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Ultraviolet-B induction of interstitial collagenase and stromelyin-1 occurs in human dermal fibroblasts via an autocrine interleukin-6-dependent loop

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Abstract Ultraviolet-B irradiation of human dermal fibroblasts has earlier been shown to induce matrix-degrading metalloproteinases, thus driving connective tissue degradation in photoaging and photocarcinogenesis. Herein, we report that Ultraviolet-B irradiation led to a dramatic increase in specific mRNA and protein levels of interstitial collagenase, stromelysin and interleukin-6. By contrast, the major tissue inhibitor of matrix-degrading metalloproteinases, TIMP-1, was unaffected. Monospecific neutralizing antibodies directed against human interleukin-6 significantly reduced the interstitial collagenase and stromelysin-1 protein levels. Taken together, our data provide the first evidence that Ultraviolet-B induction of interstitial collagenase and stromelysin-1 occurs via the synthesis and release of interleukin-6. Hence, this newly identified autocrine mechanism may contribute to dermal photodamage.

Key words: Ultraviolet-B; Interleukin-6; Collagenase; Stromelysin-1; Autocrine mechanism; Fibroblast

1. Introduction

The increase in ultraviolet (UV)-B irradiation (280–320 nm) on earth due to stratospheric ozone depletion represents a major environmental threat to the skin, substantially increasing its risk of photodamage with long-term consequences as photoaging and photocarcinogenesis [1–3]. Besides the generation of reactive oxygen species (ROS) [4,5] and the formation of potentially mutagenic DNA damage in skin cells [3,6,7], UVB irradiation induces changes in the expression of many genes [8–10] involved in signal transduction pathways, the cell cycle control, tumor promotion and progression. UVB-inducible genes comprise several proteinases, among them matrix-degrading metalloproteinases (MMP) [11,12] which have been linked to tumor invasion, metastasis [13] and cutaneous photoaging [3,12,14]. Furthermore, UVB irradiation triggers the release of various cytokines from human keratinocytes in vivo and in vitro, among them interleukin-1 (IL-1), IL-6 and tumor necrosis factor alpha (TNFα) [15–17].

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; IL, interleukin; MMP, matrix-degrading metalloproteinase; PBS, phosphate-buffered saline; ROS, reactive oxygen species; TIMP, tissue inhibitor of matrix-degrading metalloproteinases; TMB, 3,3′,5,5′-tetramethylbenzidine; TNF, tumor necrosis factor; UV, ultraviolet

In dermal fibroblasts, evidence was provided for an UVA-induced cytokine network consisting of IL-1α, IL-1β and IL-6 which, via inter-related autocrine loops, induced interstitial collagenase (MMP-1) and, thus, may contribute to the loss of dermal interstitial collagen, a hallmark in photoaging [3,18].

Even though UVA irradiation penetrates much deeper into the dermal connective tissue, UVB irradiation has been shown to reach at least the upper layers of the dermis [19]. In this study, we, therefore, addressed the questions whether (1) the cytokines IL-1α, IL-1β and IL-6 can be induced in human dermal fibroblasts by UVB irradiation and, if so, whether (2) the identified cytokine(s) are subsequently involved in the UVB-mediated up-regulation of fibroblast-derived MMP-1 and/or stromelysin-1 (MMP-3).

Our data provide several lines of direct evidence that UVB irradiation significantly induced IL-6 which, via an autocrine mechanism, regulates the increase in the steady-state protein levels of MMP-1 and MMP-3, most likely contributing to the loss of interstitial collagen and other structural proteins in dermal connective tissue. Interestingly, in contrast to the autocrine role of IL-1α and IL-1β in the UVA induction of MMP, IL-1α and IL-1β were only marginally induced after UVB irradiation, while, similar to the UVA response, IL-6 protein levels significantly increased upon UVB irradiation. These data suggest that UVB and UVA activate, at least in part, distinct pathways leading finally to the induction of MMPs and tissue degradation.

2. Materials and methods

2.1. Reagents

Cell culture medium and Trizol reagent were purchased from Life Technologies (Eggenstein, Germany) and fetal calf serum (FCS) from Biochrom (Berlin, Germany). All chemical and biochemicals were obtained from Sigma (Deisenhofen, Germany) unless otherwise indicated. The human IL-6 mRNA assay (*X*plore mRNA Quantification Systems) was purchased from Endogen (Woburn, MA, USA). Goat anti-human IL-6 neutralizing antibody and normal goat IgG isotype control were from R and D Systems (Wiesbaden, Germany). MMP-1, MMP-3 and TIMP-1 ELISA kits (Biotrak) were obtained from Amersham (Braunschweig, Germany). The IL-1, IL-6 and TNFα ELISA components (CLB, Amsterdam, The Netherlands) were purchased from Hiss Diag-nostics (Freiburg, Germany). Protein G-agarose was from Boehringer (Mannheim, Germany).

2.2. Cell culture

Dermal foreskin fibroblasts were established by outgrowth from biopsies of healthy human donors [20] with an age of 3–6 years and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with glutamine (2 mM), penicillin (400 U/ml), streptomycin

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(50 mg/ml), 10% FCS and grown on plastic tissue culture dishes in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cells were used at passages 5–15 corresponding to cumulative population doubling levels 10–32 [21].

2.3. Light source and UV irradiation

A 1000 Watt (W) Xenon high pressure UV source was used in conjunction with a monochromator with holographic grating (Dermolum UMW, Fa. Müller, Moosinning, Germany). For the experiments, the total UVB spectrum (280–320 nm) was used. Fluence rates were determined by an integrated thermopile. The fluence rates on the cell surface were 0.34 mW/cm^2 (3.4 W/m^2) for the total UVB spectrum. The resulting exposure time (t) (in s) was determined by the equation t = dose (mJ/cm^2)/fluence rate (mW/cm^2). Thus, UVB irradiation at a dose of 10 mJ/cm^2 resulted in an exposure time of 29.4 s. To guarantee a constant intensity and spectral distribution, dosimetry and spectroradiometric analyses were performed prior to experiments with an OL-754 UV/visible light spectroradiometer (Optronic, Orlando, FL, USA). Confluent fibroblast monolayer cultures were rinsed twice with pre-warmed (37°C) phosphate-buffered saline (PBS) and thereafter irradiated at a dose of 10 mJ/cm^2 of the complete UVB spectrum under a thin layer of pre-warmed PBS [22]. There was a negligible loss in viability of cell populations compared to cells held under similar conditions without irradiation [22]. Following UV irradiation, cells were washed with PBS and cultivated in DMEM without FCS for various periods of time prior to the mRNA or protein quantification.

2.4. Enzyme-linked immunoassay (ELISA)

Cytokine ELISAs were performed according to the manufacturer's protocols. Briefly, 96 well immunoplates were coated overnight at room temperature with the appropriate monoclonal anti-human cytokine antibodies (1 μg/ml, 100 μl/well) in 0.1 M carbonate/bicarbonate buffer (pH 9.5). After this and each subsequent step, plates were washed four times with PBS containing 0.005% Tween 20. After blocking the non-specific binding, serial dilutions of cytokine standards or the supernatants to be tested (100 μl/well) were incubated for 1 h at room temperature. Subsequently, plates were incubated at room temperature with 100 μl/well of the diluted biotinylated sheep anti-human cytokine antibody (1:100) and horseradish peroxidase-conjugated streptavidin (1:10 000) for 1 h and 30 min, respectively. Finally, 100 μl of 3,3′,5,5′ tetramethylbenzidine (TMB) peroxidase substrate solution (0.42 mM TMB/0.003% H_2O_2 in 0.11 M sodium acetate buffer (pH 5.5)) was added to each well and the reaction was stopped after 15 min by 100 μl of 2 M sulfuric acid per well. Optical densities were read at 450 nm using a microtiter plate reader. Concentrations of different cytokines to be tested in the samples were determined against standard curves using GraphPad software (San Diego, CA, USA). The MMP-1, MMP-3 and TIMP-1 ELISAs were performed according to the manufacturer's protocols basically corresponding to the procedure described above.

2.5. Isolation of total RNA and IL-6 mRNA quantification

Total RNA from fibroblasts was prepared using the Trizol method (Life Technologies). 0.5 μg of total RNA per sample was used for the IL-6 mRNA quantification assay according to the manufacturer's recommendations with minor modifications. The principle of this assay is based on the fact that two target-specific oligonucleotide probes hybridize to a splice junction present in the IL-6 or other specific mRNA. The 3′-end of the so called invader probe overlaps the specific sequences to which hybridization of the signal probe occurs. The cleavase VII enzyme recognizes and cleaves the 5′-end overlapping signal fragment leading to a significant signal amplification. The increase in the specific signal probe fragments is due to the isothermal reaction cycle (1 h) at a temperature (54°C) near the melting temperature of the signal probe. The accumulation of the signal fragments is directly proportional to the number of target mRNA transcripts. The accumulated signal probe fragments are subsequently captured on a streptavidin-coated plate by a biotinylated capture oligo which forms a primer-template substrate for DNA polymerase. DNA polymerase subsequently extends the signal fragments with fluorescein-dUTP (F-dUTP) (15 min, 37°C). Thereafter, the plates were washed three times and the F-dUTP was detected by an anti-fluorescein alkaline phosphatase-conjugated antibody. Here, 0.5 mM 4-nitrophenyl phosphate in 10% diethanolamine (pH 9.8) was used as a substrate for phosphatase (30 min, 37°C) and the yellow reaction product was measured at 405 nm. The precise number of IL-6 mRNA transcripts in the samples was determined by comparing the signal generated by the samples to a standard curve.

2.6. Neutralization studies

UVB irradiation of confluent human dermal fibroblasts at a dose of 10 mJ/cm^2 was performed in PBS in the presence of different concentrations of neutralizing IL-6 antibodies (5 and 25 μg/ml medium). Subsequently, the PBS/antibody solution was removed and replaced by cell culture medium supplemented with the neutralizing antibody at the same concentrations. 24 h thereafter, the supernatants were collected for determination of the MMP-1, MMP-3 and TIMP-1 protein levels. For control purposes, identical experiments were performed in parallel using equal amounts of goat control IgG.

2.7. Immunoprecipitation

24 h after treatment or mock-treatment of UVB-irradiated fibroblasts with neutralizing IL-6 antibodies, the supernatants were collected and incubated with 50 μl/ml of the homogeneous protein G-agarose suspension supernatant for 3 h at 4°C on a rocking platform. Thereafter, the antigen-antibody complexes bound to protein G were removed by centrifugation at 12 000 × g for 30 s in a microfuge. Subsequently, the IL-6 levels were determined in the supernatants as mentioned above.

3. Results

3.1. Increase in MMP-1 and MMP-3 protein levels upon UVB irradiation

Supernatants were collected from irradiated and mock-irradiated fibroblasts at 0.5, 1, 2, 4, 8, 16 and 24 h following UVB irradiation at a dose of 10 mJ/cm^2 and subjected to ELISAs for the determination of MMP-1, MMP-3 and TIMP-1 (Fig. 1). A low constitutive expression of MMP-1, MMP-3 and TIMP-1 was measured in mock-irradiated cells. 16 and 24 h following UVB irradiation, significant increases in accumulated MMP-1 and MMP-3 protein levels were observed with

Table 1
Time course analysis of the increase in IL-6, IL-1α and IL-1β protein levels after UVB irradiation (280–320 nm) of human dermal fibroblasts

Time after irradiation (h)	mean ± S.D. (pg/mg total protein)		
	IL-6	IL-1α	IL-1β
Control (C)	3.9 ± 0.5	1.4 ± 0.3	0.8 ± 0.1
0.5	3.6 ± 0.6	1.8 ± 0.3	1.6 ± 0.4
1.0	4.1 ± 1.2	1.8 ± 0.5	1.0 ± 0.4
2.0	2.8 ± 1.2	1.5 ± 0.8	1.2 ± 0.2
4.0	37.7 ± 3.0*	1.0 ± 0.2	0.9 ± 0.3
16.0	103.3 ± 11.6*	2.6 ± 0.1	1.4 ± 0.3
24.0	131.9 ± 3.4*	1.2 ± 0.4	1.3 ± 0.2

Confluent fibroblasts were irradiated at a dose of 10 mJ/cm^2 UVB. Thereafter, supernatants were collected at different time points post-irradiation. Quantitative analysis of specific protein levels was performed by ELISA using corresponding standard curves. Three independent experiments were performed. C, control (mock-irradiated cells); S.D., standard deviation; *$P < 0.0001$ (ANOVA) compared to mock-irradiated cells.

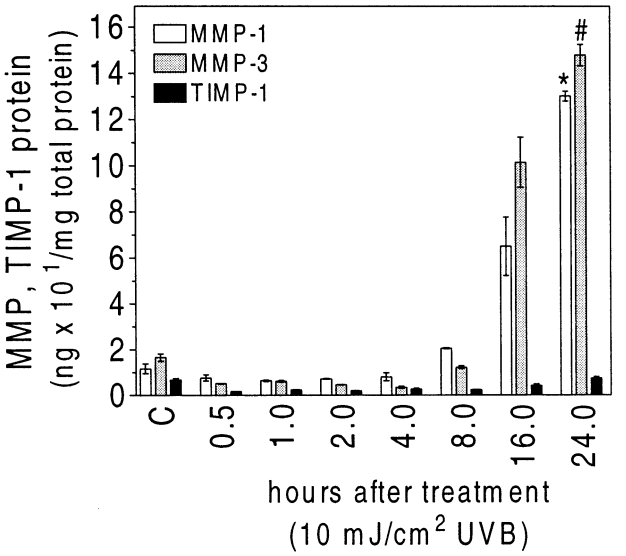

Fig. 1. The time-dependent increase in MMP-1 and MMP-3 protein levels following UVB irradiation. Confluent fibroblasts were irradiated at a dose of 10 mJ/cm^2 with the complete UVB spectrum. Thereafter, supernatants were subjected to ELISAs at different time points post-irradiation. The quantitative analysis of specific protein levels in the samples was determined using standard curves. The results are the mean ± S.D. of three independent experiments. C, control (mock-irradiated cells); S.D., standard deviation; *, #$P < 0.0001$ (two-sided t test) compared to mock-irradiated cells.

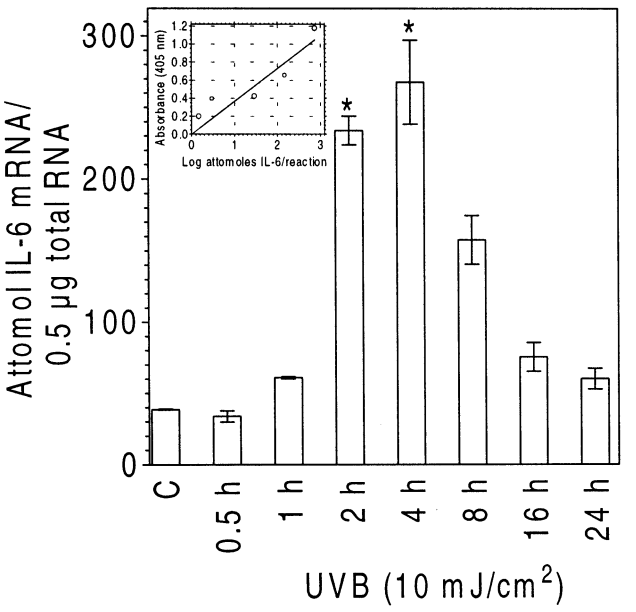

Fig. 2. Time-dependent induction of IL-6 transcription following UVB irradiation. Confluent fibroblasts were UVB-irradiated at a dose of 10 mJ/cm^2. Total RNA was isolated from fibroblasts at different time points post-irradiation and subjected to the mRNA quantification assay. Quantification of specific mRNA levels was performed using defined standard curves as described in Section 2. The results are the mean ± S.D. of three independent experiments. The inset shows the standard curve ($r^2 = 0.89$) of the IL-6 mRNA assay. C, control (mock-irradiated cells); S.D., standard deviation; *$P = 0.0019$ (ANOVA) compared to controls.

an 11- and 9-fold increase in MMP-1 and MMP-3 protein levels compared to mock-irradiated fibroblasts. By contrast, TIMP-1 protein levels were unaffected compared to the controls. These findings corroborate and extend previous data [11,22].

3.2. UVB irradiation results in an increase in the synthesis and release of IL-6 in dermal fibroblasts

To study the effect of UVB irradiation on the protein levels of specific cytokines like IL-1α, IL-1β and IL-6, time course experiments were performed (Table 1). A low constitutive protein level of all tested cytokines was detected in the supernatant of mock-irradiated cells. A significant increase in IL-6 levels was first observed at 4 h post-irradiation with a further time-dependent increase. 24 hours after UVB irradiation, a 33-fold increase in IL-6 protein levels was determined compared to that of mock-irradiated controls. By contrast, the IL-1α and IL-1β protein levels were not significantly increased upon UVB irradiation (Tab. 1). In order to further address the question whether the release of IL-6 into the supernatant was due to an increase in the IL-6 synthesis or rather resulted from the release of pre-formed IL-6, the effect of UVB irradiation on IL-6 mRNA levels was investigated (Fig. 2). For this purpose, time course experiments were performed. Following UVB irradiation, IL-6 mRNA levels significantly increased 2 h post-irradiation and peaked at 4 h post-irradiation with a maximal 9.6-fold increase in IL-6 mRNA levels (Fig. 2). Thereafter, the mRNA levels decreased and reached a similar level as the mock-irradiated cells 24 h post-irradiation. The increase in IL-6 mRNA correlated with the increase in IL-6 protein levels suggesting that the release of IL-6 was due to de novo synthesis of IL-6. This was further supported by the observation that in lysates of fibroblasts no significant amount of IL-6 was detected (data not shown).

3.3. Neutralizing antibodies against human IL-6 prevent the UVB-mediated MMP-1 and MMP-3 induction

To establish whether the UVB-induced MMP-1 and MMP-3 synthesis is regulated via an autocrine loop of IL-6, antibody neutralization experiments were performed. Interestingly, UVB induction of MMP-1 and MMP-3 on the protein level was almost completely suppressed by neutralizing antibodies directed against IL-6 (Fig. 3). Neither lower concentrations of the neutralizing antibody against IL-6 (data not

Table 2
Modulation of IL-6 protein levels after UVB irradiation by neutralizing antibodies against human IL-6

	UVB (+)			UVB (∅)
Anti-IL-6 (µg/ml SN)	0	5	25	0
IL-6 (pg/mg total protein)	154.4 ± 13.1	2.9 ± 0.2	3.0 ± 0.2	2.8 ± 0.1

Confluent fibroblasts were UVB-irradiated, incubated with neutralizing antibodies against IL-6 and thereafter, the antigen/antibody complex was removed by protein G agarose. The concentrations of IL-6 protein in the sample supernatants (SN) were determined against IL-6 standards described in Section 2 and expressed as mean ± S.D. values of three independent experiments. UVB (+), UVB irradiation at a dose of 10 mJ/cm^2; UVB (∅), mock-irradiated cells; S.D., standard deviation.

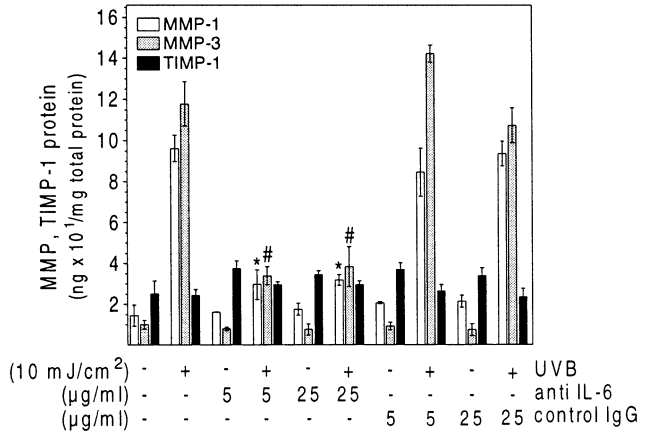

Fig. 3. IL-6 mediates the UVB induction of MMP-1 and MMP-3 via an autocrine mechanism. Confluent fibroblasts were UVB-irradiated at a dose of 10 mJ/cm^2. Supernatants from UVB-irradiated and mock-irradiated fibroblasts which had been incubated with different concentrations of anti-IL-6 and normal IgG were subjected to ELISAs, 24 h post-irradiation. Quantitative analysis of specific protein levels in defined samples was performed using established standard curves. The results are the mean ± S.D. of three independent experiments. C, control (mock-irradiated cells); S.D., standard deviation; *, #$P < 0.0001$ (ANOVA) compared to only UVB-irradiated cells.

shown) nor the normal goat IgG at identical concentrations (Fig. 3) exerted any effect on MMP protein levels. Following UVB irradiation, no significant change in TIMP-1 protein levels was observed in the presence of neutralizing antibodies against IL-6 or isotype control IgG.

3.4. *Anti-human IL-6 reduces IL-6 levels in the supernatant of UVB-irradiated cells*

To address the question whether the reduction in MMP protein levels depended on the formation of antigen(IL-6)-antibody complexes and not on non-specific effects, supernatants of UVB-irradiated fibroblasts were incubated with different concentrations of neutralizing antibodies. Finally, the antigen/antibody complexes were removed and the supernatants tested for IL-6. Table 2 shows that the IL-6 protein levels were dramatically reduced upon incubation with anti-human IL-6 antibody compared to the only UVB-irradiated controls. The IL-6 levels of UVB-irradiated fibroblasts in the presence of neutralizing antibodies were almost identical to that of the mock-irradiated controls (Table 1). This finding suggests that the reduction in MMP-1 and MMP-3 protein levels, in fact, depended on the inactivation of IL-6 by the neutralizing antibody.

4. Discussion

In vivo and in vitro studies in human dermal fibroblasts have shown that the induction of MMP and distinct members of the serine protease family play an important role in the profound quantitative and qualitative alterations underlying photodamage of the dermal connective tissue in photoaging and photocarcinogenesis [3,23,24]. Several chemical and physical agents stimulate the synthesis and activation of the MMPs, among them UVA and UVB radiation [3,11,12, 18,22,25]. Specifically, MMP-1 and MMP-3, responsible for the breakdown of dermal interstitial collagen and proteoglycans, were dose-dependently induced in vitro and in vivo following UVA and UVB irradiation [11,12,18,22,25–27].

Apart from the potential clinical implication of these findings [3,28,29], over the past years, many studies focused on the complex regulatory molecular and cellular mechanisms underlying UVA/B-mediated up-regulation of MMPs. Recently, several components of the intracellular signal transduction pathways as well as transcription factors have been identified to be involved in the UVB-mediated induction of MMPs [11,12,27]. Furthermore, UVB-generated ROS and iron appear to be central factors in the up-regulation of MMPs [12]. For UVA (340–400 nm)-dependent induction of MMP-1, we previously provided evidence that a cytokine network consisting of IL-1α, IL-1β and IL-6 induced MMP-1 via inter-related autocrine loops [18,30]. We furthermore showed that UVA generation of singlet oxygen and hydrogen peroxide preceded the synthesis and release of these cytokines [3,31].

Although there is evidence that UVB irradiation contributed to the synthesis and release of cytokines like IL-1, IL-6 and TNFα in keratinocytes in vitro and in vivo [15,17,32], only scant information exists regarding a potential effect of UVB irradiation on the cytokine stimulation in human dermal fibroblasts. Here, we report a causal autocrine relationship between UVB-mediated induction of the cytokine IL-6 and the increase in MMP-1 and MMP-3 protein levels in human dermal fibroblasts in vitro. As to our data, IL-6 protein levels accumulated up to 24 h post-irradiation, while IL-6 mRNA levels peaked at 4 h and decreased thereafter. These findings rule out that the UVB-induced increase in IL-6 expression is only mediated by a post-transcriptional mechanism by the increase in the stability of IL-6 transcripts as has previously been postulated for human keratinocytes [33]. Furthermore, our data are in line with previously published results. These data showed that normal human fetal skin fibroblasts responded to exogenously added IL-6 with an increase in the synthesis of interstitial collagenase [34,35]. Furthermore, there is evidence that human recombinant IL-6 concentrations in the range of pg/ml led to a significant increase in the collagenase activity compared to mock-treated controls [36].

In contrast to UVA-irradiated fibroblasts [18], IL-1α and IL-1β protein levels were only marginally increased upon UVB irradiation compared to the dramatic increase in IL-6 suggesting that IL-1 may not play an important role in the UVB-dependent MMP-1 and MMP-3 induction. However, exogeneously added IL-1α and IL-1β to cell cultures at much higher concentrations (≥100 pg/ml) as measured in this study induced MMP-1 [37,38] and MMP-3 [38]. Our findings are in line with previously published data showing that during inflammatory processes, mechanisms leading to the synthesis and release of IL-6 are independent on the involvement of IL-1 [39,40].

Taken together, these data contribute to a novel facet of the previously published concept which postulates that UVB irradiation of the skin leads to a keratinocyte-dependent release of growth factors and cytokines IL-1, IL-6 and TNFα which, via paracrine mechanisms, modulate the connective tissue metabolism of fibroblasts [17,41,42]. Here, we add an independent autocrine mechanism.

The intermediate molecular steps from IL-6 release to MMP-1 and MMP-3 induction after UVB irradiation are still unclear and speculative. Even high concentrations of neutral-

izing antibodies against IL-6 could not completely inhibit the UVB induction of MMP-1 and MMP-3 indicating that IL-6 plays an important but not an exclusive role in the regulation of UVB-induced MMPs. UV-induced DNA damage [8,41] and reactive oxygen species [12], respectively, might play an additive or even a synergistic role in the UVB-dependent MMP expression. Furthermore, a complex network of Janus kinases, mitogen-activated and stress-activated protein kinases are discussed to be dependent on IL-6 and to support MMP induction [12,43,44].

In conclusion, the novel finding of this study is that UVB irradiation of human dermal fibroblasts resulted in the synthesis and release of IL-6 which, via an autocrine mechanism, led to the induction of MMP-1 and MMP-3, while the major tissue inhibitor of MMPs, TIMP-1, remained almost unaffected. The resulting imbalance between MMP-1/MMP-3 and TIMP-1 may lead to an enhanced connective tissue degradation, a hallmark in various pathological states.

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