UVA-Induced Autocrine Stimulation of Fibroblast-Derived-Collagenase by IL-6: A Possible Mechanism in Dermal Photodamage?

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Like other cytokines, IL-6 has been reported to stimulate collagenase. In this study we were interested in whether IL-6 is involved in the ultraviolet (UV) mediated up-regulation of fibroblast-derived collagenase. Confluent fibroblast monolayers were irradiated under standardized conditions. Following UVA irradiation the bioactivity of IL-6 increased up to fiftyfold in the supernatants of irradiated compared to mock-irradiated fibroblasts. As determined by Northern blot analysis this was also reflected on the pre-translational level by a tenfold increase of IL-6–specific mRNA following UVA irradiation. Induction of IL-6–specific mRNA was maximal at 6 h post-irradiation, thus clearly preceding the maximal induction of collagenase mRNA at 24 h post-irradiation. To elucidate the regulatory role of IL-6 in the UVA induction of fibroblast-derived collagenase, monospecific polyclonal neutralizing antibodies directed against recombinant human IL-6 and antisense oligonucleotides specifically inhibiting the translation of IL-6 mRNA were used at various concentrations. The amount of UVA-induced collagenase mRNA was reduced in a dose-dependent manner when antibodies or specific antisense oligonucleotides were present during and after irradiation.

Taken together our data provide first evidence that UVA enhances IL-6 synthesis and secretion in fibroblasts. IL-6 induces via an autocrine mechanism collagenase and may thus contribute to the actinic damage of the dermis. Key words: UV irradiation/cytokines/MMP/extracellular matrix. *J Invest Dermatol 101:164–168, 1993*

Several intrinsic and extrinsic factors contribute to the complex phenomenon of aging. Chronologic aging affects the skin in a manner similar to other organs of the organism [1]. Superimposed on this innate process, photoaging is related to a severe ultraviolet (UV)–induced damage of the dermal extracellular matrix. There is increasing evidence that these two processes, chronologic and photoaging, have different biologic, biochemical, and molecular mechanisms [2] including differences in the histologic picture, the content of various extracellular matrix proteins, the formation of cross-links within the collagen molecule [3,4] and the capacity of fibroblasts to organize extracellular matrix molecules ([5]; Scharffetter-Kochanek K, unpublished results).

Sunbathing and tanning for cosmetic reasons as well as treatment of various dermatologic disorders by artificial UV irradiation substantially increase the risk of actinic damage of the skin. Dermal photodamage is characterized by wrinkle formation, loss of recoil capacity, increased fragility, and impaired wound healing. Quantitative and qualitative alterations of dermal extracellular matrix proteins such as elastin [6,7], glycosaminoglycans [6,8], and interstitial collagens [9] are involved. Collagen belongs to a family of closely related but genetically distinct proteins [10,11] of which collagen type I, as the major structural component of the dermis, is responsible for the physicomechanical properties of the skin providing tensile strength and stability.

There are now several morphologic and biochemical indications that collagen type I is reduced in actinically damaged skin ([12]; Trautinger F, Gruenwald C, Trenz A, Pittermann W, Kokoschka EM, Arch Dermatol Res 283:39, 1991 "[abstr]"). Because the enzymatic capacity for collagen synthesis and collagen degradation resides in the dermal fibroblast much effort has been concentrated on studying UV-induced alterations of these fibroblast-controlled processes. Beside UV-affected post-translational modifications of the newly synthesized collagen molecule [3,12,13], we and others have recently shown that fibroblast-derived collagenase—responsible for the breakdown of dermal interstitial collagen—was dose-dependently induced *in vitro* and *in vivo* by UVA irradiation [14,15]. Apart from the potential clinical implication of this finding, we have now been interested in those complex regulatory molecular and cellular mechanisms underlying UV-mediated up-regulation of fibroblast-derived collagenase.

A variety of cytokines has earlier been reported to substantially affect and modulate collagen metabolism during embryogenesis and wound healing and—in case of dysregulation—in fibrotic and atrophic disorders ([16–21]; Kulozik M, Scharffetter K, Herrmann K, Lankat-Buttgereit B, Heckmann M, Krieg T, *J Invest Dermatol* 92:465, 1989 [abstr]). Because interleukin 6 (IL-6) beside other cytokines, stimulates the synthesis of collagenase [22], we addressed the questions 1) whether IL-6 can be induced in fibroblasts by UVA irradiation and 2) whether subsequently IL-6 is involved by an autocrine loop in the UVA-mediated up-regulation of fibroblast-derived collagenase.

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Abbreviations: DTAF, dichlorotriazinyl aminofluorescein; F VIII RAG, factor VIII–related antigen; NU, neutralizing units.

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Our data provide evidence that UVA induces IL-6 in fibroblasts, which in turn via an autocrine mechanism regulates the catabolic pathway of collagen metabolism by the induction of the synthesis of collagenase, thus probably contributing to the loss of collagen type I in actinically damaged dermis.

MATERIALS AND METHODS

Cell Culture Fibroblast cultures were established by outgrowth from skin biopsies of healthy human donors. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Flow, Meckenheim, FRG) supplemented with sodium ascorbate (50 μg/ml), glutamine (300 μg/ml), penicillin (400 U/ml), streptomycin (50 μg/ml), and 10% fetal calf serum (FCS), and grown on plastic petri dishes in a humified atmosphere of 5% CO_2 and 95% air at 37°C [23].

UVA Irradiation The cells were irradiated at a distance of 40 cm by a high-intensity UVA source (UVASUN 3000 equipped with the UVASUN safety filters) emitting wavelengths in the 340–450 nm range (Mutzhas, Munich, FRG) [24]. The spectral distribution of the UVASUN 3000 source was determined with a Beckman UV 5270 spectral photometer. The incident dose at the surface of the cells was 66 mW/second. Dose rates were monitored with a combined UVA/UVB ultravioletmeter (Centra-UV-dosimeter, Osram, Munich, FRG) [25]. During irradiation, cells were incubated in phosphate-buffered saline (PBS) and maintained at 35°C–37°C in a thermostatically controlled water bath. Following irradiation, PBS was replaced by fresh medium with 10% FCS and the cells were incubated for various periods of time.

Determination of IL-6 Bioactivity The IL-6 assay was performed by measuring the IL-6–dependent proliferation of the murine plasmacytoma cell line B9 [26]. Briefly, 5×10^4 B9 cells/ml were cultured in RPMI 1640 medium supplemented with 0.05 mM 2-mercaptoethanol and 10% FCS. The assay was performed in 96 flatbottom microtiter plates in a 200 μl volume. Cells were labeled at 68–72 h with 0.5 μCi ^{3}H-thymidine (specific activity 2 Ci/mmol) (New England Nuclear, Boston, MA) and incorporated activity was quantitated by liquid scintillation counting.

One unit per milliliter is the concentration that leads to half-maximal ^{3}H-thymidine incorporation in the assay. Under these assay conditions 150–500 fg/ml of IL-6 can be detected.

Neutralization Studies To address the role of UV-induced IL-6 in the regulation of fibroblast-derived collagenase and its relationship, studies with neutralizing antibodies (anti–IL-6) were performed.

For this purpose fibroblasts were irradiated in PBS in the presence of neutralizing antibodies. Following irradiation neutralizing antibodies were added to DMEM and supplemented with 10% FCS, and cells were incubated for 24 h prior to the isolation of total RNA.

Neutralizing antibodies were tested in different concentrations using 1, 180, 360, 3600 NU/ml of anti–IL-6. Two different IL-6 antibodies were used. In one set of experiments a polyclonal monospecific antibody directed against the complete recombinant IL-6 protein was raised by immunizing rabbits with 50 μg of IL-6 in complete Freund's adjuvant at 2-week intervals for a period of 2 months [27]. In control experiments corresponding amounts of rabbit serum were added to the cultures as indicated above.

In another set of experiments, commercially available affinity-purified rabbit anti-human IL-6 (Endogen, Boston, MA) was used. This polyvalent neutralizing antibody exhibits a highly specific immunologic reactivity with human IL-6 and does not bind other human cytokines. For control purposes identical experiments were performed in parallel using equal amounts of pre-immune rabbit control IgG (Endogen, Boston, MA).

RNA Extraction and Northern Blot Analysis Total RNA was isolated from cells following established procedures [28]. Briefly, fibroblast monolayer cultures were washed twice with PBS, denatured in 5 M guanidine-isothiocyanate (1 ml), and separated over a cesium chloride cushion (3 ml) at 35,000 rpm for 18 h.

The pellet was rinsed in 70% ethanol, extracted with chloroform/phenol, precipitated in 80% ethanol, and finally dissolved in 20–100 μl TE (10 mM Tris HCl, 1 mM EDTA, pH 8.0). The amount of total RNA was determined photometrically at 260 nm. For Northern blot analysis, 2–8 μg of total RNA were separated by gel electrophoresis on 1% agarose gels under denaturing conditions and subsequently blotted onto nitrocellulose (Bio Rad, Munich, FRG). For dot-blot analysis, serial dilutions of total RNA (3, 1.5, 0.75, 0.375, 0.15 μg) were spotted onto nitrocellulose using a filtration manifold (Bio Rad, Munich, FRG). Filters were baked in a vacuum oven at 80°C for 2 h. Following pre-hybridization at 42°C in 50% formamide, 50 mM sodium phosphate, 5 × Denhardt's (1 × Denhardt is 0.2% bovine serum albumine, 0.02% polyvinyl pyrrolidone, and 0.02% ficoll), and 0.5% sodium dodecylsulfate (SDS) for 2–12 h, hybridization was carried out using deoxyadenosine-5′-[^{32}P]-triphosphate oligolabeled cDNA probes (Megaprime DNA Labelling System, Amersham, Braunschweig, FRG) in 50% formamide, 50 mM sodium phosphate (pH 6.5), 5 × SSPE (0.18 M sodium chloride, 10 mM sodium phosphate, 1 mM ethylenediaminetetraacetic acid, pH 7.4), 5 × Denhardt's and 250 μg/ml salmon sperm DNA (Calbiochem, Bad Soden, FRG) at 55°C for 24 h. For oligolabeling the following clones were used: a 920-bp–long fragment of the original clone K4 corresponding to the 3′ terminal end of the coding sequence and the 3′ untranslated part of collagenase RNA [29,30], a 450-bp–long cDNA fragment of human β-actin [31] and a 1121-bp Eco RI fragment of the human IL-6 cDNA clone (pBSF 2.38.1) (Zimmermann R, Bill E, Northoff H, Heinrich PC, Biol Chem Hoppe-Seyler 369:950–951, 1988 [abstr]). Following hybridization, filters were washed twice in 2 × SSC (1 × SSC is 0.15 M sodium chloride, 0.015 M tri-sodiumcitrate), 0.1% SDS at room temperature for 15 min and twice in 0.1 × SSC, 0.1% SDS at 50°C to 60°C for 15 min. Filters were then exposed with intensifying screens to x-ray film (Kodak X-Omat AR) at −80°C. After development, intensity of dots was measured by densitometry and calculated as percentage of control values derived from RNA of non-irradiated cells.

Immunofluorescence Studies 0.5 ml of a cell suspension (2×10^5 cells/ml) were seeded on a slide and grown for 24 h. After being washed in PBS the cells were fixed in 3% paraformaldehyde (Sigma, Deisenhofen, FRG) in PBS, pH 7.6, for 5 min at room temperature. Immunofluorescence staining was carried out as described elsewhere [32]. Briefly, incubation was performed with the primary antibody (either anti–factor VIII–related antigen [F VIII RAG] or anti–keratin K14, anti–IL-6, anti–IL-6 receptor [IL-6R]) in a humid chamber overnight at room temperature using the following dilutions: anti–keratin K14 1:2000, anti–F VIII RAG 1:1000, anti–IL-6 1:200, anti–IL-6R 1:200. After being washed in PBS, the slides were incubated with the appropriate second antibodies conjugated either with dichlorotriazinyl aminofluorescein (DTAF) or Texas Red at a dilution of 1:200 for 30 min (Dianova, Hamburg, FRG). After further rinsing in PBS, each slide was mounted in 50% glycerol in PBS and cells were then examined under a Zeiss immunofluorescence microscope equipped with appropriate filters. 10^6 cells for each experiment were screened for contamination with human endothelial cells or human keratinocytes. Human endothelial cells isolated from umbilical cords of newborn and normal human skin served as positive controls for the expression of F VIII RAG and for the expression of keratin K14.

Inhibition of Translation with Anti-sense Oligonucleotides To specifically inhibit translation of IL-6 mRNA human dermal fibroblasts were incubated with anti-sense phosphorothioate DNA oligonucleotides (Biometra, Göttingen, FRG) (0.1, 0.2, 1, and 5 μM) in PBS during irradiation and in DMEM supplemented with 10% FCS post-irradiation. Phosphorothioate DNA oligonucleotides with randomized sequences were used as a negative control. Total RNA was isolated 24 h post-irradiation and subjected to Northern blot analysis using oligolabeled cDNA probes for human collagenase and β-actin as an internal control.

RESULTS

Induction of IL-6 and Collagenase mRNA Following UVA Irradiation To study the effect of UVA irradiation on IL-6 and collagenase mRNA induction time course experiments were performed. Total RNA was isolated 0, 1, 3, 6, 12, and 24 h following UVA irradiation and subjected to dot-blot and Northern blot analysis. A low constitutive expression of IL-6 and collagenase was detected in non-irradiated cells.

Induction of IL-6 mRNA was observed already 1 h following irradiation. Induction of IL-6 mRNA was highest at 3 h post-irradiation, thus preceding the maximal induction of collagenase mRNA 24 h after UVA exposure (Fig 1). To rule out the possibility that contaminating lipopolysaccharides (LPS) was responsible for the induction of IL-6 mRNA similar experiments were repeated in the presence of polymyxin B at a concentration of 12.5 μg/ml. The UVA induction of IL-6 mRNA was not altered by the addition of polymyxin B, which inhibited completely the IL-6–inducing effect of lipopolysaccharides (data not shown).

Detection of Bioactivity of Various Cytokines in the Supernatants of Irradiated Cells When culture supernatants of non-irradiated fibroblasts were tested for IL-6 synthesis, low IL-6 activity was detected in the absence of added stimuli. UVA irradiation augmented IL-6 production fiftyfold compared to the mock-irradiated control (Fig 2).

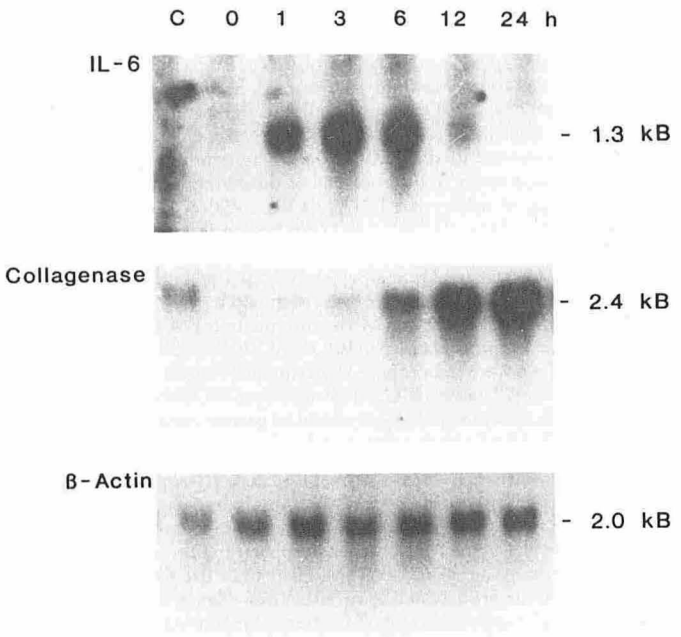

Figure 1. Determination of specific mRNAs by Northern blot analysis. Total RNA from fibroblast monolayer cultures was isolated as detailed in *Materials and Methods* 0, 1, 3, 6, 12, and 24 h after UVA irradiation (C, mock-irradiated control) with the UVASUN 3000 source (30 J/cm²). The RNA was fractionated by electrophoresis under denaturing conditions and blotted onto nitrocellulose. After sequential hybridization with cDNA probes for human IL-6, human collagenase, and human β-actin, the filter was processed for autoradiography and relative amounts of specific mRNAs were determined densitometrically.

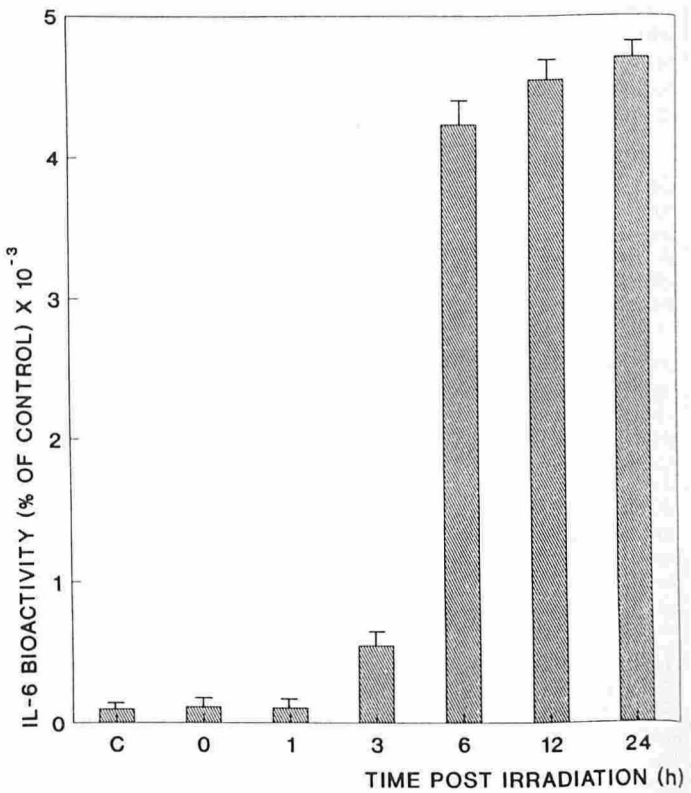

Figure 2. Determination of IL-6 bioactivity in the supernatants of fibroblast monolayer cultures. The bioactivity of IL-6 was determined in the supernatants of fibroblasts at different time points following UVA irradiation by means of the proliferation assay using the plasmacytoma cell line B9 as described in *Materials and Methods*. The bioactivity of IL-6 was expressed as percentage of the non-irradiated control (C).

Effect of Neutralizing Antibodies Against IL-6 on the UVA Induction of Collagenase mRNA To establish whether UVA-induced collagenase synthesis was affected by IL-6, antibody-neutralization experiments were conducted. Table I shows that the presence of various concentrations of anti-IL-6 during and after irradiation reduced the steady-state levels of collagenase mRNA in a dose-dependent manner. However, even at high concentrations, a reduction of collagenase mRNA levels equivalent to the low steady-state levels in non-irradiated cells was not obtained. Pre-immune rabbit IgG at identical concentrations did not alter collagenase mRNA levels. Similar data were obtained using rabbit serum containing anti-human IL-6. In contrast, normal rabbit serum did not exert any effect on collagenase mRNA.

Effect of Anti-Sense IL-6 Phosphorothioate Oligonucleotides on the Steady-State Level of Collagenase mRNA To clarify whether an early intracellular action of IL-6 mediates the UVA induction of collagenase antisense oligonucleotides have been used to specifically inhibit the translation of IL-6 mRNA. Although there was a dose-dependent downregulation of the steady-state level of collagenase mRNA, even high concentrations of anti-IL-6 antisense oligonucleotides could not inhibit the UVA induction of collagenase (Fig 3).

Immunostaining with Antibodies Against F VIII RAG, Keratin K14, IL-6 and IL-6 *R* To detect contaminations of fibroblast monolayer cultures with keratinocytes or endothelial cells, aliquots of 2×10^6 cells of the suspension that was used for the experiments were screened for F VIII RAG and keratin K14 expression. No specific immunofluorescence was found. As positive controls human umbilical cord vein endothelial cells (F VIII RAG) and cryostat sections of normal skin (keratin K14) were used (data not shown). Furthermore, both IL-6 as well as IL-6 receptor were found to be expressed in human dermal fibroblasts (data not shown).

DISCUSSION

Photoaging is due to chronic sun exposure or therapeutic irradiation and is characterized by profound quantitative and qualitative alterations of the dermal connective tissue.

Several extracellular dermal proteins are involved [2,6,7,8], among them collagen, the major structural component of the dermis. However, conflicting data had been published [33]. In most studies collagen has been found to be reduced in actinically damaged skin ([6,12], Trautinger F, Gruenwald C, Trenz A, Pittermann W, Kokoschka EM, Arch Dermatol Res 283:39, 1991 [abstr]). The net accumulation of collagen reflects the balance of the rate of synthesis and degradation. Therefore understanding of the UV-induced quantitative alterations of collagen requires knowledge of the mechanisms of how UV irradiation affects these fibroblast-controlled processes. UVA irradiation is known to reach the reticular dermis making fibroblasts an accessible target [34].

Table I. Effect of Anti-IL-6 on the UVA Induction of Collagenase

Treatment	Dose (NU/ml)	Collagenase (β-Actin)[a]
Control		1.0
UVA		8.6
UVA + control IgG	(360 μg/ml)	8.7
UVA + anti-IL-6	1 (0.1 μg/ml)	8.0
UVA + anti-IL-6	180 (18 μg/ml)	6.7
UVA + anti-IL-6	360 (36 μg/ml)	4.5
UVA + anti-IL-6	3600 (360 μg/ml)	2.4

[a] Densitometrically established ratios of collagenase/β-actin.

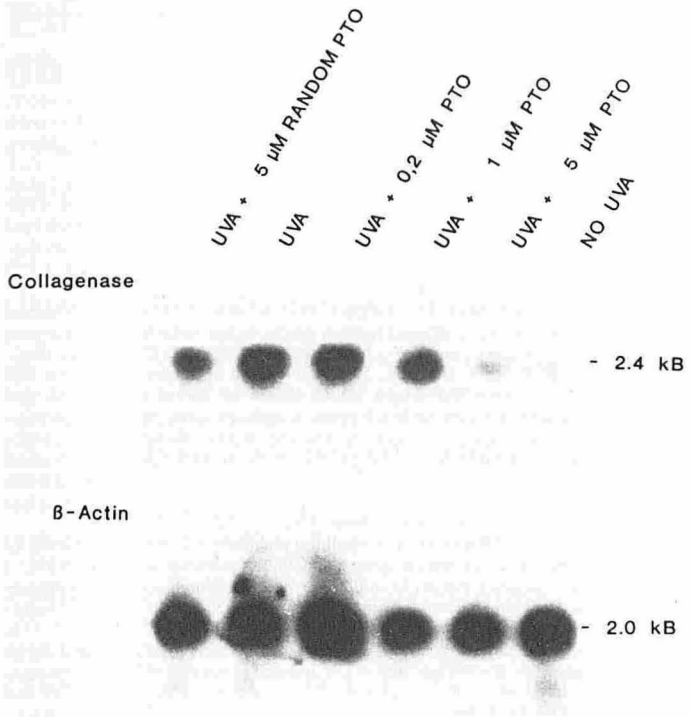

Figure 3. Effect of anti-sense IL-6 phosphorothioate oligonucleotides on the steady-state level of collagenase mRNA. To specifically inhibit the translation of IL-6 mRNA fibroblast monolayer cultures were incubated with anti-sense phosphorothioate DNA oligonucleotides at the indicated concentrations during and after irradiation. Phosphorothioate DNA oligonucleotides with randomized sequences were used as a negative control. Total RNA was isolated 24 h post-irradiation and subjected to Northern blot analysis using cDNA probes for human collagenase and human β-actin.

Recently, we have shown that UVA irradiation induces the interstitial fibroblast-derived collagenase *in vitro* and *in vivo* [14]. The interstitial fibroblast-derived collagenase (EC 3.4.24.7) belongs to a family of matrix-degrading metalloproteinases and is characterized by its distinctive ability to cleave alpha chains of interstitial collagens. Fibroblast-derived collagenase cleaves collagen type I at a single site, revealing fragments approximately three quarters and one quarter the size of the original molecule. In contrast to others [35] we and Petersen *et al* have demonstrated that up-regulation of collagenase mRNA could be attributed to wavelengths of the UVA spectrum (320–400 nm) [14,15]. In this contribution we report on the underlying regulatory molecular and cellular mechanisms of the UVA induction of fibroblast-derived collagenase. An UV-induced extracellular factor from human fibroblasts has been reported to communicate the UV response to non-irradiated cells [36]. However, the exact nature of this factor has not been determined. During the last years much information has accumulated on a group of signaling peptides comprising growth factors, cytokines, and interleukins [37], which had been implicated in tissue remodeling and degradation in a variety of physiologic and pathologic processes [20,21,38]. Among them IL-6 has been reported to stimulate collagenase in dermal human fibroblasts when added exogeneously [22].

Based on these observations we investigated the role of IL-6 in the regulation of UVA-induced collagenase. Northern blot analysis revealed that the specific IL-6 mRNA was induced in a time-dependent manner following UVA irradiation of fibroblasts. The UVA induction of specific IL-6 mRNA results in the translation and secretion of functionally active IL-6. Our data complement earlier findings that UV irradiation enhances the activity of NF-kappa-B [35], which is known to stimulate the transcription of the gene encoding IL-6 [39]. Although the induction of IL-6 occurs prior to the induction of fibroblast-derived collagenase, a causal relationship in the regulation of these proteins has not been revealed. To improve the understanding of the regulatory role of IL-6 in the UVA induction of fibroblast-derived collagenase, antibody-neutralization experiments were performed. Even at high concentrations of neutralizing antibodies against IL-6 — which totally neutralize IL-6 activity in the supernatants of UVA irradiated fibroblasts — the UVA induction of fibroblast-derived collagenase could not be completely abrogated. This might be due to an early intracellular action of IL-6 — a mechanism that has previously been postulated for platelet-derived growth factor [40].

In an attempt to address this issue, anti-sense oligonucleotides have been used to specifically inhibit the translation of IL-6 mRNA. By the specific inhibition of gene expression, the anti-sense technique allows the dissection of the role of individual gene products. This approach has previously been employed to investigate the function of cellular oncogenes [41,42] and the mechanisms of viral replication [43,44]. Incubation of UVA-irradiated cells with IL-6 anti-sense oligonucleotides resulted in a significant but not in a complete down-regulation of UVA-induced collagenase. This indicates that protein synthesis of IL-6 is required, whereas the release of preformed intracellular IL-6 does not substantially participate in the UVA induction of collagenase.

However, even high concentrations of antisense oligonucleotides or neutralizing antibodies against IL-6 could not completely inhibit the UVA induction of collagenase, indicating therefore that IL-6 plays an important but not an exclusive role in the regulation of UVA-induced collagenase. Other cytokines or UV-induced DNA damage as earlier postulated by Stein and co-workers [35] might play an additive or even a synergistic role in the induction of collagen degradation. Furthermore, recent data shed some doubt that IL-6 stimulates the fibroblast-derived collagenase [45]. Even though our data cannot contribute to the resolution of this discrepancy, IL-6 may play a synergistic role in the induction of collagenase possibly by enhancing the effect of an additional cytokine.

The potential role of additional cytokines in the UV response of fibroblasts is currently under investigation. Paracrine modulation of the fibroblast and its collagen metabolism by cytokines released from non-fibroblastic cells has been reported to affect tissue remodeling substantially during morphogenesis, repair, degradation, and fibrosis ([16,17]; Kulozik M, Scharffetter K, Herrmann K, Lankat-Buttgereit B, Heckmann M, Krieg T, J Invest Dermatol 92:465, 1989 [abstr]). We provide several lines of evidence that an autocrine mechanism is involved in the complex UV response of dermal fibroblasts. First, immunofluorescence studies did not detect any contamination of fibroblast cultures with other cell types that might be potential sources of UV-induced cytokines. Second, the UV induction of fibroblast-derived collagenase could partly be inhibited when the synthesis or the function of IL-6 was blocked by anti-sense or antibody techniques. Finally, we were able to confirm the finding that IL-6 and its receptor are concomittantly expressed in fibroblast cultures. Thus the prerequisites for autocrine regulations — defined as the ability of either a cell population or a single cell to produce and to respond via specific receptors to their (its) own factor(s) — are fulfilled. As to our results UVA irradiation induces IL-6 in fibroblasts that via an autocrine loop regulates the catabolic pathway of collagen metabolism by the induction of interstitial fibroblast-derived collagenase. Furthermore, interstitial collagenase degrades collagen types I, III, and also IV, which plays an important role in the maintenance of the dermoepidermal junction. UV induction of fibroblast-derived collagenase may contribute as well to the loss of interstitial collagen as to the dissolution of the basement membrane zone. Blister formation following UVA or PUVA treatment [46,47] or after tanning on sun beds [48–53] has repeatedly been published. In fact, the induction of suction blisters is much easier in psoralen plus UVA–treated compared to control persons, underlining the pathogenetic role of the UV damage of the dermoepidermal junction in blister formation [46].

Our present findings contribute on a molecular level to the understanding of those clinical features collectively known as photoaging. However, even though UVA induces collagenase mRNA

in vivo as shown by *in situ* hybridization [14] it is difficult to fully evaluate the mechanism of collagenase activation in the dermis.

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