Ultraviolet A Irradiation Upregulates Type VII Collagen Expression in Human Dermal Fibroblasts

Mei Chen,* Marta J. Petersen†, Hai-Li Li†, Xiao-Yan Cai,* Edel A. O'Toole,* and David T. Woodley*

*Department of Dermatology, Northwestern University Medical School, Chicago, Illinois, U.S.A.; and †Department of Dermatology, University of Utah School of Medicine, Salt Lake City, Utah, U.S.A.

Type VII collagen, a major component of skin-anchoring fibrils, is synthesized by both fibroblasts and keratinocytes, the two principal cell types in the skin. In this study, we examined the effects of ultraviolet A (UVA) irradiation on the expression of type VII collagen in human fibroblasts. UVA irradiation (0–15 J/cm^2) caused a dose-dependent increase (5- to 10-fold) in type VII collagen mRNA levels as detected by northern blot analysis. The UVA-induced enhancement of type VII collagen gene expression correlated with an increase in its protein level by immunoblot analysis of proteins secreted into the conditioned medium. The effect of UVA was observed at 12 h and reached its maximum by 18 h. Under these conditions, however, the expression of fibronectin, a major dermal matrix protein, remained unchanged, suggesting that the induction of type VII collagen expression was selective. Actinomycin D, a transcription inhibitor, blocked the UVA-mediated induction of type VII collagen gene expression, whereas cycloheximide, a protein synthesis inhibitor, superinduced the expression of type VII collagen, suggesting that *de novo* protein synthesis was not required for the action of UVA. Interestingly, in contrast to the increased type VII collagen expression in fibroblasts in response to UVA, a slight decrease in type VII collagen mRNA level was observed in the UVA-irradiated keratinocytes, suggesting that the effect of UVA on the type VII collagen expression is cell type specific. *Key words: northern blot/anchoring fibrils/basement membrane/keratinocyte/skin. J Invest Dermatol 108:125–128, 1997*

Chronic exposure to ultraviolet radiation results in marked changes in the connective tissue of the dermis. Sunbathing, tanning, and therapeutic UV irradiation substantially increase the risk of actinic damage of the skin, resulting in wrinkle formation, reduced recoil capacity, increased fragility, and impaired wound healing. These clinical manifestations (collectively known as photoaging) are thought to be due to both quantitative and qualitative alterations of dermal extracellular matrix proteins (Smith *et al*, 1962; Lavker, 1979; Oikarinen *et al*, 1985; Kligman, 1986). For example, type I collagen, a major component of the dermis, is reduced in actinically damaged skin (Talwar *et al*, 1995). Because fibroblasts are known to be responsible for both collagen synthesis and degradation, a number of studies have examined the UV-induced alteration of matrix-metalloproteinases, a family of enzymes that degrade extracellular matrix including collagens. It is clear that ultraviolet A (UVA) promotes connective tissue catabolism by upregulating the expression of metalloproteinases that degrade the extracellular matrix proteins and by downregulating the expression of the various extracellular matrix components (Scharffetter *et al*, 1991; Petersen *et al*, 1992; Herrmann *et al*, 1993). Taken together, these studies suggest that collagenase plays a role in the pathophysiology of the connective tissue alterations observed in chronically sun-exposed skin.

Manuscript received February 23, 1996; revised August 6, 1996; accepted for publication October 30, 1996.

Reprint requests to: Dr. David T. Woodley, M.D., Department of Dermatology, Northwestern University Medical School, 303 East Chicago Avenue, Chicago IL, 60611.

This work was presented at the annual national meeting of the Society of Investigative Dermatology, May 24–28, 1995, in Chicago, Illinois.

Type VII collagen is the major component of anchoring fibrils, attachment structures that play an essential role in stabilizing the association of the basement membrane zone to the underlying papillary dermis (Sakai *et al*, 1986; Keene *et al*, 1987). It is synthesized and secreted by both human epidermal keratinocytes and dermal fibroblasts, the two principal cell types in the skin (Stanley *et al*, 1985; Woodley *et al*, 1985; Ryynänen *et al*, 1992). Expression of type VII collagen is increased in the skin of patients with systemic sclerosis (Rudnicka *et al*, 1994). Although the effects of UVA visible light exposure on anchoring fibrils has not been reported, a number of research groups have demonstrated a reduction in the interstitial dermal collagens, type I and III, in photodamaged skin (Griffiths *et al*, 1993; Schwartz *et al*, 1993; Talwar *et al*, 1995). The purpose of this study was to examine the effect of UVA on type VII collagen expression and production in cultured human skin cells. We showed that UVA upregulates type VII collagen gene expression in human dermal fibroblasts, whereas it downregulates its expression in human keratinocytes.

MATERIALS AND METHODS

Cell Culture Cultures of human fibroblasts were initiated from neonatal foreskins and adult skin samples and cultured as previously described (Petersen *et al*, 1987). Fibroblasts were grown to near-confluency in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Human neonatal keratinocytes were obtained from neonatal foreskins and cultured under serum-free, low calcium (0.15 mM) conditions according to Boyce and Ham (1983), as modified by O'Keefe and Chiu (1988). Keratinocytes in passage 3–4 were used for the experiments.

Irradiation of Cells Confluent cultures of fibroblasts were irradiated with UVA, through a thin layer of clear phosphate-buffered saline and the lid of the tissue culture dish. The UVA light source consisted of 1100-A two-foot fluorescence lamps that emit a continuous spectrum of ultraviolet

0022-202X/97/$10.50

radiation in the range of 320 nm–400 nm (National Biological Company, Twinsburg, OH). Control cultures were sham irradiated for the length of time needed to administer the highest dose of UVA. The amount of UVA administered was assessed with a LMA-302 radiometer (United States Biochemical Corp.) with a UVA probe. Following irradiation, phosphate-buffered saline was replaced by fresh Dulbecco's modified Eagle's medium with 10% fetal bovine serum, and the cells were incubated for various periods of time before being subjected to northern blot (mRNA) and immunoblot (protein) analysis for the expression of type VII collagen.

For inhibition studies, fibroblast cultures were irradiated with 15 J UVA per cm^2 at time 0 or sham-irradiated. After irradiation, cells were treated with cycloheximide (5 μg per ml) and actinomycin D (5 μg per ml), and RNA was isolated after 18 h, as described in the following section.

Isolation and Analysis of RNA Total RNA was isolated by $CsCl_2$ density gradient centrifugation (Wang and Gudas, 1983). Northern filters were hybridized with a 2.4-kb human type VII collagen cDNA containing nine fibronectin type III-like repeats. The same filters were rehybridized with a human fibronectin (GIBCO BRL, Gaithersburg, MD) cDNA, as well as with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA used as a control. cDNA probes were labeled by random-primed kit (Boehringer Mannheim, Indianapolis, IN). The [^{32}P] cDNA-mRNA hybrids were detected by autoradiography and quantified by densitometry.

Western Immunoblotting After UVA irradiation, conditioned medium containing 150 μM ascorbic acid was collected after 40 h. It was then equilibrated to 5 mM ethylenediamine tetraacetic acid, 50 μM phenylmethylsulfonyl fluoride, and N-ethylmaleimide and concentrated in Centricon-100 (Amicon, Beverly, MA). Equal amounts of protein were resolved by 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and incubated with a rabbit polyclonal antibody (1:1000), developed in our laboratory, that recognizes the noncollagenous (NC1) domain of type VII collagen. The immunoreactive type VII collagen protein was visualized by using horseradish peroxidase–conjugated secondary antibody (1:2000) (Organon, Teknika-Cappel, Durham, NC) and enhanced chemiluminescence detection reagent (ECL, Amersham, Little Chalfont, U.K.).

RESULTS

UVA Irradiation Induces Type VII Collagen Expression in Human Fibroblasts To examine whether or not UVA affects the expression of type VII collagen in human fibroblasts, total RNA was isolated from fibroblast cultures irradiated with 0–15 J of UVA per cm^2 after 18 h and subjected to northern blot analysis using cDNA probes specific for human type VII collagen and fibronectin mRNAs. As shown in **Fig 1**, low but detectable levels of type VII collagen transcripts were observed in the unirradiated cells. UVA irradiation, however, significantly augmented type VII collagen expression in a dose-dependent manner. This increased expression could be detected in the cells treated with as low as 6 J irradiation per cm^2, and the maximum increase (5- to 10-fold) was achieved after exposure to 15 J per cm^2. When the same blot was rehybridized using human fibronectin and control GAPDH cDNA probes, no significant change between sham-irradiated and UVA-irradiated cells was observed in either of these genes, indicating that the induction of type VII collagen expression by UVA irradiation was specific. **Fig 2** shows the results from three experiments with cells from three different donors. In this set of experiments, densitometric analysis indicated that UVA irradiation increased the type VII collagen mRNA between 5- to 10-fold.

We also examined the time course of induction of type VII gene expression using 15 J of UVA per cm^2. As shown in **Fig 3**, a time-dependent increase in type VII expression, which appeared as early as 12 h and reached a maximum by 18 h after UVA irradiation, was clearly detected,. Under these conditions, cells were morphologically normal and remained viable as determined by trypan dye exclusion (data not shown).

***De Novo* Protein Synthesis Is Not Required for UVA-Induced Type VII Collagen Expression** To study the possible mechanism by which UVA induces type VII expression, we tested the effects of cycloheximide, a protein synthesis inhibitor, and actinomycin D, a transcription inhibitor, on the expression of type VII collagen. As shown in **Fig 4**, treatment of the cells with actinomycin D completely abolished the UVA-stimulated induction of type VII collagen gene expression. These data suggest that

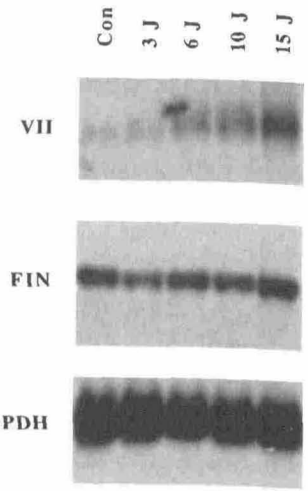

Figure 1. Effect of UVA irradiation on type VII collagen and fibronectin mRNA expression in fibroblasts. Confluent fibroblast cultures were exposed to the indicated doses of UVA or sham-irradiated (Con) and RNA was isolated after 18 h. Northern blot analysis was carried out with 20 μg of total RNA, and the blot was hybridized with a ^{32}P-labeled cDNA probe representing type VII collagen (VII), fibronectin (FIN), and GAPDH. GAPDH mRNA was used as a control for the amount of RNA loaded per lane.

the increase in type VII collagen mRNA level after UVA irradiation results from transcriptional activation of the gene. Interestingly, whereas cycloheximide treatment alone resulted in a slight but detectable increase in type VII collagen mRNA (as normalized to GAPDH RNA), UVA and cycloheximide together caused a super-induction of type VII collagen mRNA. Thus, these data indicate that the induction of type VII collagen mRNA is independent of new protein synthesis, and the factors that mediate the UVA response are already present in the cell prior to the irradiation.

UVA Irradiation Enhances Secretion of Type VII Collagen To examine whether or not the elevated type VII collagen mRNA levels by UVA will indeed result in an enhanced production of type VII collagen protein, we performed immunoblot analysis of the type VII collagen secreted into the conditioned medium. As shown in **Fig 5**, the conditioned medium of sham-irradiated cells did not

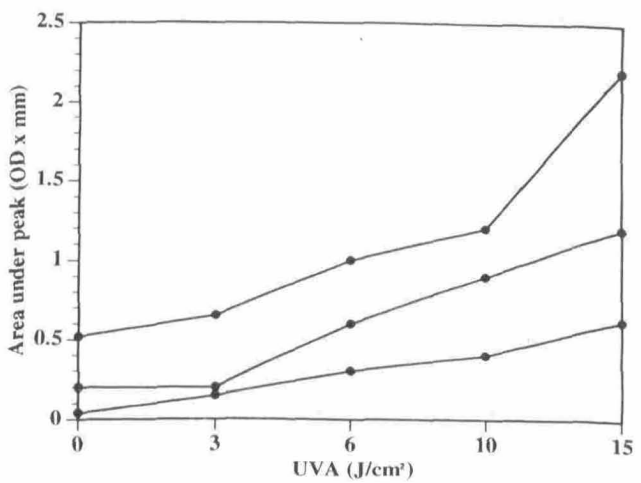

Figure 2. UVA-induced increase of type VII collagen mRNA level by fibroblasts in three experiments. Densitometric analysis of the type VII collagen mRNA bands from three experiments in which fibroblasts were irradiated with UVA as described in **Fig 1**. In this set of experiments, 15 J of UVA irradiation per cm^2 increased type VII collagen mRNA expression in cultured fibroblasts 5- to 10-fold.

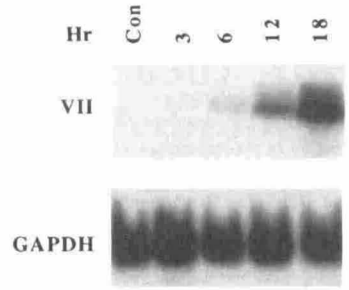

Figure 3. Kinetics of UVA-induced expression of type VII collagen mRNA. Confluent fibroblast cultures were irradiated with 15 J of UVA per cm^2, and total cellular RNA was extracted at the indicated times. Twenty micrograms of RNA were size-fractionated, and northern blot was hybridized to type VII collagen probe. Hybridization of the probe for GAPDH demonstrates equivalent loading of lanes.

contain detectable levels of immunoreactive type VII collagen. In contrast, irradiation with UVA (0–15 J/cm^2) produced a dose-dependent increase in the immunoreactive 290-kDa type VII collagen α-chain. The dose-dependent increases in type VII collagen at the protein level correlated well with the increases in type VII collagen mRNA levels (see **Figs 1, 2**). The maximum increase (4- to 7-fold) in the secreted type VII collagen protein was observed when the fibroblasts were exposed to 15 J of UVA per cm^2 in two independent experiments **(Fig 6)**. Thus, these results demonstrate that UVA enhances both the transcription of the type VII collagen gene and the synthesis and secretion of its protein product.

UVA Downregulates Type VII Collagen mRNA in Human Keratinocytes Previous studies have shown cell type–specific regulation of collagenase gene expression in response to UVA irradiation (Petersen *et al*, 1992). To determine whether UVA-induced type VII expression is also cell type specific, we conducted similar experiments using cultured human keratinocytes. Monolayer culture of keratinocytes were irradiated with 0–20 J of UVA per cm^2, and total RNA was isolated and subjected to northern blot analysis. Again, the cells remained viable for at least 18 h after the irradiation. In contrast to the UVA-induced upregulation of type VII collagen expression observed in fibroblasts, the mRNA levels of type VII collagen were slightly decreased in keratinocytes after irradiation with ≥ 10 J of UVA per cm^2 **(Fig 7)**. Rehydribization of the filter with a fibronectin cDNA probe demonstrated that, similar to fibroblasts, fibronectin mRNA levels did not change significantly. Furthermore, mRNA levels of control GAPDH were not

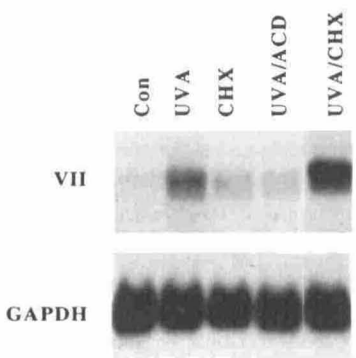

Figure 4. Effects of cycloheximide and actinomycin D on type VII collagen mRNA induction by UVA. Five micrograms of cycloheximide (CHX) per ml and 5 μg of actinomycin D (ACD) per ml were added to fibroblasts for 18 h after irradiation with 15 J of UVA per cm^2 or to sham-irradiated control cells (Con) as indicated. Total RNA was extracted and analyzed by northern blot analysis with cDNA probe specific for type VII collagen mRNAs. Similar results were obtained from two other independent experiments.

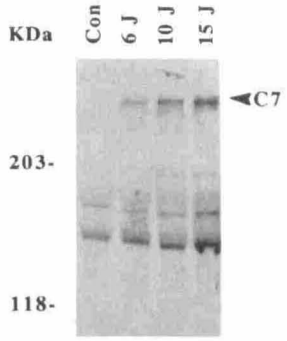

Figure 5. Immunoblotting of type VII collagen from conditioned medium of UVA-irradiated fibroblasts. Fibroblast cultures were irradiated with the indicated doses of UVA, and the conditioned medium was collected 40 h post-irradiation. The conditioned medium was concentrated, and equal amounts of each sample were separated on a 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by immunoblotting against a rabbit polyclonal antibody against the NC1 domain of type VII collagen. The positions of molecular weight marker and type VII collagen (C7) are indicated.

altered during UVA irradiation. Although the mechanism remains unknown, these results indicated that the effect of UVA irradiation on type VII collagen gene expression is cell type–specific.

DISCUSSION

In this study, we demonstrated that UVA irradiation induces human dermal fibroblasts to upregulate type VII collagen gene expression at both the mRNA level and at the protein level. The UVA modulation was observed in a time- and dose-dependent manner whereas the mRNA levels of fibronectin, a large dermal glycoprotein, and a house-keeping gene, GAPDH, were not altered, suggesting selective induction of type VII collagen mRNA. Inhibition of RNA synthesis by actinomycin D abolished the UVA-mediated increase in type VII collagen mRNA, which strongly suggests that the stimulatory effect of UVA is dependent upon ongoing gene transcription. In contrast, inhibition of protein synthesis by cycloheximide superinduced levels of type VII collagen mRNA during UVA irradiation, indicating that *do novo* protein synthesis is not required for type VII collagen induction by UVA.

There are numerous reports of the cycloheximide induction of mRNAs for various cytokines and lymphokines (Greenberg *et al*, 1986, Mahadevan and Edwards, 1991). In this study, we showed

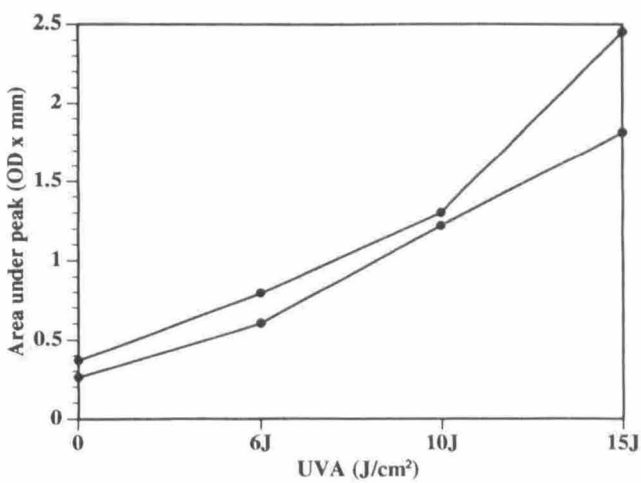

Figure 6. UVA-induced increase of type VII collagen production by fibroblasts in two experiments. Densitometric analysis of the 290-kDa type VII collagen bands from two experiments in which fibroblasts were irradiated with UVA as described in **Fig 6**. UVA irradiation (15 J/cm^2) increased type VII collagen production 4- to 7-fold.

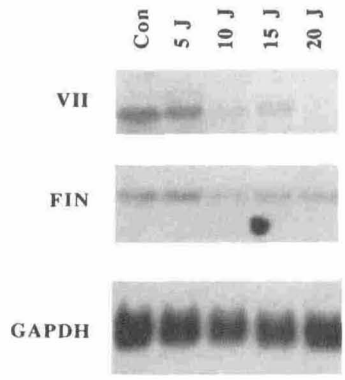

Figure 7. Effect of UVA irradiation on the type VII collagen mRNA expression in keratinocytes. Keratinocyte cultures were exposed to the indicated doses of UVA, and total RNA was isolated after 18 h. Northern blot analysis was performed with cDNA probes for type VII collagen (VII), fibronectin (FIN), and GAPDH. This experiment was performed twice with very similar results.

that UVA-mediated induction of type VII collagen mRNA was superinduced by cycloheximide. In keeping with this observation, we have previously demonstrated that induction of type VII collagen by phorbol myristate acetate is also superinduced by cycloheximide (Chen *et al*, 1995). These results suggest enhanced transcription or stabilization of accumulated type VII collagen mRNA by cycloheximide. Accumulation of transcripts may occur by inhibition of labile, negative transcription factors(s) or by inhibition of synthesis of protein(s) that promote degradation of type VII collagen transcripts and other selected RNA species (Mahadevan and Edwards, 1991).

UVA is a long-wave ultraviolet light that readily penetrates through the skin into the subcutaneous fat. Therefore, dermal fibroblasts would readily be exposed to this type of light. Moreover, in sunlight, there is much more UVA irradiation than other types of ultraviolet light. Unlike the shorter UVB sunburn spectrum of sunlight, UVA is much less erythemogenic. Consequently, humans routinely receive much more UVA than UVB. It should be emphasized here that the UVA-induced effects observed herein are short-term effects of UVA irradiation on dermal fibroblasts and could, perhaps, be viewed as an acute injury response. The effects of chronic, long-term, ultraviolet light on human skin or *in vitro* on cultured skin cells have not been studied and may be quite different.

Our studies show that keratinocytes do not respond to UVA irradiation in the same way as fibroblasts. In contrast to the induction of type VII collagen expression by fibroblasts, a slight decrease in the level of type VII collagen mRNA was observed in UVA-irradiated keratinocytes. Petersen *et al* (1992) also demonstrated a differential effect on the modulation of collagenase by UVA between human fibroblasts and keratinocytes. These results may indicate that modulation of type VII collagen gene expression in fibroblasts and keratinocytes involves different regulatory pathways.

The precise mechanisms by which UVA irradiation induces expression of type VII collagen remain unknown. There is a strong link between cytokines and UVA irradiation that can affect the local cytokine milieu in the tissue. For example, recent studies have shown that UVA irradiation stimulates fibroblasts to synthesize and release interleukin (IL)-1α and IL-1β. These cytokines then stimulate the synthesis and secretion of IL-6 and subsequently induce collagenase/matrix metalloproteinase-1 synthesis (Wlaschek *et al*, 1993, 1994). IL-1α and IL-1β have also been shown to induce increased production of type VII collagen in fibroblasts (Mauviel *et al*, 1994). It is interesting to note that the maximal UVA induction of IL-1–specific mRNA occurs at 6 h post-irradiation, which clearly precedes the maximal UVA induction of type VII collagen mRNA that occurs at 18 h post-irradiation **(Fig 3)**. Whether IL-1 is involved in the UVA-mediated induction of type VII collagen by human dermal fibroblasts is not known.

We thank Dr. Wei Li (Ben May Institute for Cancer Research, University of Chicago) for critical reading of the manuscript. The work was supported by grants PO1 AR 41045 and RO1 A33625 (Dr. Woodley). Dr. O'Toole is a Howard Hughes Medical Institute Physician Postdoctoral Fellow.

REFERENCES

Boyce ST, Ham RG: Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *J Invest Dermatol* 81S:33–40, 1983

Chen M, Quintans J, Fuks Z, Thompson C, Kufe DW, Weichselbaum RR: Suppression of *Bcl-2* messenger RNA production may mediate apoptosis after ionizing radiation, tumor necrosis factor a, and ceramide. *Cancer Res* 55:991–994, 1995

Greenberg ME, Hermanowski AL, Ziff EB: Effect of protein synthesis inhibitors on growth factor activation of c-fos, c-myc, and actin transcription. *Mol Cell Biol* 6:1050–1057, 1986

Griffiths CE, Russman AN, Majmudar G, Singer RS, Hamilton TA, Voorhees JJ: Restoration of collagen formation in photodamaged human skin by tretinoin (retinoic acid). *N Engl J Med* 30:2038–2039, 1993

Herrmann G, Wlaschek M, Lange TS, Prenzel K, Goerz G, Kochanek-Scharffetter K: UVA irradiation stimulates the synthesis of various matrix-metalloproteinase (MMPs) in cultured human fibroblasts. *Exp Dermatol* 2:92–97, 1993

Keene DR, Sakai LY, Lundstrum GP, Morris NP, Burgeson RE: Type VII collagen forms an extended network of anchoring fibrils. *J Cell Biol* 104:611–621, 1987

Kligman LH: Photoaging: manifestation, prevention, and treatment. *Dermatol Clin* 4:517–528, 1986

Lavker RM,: Structural alterations in exposed and unexposed aged skin. *J Invest Dermatol* 73:59–66, 1979

Mauviel A, Lapiere JC, Halcin C, Evans CH, Uitto J: Differential cytokine regulation of type I and type VII collagen gene expression in cultured human dermal fibroblasts. *J Biol Chem* 269:25–28, 1994

Mahadevan LC, Edwards DR: Signaling and superinduction. *Nature* 349:747–748, 1991

Oikarinen A, Karvonen J, Uitto J, Hannuksela M: Connective tissue alterations in skin exposed to natural and therapeutic UV-irradiation. *Photodermatology* 2:15–26, 1985

O'Keefe EJ, Chiu ML: Stimulation of thymidine incorporation in keratinocytes by insulin, epidermal growth factor, and placental extract: comparison with cell number to assess growth. *J Invest Dermatol* 90:2–7, 1988

Petersen MJ, Hansena C, Craig S: Ultraviolet A irradiation stimulates collagenase production in cultured human fibroblasts. *J Invest Dermatol* 99:440–444, 1992

Petersen MJ, Woodley DT, Stricklin GP, O'Keefe EJ: Production of procollagenase by cultured human keratinocytes. *J Biol Chem* 262:835–840, 1987

Rudnicka L, Varga J,Christiano AM, Iozzo RV, Jimenez SA, Uitto J: Elevated expression of type VII collagen in the skin of patients with systemic sclerosis: regulation by transforming growth factor-b. *J Clin Invest* 93:1709–1715, 1994

Ryynanen J, Sollberg S, Parente MG, Chung LC, Christiano AM, Uitto J: Type VII collagen gene expression by cultured human cells and in fetal skin. Abundant mRNA and protein levels in epidermal keratinocytes. *J Clin Invest* 89:163–168, 1992

Sakai LY, Keene DR, Morris NP, Burgeson RE: Type VII collagen is a major structural component of anchoring fibrils. *J Cell Biol* 103:1577–1586, 1986

Scharffetter KM, Wlaschek A, Hogg K, Bolsen A, Schothorst G, Goerz T, Plewig G: UVA induces collagenase in human dermal fibroblasts *in vitro* and *in vivo*. *Arch Dermatol Res* 283:506–511, 1991

Schwartz E, Cruickshank FA, Christensen CC, Perlish JS, Lebwohl M: Collagen alterations in chronically sun-damaged human skin. *Photochem Photobiol* 58:841–844, 1993

Smith JG, Davidson EA, Sams WM, Clark RD: Alterations in human dermal connective tissue with age and chronic sun-damage. *J Invest Dermatol* 39:347–350, 1962

Stanley JR, Rubinstein N, Klaus-Kovtun V: Epidermolysis bullosa acquisita antigen is synthesized by both human keratinocytes and human dermal fibroblasts. *J Invest Dermatol* 85:542–545, 1985

Talwar HS, Griffiths CE, Fisher GJ, Hamilton TA, Voorhees JJ: Reduced type I and type III procollagens in photodamaged adult human skin. *J Invest Dermatol* 105:285–290, 1995

Wang SY, Gudas LJ: Isolation of cDNA clones specific for collagen IV and laminin from mouse tertocarcinoma cells. *Proc Natl Acad Sci USA* 80:5880–5884, 1983

Wlaschek M, Heinen G, Poswig A, Schwarz A, Krieg T, Scharffetter-Kochanek K: UVA-induced autocrine stimulation of fibroblast-derived collagenase/MMP-1 by interrelated loops of interleukin-1 and interleukin-6. *Photobiochem Photobiol* 59:550–556, 1994

Wlaschek M,Bolsen K, Herrmann G, Schwarz A, Wilmroth F, Heinrich PC, Goerz G, Scharffetter-Kochanek: UVA-induced autocrine stimulation of fibroblast-derived collagenase by IL-6: a possible mechanism in dermal photodamage. *J Invest Dermatol* 93:164–168, 1993

Woodley DT, Briggaman RA, Gammon WR, O'Keefe EJ: The epidermolysis bullosa acquisita antigen is synthesized by human keratinocyte cultured in serum-free medium. *Biochem Biophys Res Commun* 130:1267–1272, 1985