN collagen (Figure 4c). However, reduced procollagen levels were not observed at doses below 1 MED.

Involvement of c-Jun in UV inhibition of type I procollagen synthesis. The data presented above indicate that UV irradiation inhibits type I and type III procollagen gene expression in human skin in vivo. We next sought to gain some insight into the mechanism(s) responsible for this inhibition. Type I and type III procollagen genes are subject to both positive and negative regulation by a large number of different tran-
We have reported previously that UV irradiation causes sustained induction (at least 24 hours) of c-Jun in human skin in vivo (21). We therefore investigated the role of c-Jun in the observed inhibition of procollagen gene expression by UV irradiation. For these studies we used primary adult human skin fibroblasts. UV irradiation of the fibroblasts caused a transient induction of c-Jun protein (Figure 5a). We found that c-Jun was induced (fivefold) within 4 hours, remained elevated for at least 8 hours, and returned to near baseline by 16 hours after UV irradiation. Human skin fibroblasts were transfected with a CAT reporter gene under the control of type I(α2) procollagen proximal gene promoter (−772 to +58bp), containing the critical AP-1–binding site. This reporter was constitutively active in human fibroblasts (Figure 5b). Promoter activity was significantly reduced 8 hours after exposure of the fibroblasts to UV irradiation. Overexpression of wild-type c-Jun further suppressed procollagen I(α2) promoter activity in UV-irradiated fibroblasts. In contrast, overexpression of dominant-negative mutant c-Jun abrogated UV-induced
inhibition of type I procollagen promoter activity (Figure 5b). Northern analyses revealed that UV irradiation reduced endogenous type I(α1), type I(α2), and type III(α1) procollagen gene expression in cultured human skin fibroblasts. Eight hours after UV irradiation, type I(α1), type I(α2), and type III(α1) procollagen mRNA levels were reduced 37 ± 9% (n = 5, P < 0.01); 52 ± 5% (n = 8, P < 0.01); and 46 ± 6% (n = 3, P < 0.01), respectively. These data support the involvement of UV-induced c-Jun in UV inhibition of type I(α2) procollagen gene expression. All-trans retinoic acid protects against UV-induced inhibition of procollagen synthesis in human skin in vivo. Pretreatment of skin with all-trans retinoic acid inhibits accumulation of c-Jun protein in UV-irradiated human skin (21). The data presented implicate c-Jun induction in UV-irradiation inhibition of procollagen synthesis. We therefore investigated whether pretreatment of human skin with all-trans retinoic acid could protect against UV-induced inhibition of type I and type III procollagen synthesis. Human skin was treated with vehicle or all-trans retinoic acid (0.1%) 24 hours before UV irradiation (2 MED). Skin samples were obtained 24 hours after irradiation and analyzed for type I(α1) and type III(α1) procollagen gene expression by in situ hybridization, and protein levels were analyzed using the Western blot method. As expected, type I and type III procollagen mRNA (Figure 6) and protein (Figure 7) expression were significantly reduced in vehicle-treated, UV-irradiated (compared with vehicle-treated, nonirradiated) human skin. Pretreatment of skin with all-trans retinoic acid substantially protected against loss of type I and type III procollagen mRNA and protein. Treatment of skin with all-trans retinoic acid alone had no effect on type I or type III procollagen mRNA or protein levels.

Discussion

The data demonstrate that UV irradiation causes reduction of type I and type III procollagen mRNA and protein expression in fibroblasts in human skin in vivo. UV reduction of procollagen mRNA and protein expression was most prominent in the upper one-third of skin connective tissue. This localization likely reflects the depth of UV penetration into the skin, which increases with increasing wavelength. We used a UVB/UVA source that emits wavelengths primarily between 290–340 nm, which penetrates into the upper region of the connective tissue (33).

In nonirradiated human skin, type I and III procollagen proteins are present within fibroblasts and extracellularly. In the upper dermis, this procollagen pool is substantially reduced within 8 hours and essentially absent within 24 hours after UV irradiation. This depletion of existing procollagen protein presumably reflects ongoing conversion into mature collagen coupled with decreased new procollagen synthesis due to reduced procollagen mRNA levels. However, our data do not allow us to directly ascertain the fate of the existing procollagen protein pool. It is possible that UV irradiation stimulates degradation and/or processing of procollagen.

We have reported previously that UV irradiation induces collagenase and other matrix metalloproteinases in human skin in vivo (19, 20). Collagenase is induced 12–16 hours after UV irradiation, trailing the initial loss of procollagen protein, which is observed 8 hours after UV irradiation. This difference in the time of onset of collagenase induction and reduction of procollagen protein makes it unlikely that initial loss of procollagen results from UV-induced, collagenase-mediated degradation. In general, however, the time course for UV induction of matrix-metalloproteinase mRNA and pro-
tein expression inversely mirrors that for UV reduction of procollagen mRNA and protein expression. During the first 24 hours after UV exposure, matrix-metalloproteinase mRNAs and proteins are induced, whereas procollagen mRNAs and proteins are reduced. During the second and third days after UV exposure, matrix-metalloproteinase expression declines, whereas procollagen expression increases. Multiple UV exposures cause sustained elevations of matrix-metalloproteinase mRNAs and proteins (19) and sustained repression of procollagen mRNAs and proteins. Simultaneous reduction of procollagen expression and induction of mature collagen degradation maximizes mature collagen loss during the initial 24 hours after a single UV exposure. During the succeeding 48 hours (i.e., 2–3 days after UV exposure) matrix-metalloproteinase expression wanes as procollagen expression rises. This temporal coordination of matrix metalloproteinase–mediated collagen breakdown and procollagen mRNA and protein expression protects newly synthesized mature collagen (and procollagen) from rapid degradation.

Evidence suggests that transcriptional control is the major mechanism of regulation of type I procollagen expression (26, 29, 34–37). Transcription factor AP-1, which is composed of Jun and Fos proteins, negatively regulates both type I(α1) and type I(α2) procollagen gene transcription (31, 32, 37). UV irradiation rapidly upregulates AP-1 in human skin in vivo. This induction of AP-1 results primarily from increased c-Jun expression, since c-Fos is constitutively expressed in human skin (20, 21). c-Jun remains maximally elevated for at least 24 hours after UV irradiation in human skin in vivo. This sustained induction of c-Jun is consistent with the observed time course for repression of procollagen gene expression. These observations led us to investigate the role of c-Jun in UV inhibition of type I procollagen gene expression in primary adult human skin fibroblasts. UV irradiation of skin fibroblasts rapidly induced c-Jun and inhibited endogenous type I and type III procollagen gene expression. We found that human type I(α1) procollagen-promoter CAT-reporter constructs, which were previously characterized in mouse 3T3 fibroblasts (29), were not transcriptionally active in adult human skin fibroblasts and therefore could not be studied. In contrast, the human type I(α2) procollagen promoter CAT reporter (containing 714 bp upstream from the transcription start site) was active in human skin fibroblasts. Promoter activity was reduced by UV irradiation and further reduced by overexpression of wild-type c-Jun. Overexpression of dominant-negative mutant c-Jun completely abrogated UV inhibition of promoter activity. In cultured fibroblasts, c-Jun remained elevated for at least 8 hours after UV irradiation (as opposed to 24 hours in human skin in vivo). In cultured fibroblasts, UV inhibition of endogenous type I(α2) procollagen gene expression and type I(α2) procollagen promoter reporter was maximal 8 hours after UV exposure. Therefore, the kinetics of induction of c-Jun and repression of type I(α2) procollagen promoter activity coincided. These data indicate that UV inhibition of type I(α2) procollagen gene expression in adult human fibroblasts is mediated, at least in part, by UV-induced c-Jun. However, these data do not rule out the possibility that there may be other factors, including reduced mRNA stability, that contribute to UV inhibition of procollagen gene expression.

Treatment of human skin in vivo with all-trans retinoic acid before UV irradiation protected against UV-induced loss of type I and type III procollagen transcripts and proteins. Protection by all-trans retinoic acid was observed after 24 hours of pretreatment, but not after 8 hours of pretreatment (data not shown). We have shown previously that pretreatment of human skin with all-trans retinoic acid for 24 hours, but not 8 hours, inhibits induction of c-Jun and AP-1 (19–21). These data support the involvement of c-Jun in UV inhibition of type I and type III procollagen expression.
in human skin in vivo. The requirement for prolonged pretreatment with all-trans retinoic acid presumably reflects a mechanism that involves new gene expression, however, this mechanism remains to be clarified.

Type I procollagen and type III procollagen are reduced in chronically photodamaged human skin, not recently exposed to UV (5, 6, 11). Treatment of such photodamaged skin with all-trans retinoic acid increases type I procollagen expression (11). The mechanism of this increase is not known, however, it may involve increased expression or activation of TGF-β (38, 39). In addition, treatment of skin with all-trans retinoic acid before UV exposure inhibits collagen breakdown by matrix metalloproteinases (19, 20) and protects against UV-induced reduction of procollagen expression, as demonstrated in this study. Therefore, all-trans retinoic acid and its metabolic precursor all-trans retinol (vitamin A) should have the capacity to both repair existing photo-damage and retard accumulation of new photodamage.

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