Interleukin-10 Production by Cultured Human Keratinocytes: Regulation by Ultraviolet B and Ultraviolet A1 Radiation

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Keratinocytes are the primary cellular target for ultraviolet radiation in human skin, and ultraviolet radiation–induced therapeutical effects may thus be mediated by keratinocyte-derived, antiinflammatory mediators. Interleukin-10 is capable of exerting anti-inflammatory effects by virtue of its capacity to suppress the production of interferon-γ. The present study therefore assessed the ability of cultured human keratinocytes to produce interleukin-10 following ultraviolet irradiation. Exposure of long-term cultured normal human keratinocytes to ultraviolet B (280–320 nm) or to ultraviolet A1 (340–400 nm) radiation caused a time- and dose-dependent induction of interleukin-10 mRNA expression and interleukin-10 protein secretion, with ultraviolet A1 radiation being the strongest stimulus. Ultraviolet radiation–induced interleukin-10 production by normal human keratinocytes was enhanced by a factor of two, when cells were cultured in high- rather than low-calcium medium. Neither addition of the ultraviolet radiation–inducible cytokines tumor necrosis factor-α or interleukin-1α to unirradiated keratinocytes nor presence of their respective neutralizing antibodies in cultures of irradiated keratinocytes induced or inhibited interleukin-10 synthesis. Modulation of eicosanoid production by addition of prostaglandin E_2 to keratinocyte cultures or disturbance of cyclooxygenase activity by indomethacin did not affect interleukin-10 production in resting or irradiated cells. These studies demonstrate that cultured human keratinocytes are capable of producing interleukin-10. Human keratinocyte interleukin-10 production is dependent on the differentiation state of the cell and induced by ultraviolet B and, in particular, ultraviolet A1 radiation exposure. This novel property of ultraviolet radiation may account at least in part for the efficacy of phototherapy in inflammatory skin diseases. *J Invest Dermatol 104:3–6, 1995*

Ultraviolet (UV) irradiation represents a well-established modality for the treatment of inflammatory skin diseases. For example, UVB (280–320 nm) and/or UVA1 (340–400 nm) radiation may be effectively used to treat patients with psoriasis or atopic dermatitis [1,2]. The anti-inflammatory effects of UV phototherapy have been at least in part ascribed to the immunomodulatory capacities of UV radiation, e.g., UV radiation induced inhibition of epidermal Langerhans cell antigen-presenting cell function [3].

In addition to Langerhans cells, keratinocytes constitute an important cellular target for UV radiation–induced immunomodulation [4]. Previous studies clearly indicate that UVB irradiation may profoundly affect the capacity of keratinocytes to synthesize immunomodulating cytokines. Accordingly, *in vitro* irradiation of keratinocytes with UVB radiation leads to release of tumor necrosis factor-α (TNFα), interleukin-1α (IL-1α), and IL-6 [5–8]. A common property of these UVB-inducible cytokines, however, is their capacity to exert pro-inflammatory effects, and UV radiation–induced release of these mediators may account for some of the symptoms associated with a sunburn reaction [9], but fails to explain the anti-inflammatory effects associated with UV phototherapy. Recently, phototherapy with UVB, and in particular with UVA1 radiation, was found to significantly improve eczema in patients with atopic dermatitis, and this clinical improvement was associated with significant downregulation of *in situ* expression of the pro-inflammatory cytokine interferon-γ (IFNγ) in eczematous lesions of these patients [2,10]. Downregulation of *in situ* expression of IFNγ after phototherapy may have been due either to direct effects of UV radiation on IFNγ-producing T cells, or to indirect effects, e.g., the release of a keratinocyte-derived mediator, which is capable of suppressing IFNγ production. A likely candidate for the latter effect could be the recently identified cytokine IL-10 [11,12]. IL-10, which previously has been described as cytokine synthesis inhibitory factor, is a potent inhibitor of Th_1-mediated immunomodulatory events and acts in part by suppression of T-cell–derived IFNγ. Therefore, in the present *in vitro* study, the capacity of cultured human keratinocytes to synthesize and release IL-10 following exposure to therapeutically relevant doses of UVB and/or UVA-1 radiation was assessed.

MATERIALS AND METHODS

Chemicals All chemicals used for high-performance liquid chromatography were of analytical grade.

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Abbreviation: HNK, long-term cultured normal human keratinocytes.

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Cytokines, Antibodies, Eicosanoids Recombinant human (rh) TNFα was kindly provided by Boehringer Mannheim, Mannheim, Germany. The polyclonal rabbit anti-TNFα antibody IP 300 (IgG and IgM), which was purchased from Genzyme Corporation, Boston, MA, was used at a concentration of 10 μg/ml, which corresponds to a neutralizing activity of 10,000 units TNFα bioactivity, as assessed by the L929 cell cytotoxicity assay. Neutralizing anti-IL-1α antibody was from Genzyme Corporation, Boston, MA. The blocking anti-IL-1 receptor type I antibody M 4 was kindly provided by J.E. Sims, Immunex Corporation, Seattle, WA [13]. Mouse and rat isotype control antibodies were from Dianova, Hamburg, Germany. As a control for the polyclonal rabbit antibodies, a rabbit pre-immune serum was used (kindly provided by Dr. M. Brockhaus, Hoffmann-LaRoche LTD, Basel, Switzerland). Prostaglandin E_2 and indomethacin were from Sigma, Munich, Germany.

Cell Culture The human carcinoma cell line KB (American Type Culture Collection, Rockville, MD) was maintained in monolayer cultures in Dulbecco's modified Eagle's medium (Gibco, Berlin, Germany) containing 10% fetal bovine serum (Gibco, Berlin, Germany) in a humidified atmosphere containing 5% CO_2 as previously described. Supplementation of medium with 10% FBS did not increase basal IL-10 release by cultured KB cells and did not interfere with the IL-10 enzyme-linked immunosorbent assay (ELISA). This cell line was derived from an epidermoid carcinoma in the mouth of an adult man and has previously been used as a model for transformed human keratinocytes [7,8,14].

Long-term cultures of normal human keratinocytes (HNK) were prepared from neonatal foreskin as described previously and maintained in culture using a defined keratinocyte growth medium (KGM, Clonetics Corp., San Diego, CA) [15]. For all experiments, fourth- and fifth-passage cells at subconfluency were used. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. For stimulation, cells were seeded 24 h before irradiation into 25-mm tissue-culture plates (Falcon, Lincoln Park, NJ) at a density of 2.5×10^5 cells/plate. In some experiments, cells were incubated in KGM containing 2 mM Ca^{++} rather than 0.09 mM Ca^{++} 48 h prior to irradiation experiments.

Ultraviolet Radiation For UV radiation, medium was replaced by phosphate-buffered saline, lids were removed, and cells were exposed as previously described to UVB radiation (100 J/m^2) using a bank of 4 FS20 sunlamp bulbs (Westinghouse Electric Corp., Pittsburgh, PA), which are known to primarily emit in the UVB range (280–320 nm) [14,15]. The UVB output was monitored by means of an IL-1700 research radiometer and SEE 240 UVB photodetector (International Light, Newburyport, MA) and was approximately 24×10^{-5} W/cm^2 at a tube to target distance of 22 cm. This UVB dose previously was found to enhance several cellular functions including the expression of cytokine and adhesion molecule mRNA or prostaglandin synthesis and does not affect cell viability as assessed by trypan blue exclusion experiments [7,8,14,15]. To assure that irradiation effects were due to the UVB portion of the emission spectrum of FS20 sunlamps, cells were irradiated through window glass, which completely absorbed wavelengths below 320 nm as measured spectrophotometrically. After irradiation, cells were washed and cultured in medium in the presence or absence of the indicated antibodies. As controls, cells were washed and allowed to sit in phosphate-buffered saline for the irradiation period. For UVA1 irradiation cells were exposed to a UVASUN 5000 Biomed irradiation device (Mutzhas, Munich, Germany). The emission was filtered with UVACRYL (Mutzhas), and UG1 (Schott Glaswerke, Munich, Germany), and consisted exclusively of wavelengths greater than 340 nm. The UVA1 output was determined with a UVAMETER (Mutzhas) and found to be approximately 150 mW/cm^2 UVA1 at a tube to target distance of 30 cm.

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) IL-10 mRNA expression was measured by RT-PCR as described previously [10,16]. Specifically, 1 μg of total RNA was reverse transcribed using mouse maloney leucemia virus reverse transcriptase and an oligo-dT_{18} primer. Linear amplification conditions for each primer pair used were determined as described in detail previously [16]. In brief, a) identical amounts of cDNA were subjected to increasing cycle numbers of PCR to obtain the linear amplification range, then b), increasing amounts of cDNA (for up to 64 times the starting amount) were subjected to PCR of a given cycle number within the linear range to exclude that increased amounts of a specific cDNA lead to disturbance of the linearity in PCR amplification. Amplification was found to be linear in the range for up to 31 cycles for the IL-10 primer pair and up to 25 cycles for the GAPDH primer pair. Therefore, for investigation of IL-10 mRNA, routinely one tenth of the total cDNA was subjected to 28 PCR cycles by employing a primer pair commercially available from Stratagene, Heidelberg, Germany. For investigation of GAPDH mRNA as house-keeping gene, routinely one tenth of the total cDNA was subjected to 22 PCR cycles by employing a primer pair commercially available from Stratagene. Each PCR of each sample for each primer pair was carried out at least two times. Identity of products was established by performing specific endonuclease digestion assays of amplification products. For quantification, amplification products were directly subjected to ion-exchange chromatography connected to an on-line UV spectrophotometer (A260 nm), as described previously [16]. Because GAPDH expression was used as house-keeping gene, amplification signals for IL-10 mRNA were normalized to the respective amplification signals of GAPDH mRNA, as has been described previously [16]. Based on cell numbers, amplification signals for GAPDH mRNA did not change by more than ±12% between culture dishes regardless of their treatment.

IL-10 ELISA For determination of IL-10 protein production two ELISA systems specific for human IL-10 were employed: the Interleukin-10 ELISA kit from Laboserv, Gießen, Germany, based on a murine anti-human IL-10 antiserum, and an IL-10 ELISA kit from Genzyme Corporation, Boston, MA, based on a rabbit anti-human IL-10 antiserum. Both ELISA kits had a sensitivity of about 10 pg IL-10/ml culture supernatant. Measurements were carried out in triplicates.

RESULTS

Irradiation of HNK with 100 J/m^2 UVB radiation resulted in a time-dependent increase of IL-10 protein production as well as in increased intracellular transcription levels of IL-10 specific mRNA **(Fig 1)**. After a lag phase of up to 4 h, both IL-10 mRNA and protein expression increased, reaching a plateau phase at approximately 8 h, which lasted for 24 to 48 h. If HNKs were exposed to FS20 sunlamp radiation filtered through window glass, no enhanced IL-10 protein content was detectable in supernatants 8 h and 24 h after irradiation **(Table I)**. Essentially identical results were obtained when cells from the epidermoid carcinoma cell line KB rather than HNKs were irradiated with UVB radiation (data not

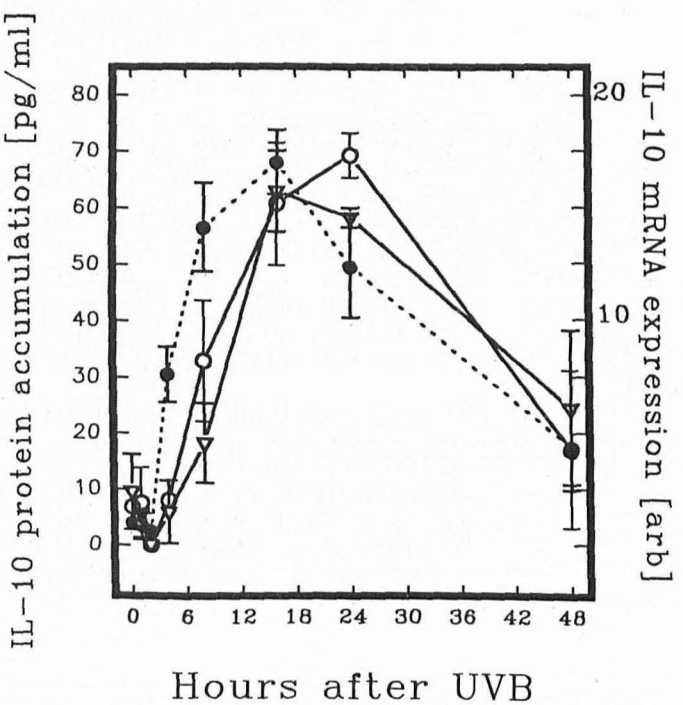

Figure 1. IL-10 expression by HNK at the protein and mRNA level. HNK were kept in culture and irradiated with 100 J/m^2 UVB as described in *Materials and Methods*. IL-10 protein accumulating in culture supernatants was assessed using two different ELISA systems based on distinct antisera (○, murine anti-human IL-10 antiserum; ▽, rabbit anti-human IL-10 antiserum). IL-10 mRNA expression (●) was assessed by RT-PCR as described in *Materials and Methods* and was normalized to the expression of the house-keeping gene GAPDH, which did not differ after any of the treatments by more than ±12% as compared to untreated cells. Interleukin-10 mRNA expression is given in fold expression as compared to unirradiated HNK (unirradiated HNK = 1). Data are given as mean ± SD of three independent experiments.

Table I. Induction of IL-10 Protein Expression in Cultured Normal Human Keratinocytes Following *In Vitro* UVB Radiation Exposure[a]

	Treatment		
Time	Untreated	FS20 sunlamps	FS20 Sunlamps (filtered)
0 h	5.0 pg ± 2.1 pg	6.3 pg ± 2.7 pg	4.7 pg ± 3.1 pg
8 h	4.1 pg ± 0.7 pg	37.6 pg ± 6.8 pg	7.3 pg ± 4.2 pg
24 h	2.9 pg ± 2.5 pg	59.0 pg ± 8.7 pg	9.4 pg ± 2.6 pg

[a] Long-term cultured, normal human keratinocytes were exposed *in vitro* to either unfiltered or window glass–filtered UV radiation (100 J/cm^2) from FS20 sunlamps or left untreated. Supernatants were harvested at the indicated time points and IL-10 protein content determined as described in *Materials and Methods*. Data represent one of two essentially identical experiments.

shown). The capacity to induce keratinocyte IL-10 production was not restricted to short-wave UV (UVB) radiation, but could also be observed upon exposure of cells to long wave UVA1 radiation (32 J/cm^2) **(Fig 2)**. These UVB and UVA1 radiation doses were used because they represent the maximal doses that can be administered without decreasing keratinocyte viability. In fact, UVB versus UVA1 radiation–induced IL-10 production showed essentially identical time kinetics, but at every time point investigated UVA1-induced IL-10 production exceeded that induced by UVB radiation by a factor of two.

To determine the effect of keratinocyte differentiation on IL-10 production, in the following experiments HNKs were grown either in low- or high-calcium medium and then exposed to UVB radiation. Again, a similar time kinetic of IL-10 protein and mRNA expression could be observed for HNKs grown in high-calcium

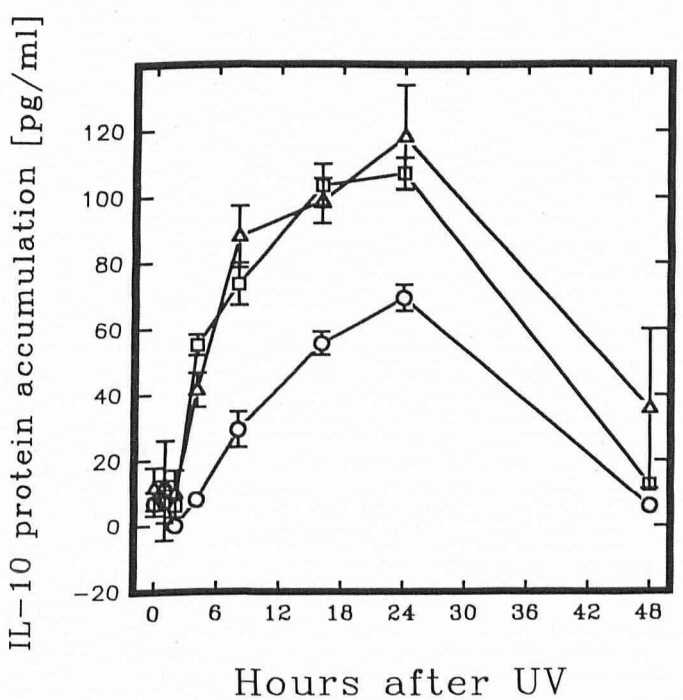

Figure 2. Increase of HNK IL-10 expression by high-calcium concentrations and UVA1 irradiation. HNK were kept in culture and irradiated with 100 J/m^2 UVB (○) or 32 J/cm^2 UVA1 (□), respectively, or were kept in culture medium containing 2 mM rather than 0.09 mM Ca^{++} and irradiated with 100 J/m^2 UVB (△) as described in *Materials and Methods*. IL-10 protein accumulating in culture supernatants was assessed using the ELISA based on the murine anti-human IL-10 antiserum. Data are given as mean ± SD of three independent experiments.

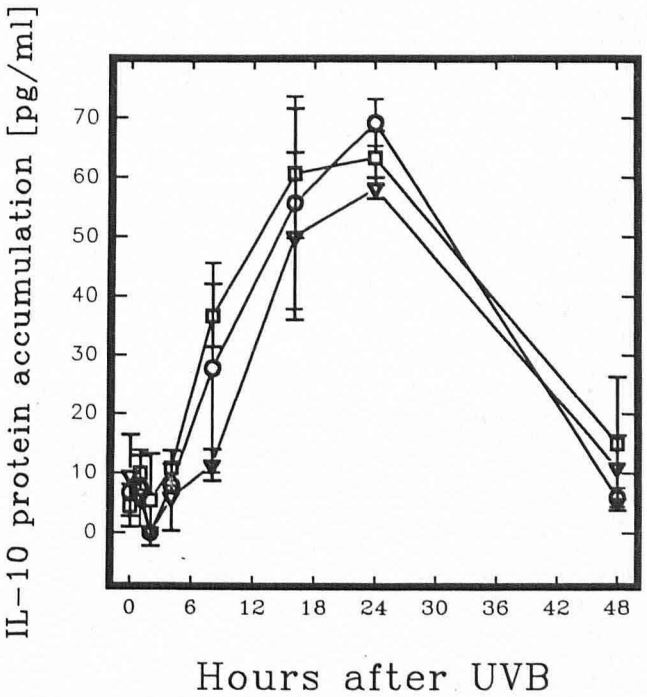

Figure 3. UVB irradiation–caused IL-10 expression is not inhibited by blocking the effects of IL-1α or TNFα. HNK were kept in culture and irradiated with 100 J/m^2 UVB in the presence of neutralizing anti-TNFα antibodies (○), neutralizing anti–IL-1α antibodies (□), or IgG control antibodies (▽). IL-10 protein accumulating in culture supernatants was assessed using the ELISA based on the murine anti-human IL-10 antiserum. Data are given as mean ± SD of three independent experiments.

medium as compared to those cultured in low-calcium medium, but at every time point investigated, high-calcium–cultured keratinocytes released two times more IL-10 than low-calcium–cultured keratinocytes **(Fig 2)**.

The next experiments addressed the question whether UV radiation–inducible cytokines or eicosanoids are able to influence keratinocyte IL-10 production. Neither addition of TNFα nor of IL-1α to unirradiated HNK resulted in release of detectable amounts of IL-10 protein into the culture supernatants. However, in some samples of HNKs used for these experiments, a slight upregulation of IL-10 mRNA could be observed after treatment with these two cytokines (data not shown). To test whether UVB-induced, keratinocyte-derived TNFα or IL-1α might be instrumental in UVB-induced IL-10 production, irradiated keratinocytes were cultured in the presence of neutralizing anti-TNFα, anti–IL-1α, and blocking anti–IL-1 receptor type I antibodies, respectively. Although presence of each of these antibodies was able to significantly inhibit UVB-induced PGE_2 synthesis [14] they did not interfere with keratinocyte IL-10 production **(Fig 3)**.

Similarly, addition of PGE_2, PGD_2 (each 1 μM or 10 μM), or indomethacin (20 μM) did not have any effect on IL-10 production, neither by irradiated nor by unirradiated HNK (data not shown).

DISCUSSION

The presented data clearly demonstrate that human keratinocytes are capable of IL-10 production after treatment with ultraviolet radiation. IL-10 protein could be detected by two independent ELISA systems based on different antisera, resulting in comparable amounts of IL-10 detected in culture supernatants. This IL-10 production is at least in part due to enhanced IL-10–specific mRNA content in stimulated cells. The observed IL-10 synthesis is very unlikely due to other cell contaminants because fourth- to sixth-passage cells have been used in these experiments. In addi-

tion, cells from the epidermoid carcinoma cell line KB were also found to synthesize IL-10 (data not shown). These observations are in line with previous findings demonstrating IL-10 production by murine keratinocytes [17,18].

Interleukin-10 expression induced by irradiation with FS20 sunlamps was due to the UVB portion of the emission spectrum of these lamps, because removal of UVB radiation by glass filters resulted in complete loss of IL-10 induction. Exposure of keratinocytes to UVB radiation led to IL-10 production after a lag phase of approximately 4 h. This kinetic behavior suggested the possible involvement of a UVB-induced, endogenously produced, keratinocyte-derived, soluble mediator responsible for subsequent induction of IL-10 synthesis. Two UVB-inducible cytokines, TNFα and IL-1α, which are induced less than 4 h after UVB irradiation [5–7] did not appear to be inducers of keratinocyte IL-10 production. However, in some experiments performed, stimulation of keratinocytes with TNFα or IL-1α caused a slight but significant induction of IL-10 mRNA, which was not associated with detectable synthesis of IL-10 protein. These observations prompted us to incubate UVB-irradiated keratinocytes in the presence of neutralizing antibodies against TNFα, IL-1α, and blocking antibodies against the IL-1 receptor type I in biologically effective concentrations [14]. None of the antibodies, however, reduced UVB-induced IL-10 production. In addition, another mediator linked to UVB-induced cytokine production, namely PGE_2, neither interfered with UVB induction of IL-10 nor was capable of IL-10 induction by itself. An inducing or suppressing effect of UVB-induced prostanoid synthesis could also be excluded by inhibition of cyclooxygenase activity with indomethacin, which had no effect on IL-10 production. Although these observations do not exclude the involvement of soluble, keratinocyte-derived mediators in UVB-induced IL-10 production, they are more supportive for the assumption of a direct, independent effect of UVB radiation on IL-10 synthesis.

IL-10 production of HNK after UVB irradiation appeared to be regulated by the differentiation state of the cells. Accordingly, IL-10 synthesis by HNK was increased if cells were cultured in high- rather than low-calcium medium. It may be speculated that this behavior reflects an increased UVB susceptibility of keratinocytes in the upper layers of the epidermis, which absorb the highest amount of incoming UV radiation.

The amount of IL-10 produced after UV irradiation also depends on the type of UV radiation employed. Long-wave UVA1 irradiation of keratinocytes led to a nearly twofold higher IL-10 protein synthesis as compared to UVB-irradiated keratinocytes. Interestingly, UVA1 irradiation previously was found to be superior to UVB irradiation in the treatment of atopic dermatitis [2], and the clinical improvement induced by UVA1 irradiation appeared to be due to reduction of lesional IFN-γ expression in atopic eczema [10]. It is therefore tempting to speculate that UVA1-induced expression of IL-10 by keratinocytes accounts at least in part for downregulation of T-helper-1 cell–derived cytokines in inflammatory skin diseases. However, *in vivo* studies employing *in situ* techniques detecting IL-10 expression in human skin are required to prove the involvement of UVR-induced IL-10 expression in the treatment of inflammatory skin diseases.

In summary, human keratinocytes, depending on their differentiation state, are able to respond upon UVB and in particular upon UVA1 irradiation with synthesis of IL-10. Because in the present study a second immunomodulatory mediator for IL-10 production could not be identified, it is assumed that IL-10 synthesis is a direct result of UV irradiation. It is proposed that UV radiation–induced keratinocyte IL-10 expression may at least in part account for the therapeutic effectiveness of UV phototherapy in inflammatory skin diseases.

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