Early Phase of UVB-induced GM-CSF Upregulation in Epithelial Cell Line is not Totally Dependent on IL-1α

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Backgounds **: It was demonstrated that ultraviolet(UV) B light induces the release of IL-1α in cultured human epithelial cell line and augmentation of GM-CSF production by UVB is reported to be mediated by IL-1α in the murine keratinocyte cell line Pam 212.**

Objective **: The purpose of this study was to evaluate the effects of UVB on kinetic profile of IL-1 and GM-CSF mRNA expression and to see whether synthesis of GM-CSF by UVB can be completely inhibited by blocking IL-1α mediated pathway.**

Method **: We used a competitive RT-PCR for measuring cytokine gene expression in epithelial cell line after UV radiation.**

Results **: The IL-1α mRNA increased as early as 1h after UV irradiation, and then decreased at 3h after the irradiation. Thereafter, the response of IL-1α mRNA was upregulated with a second peak at 6h after the UV irradiation. However, mRNA for GM-CSF increased at 1h after UV light exposure and anti-IL-1α antibodies could only partially inhibit UV-augmented GM-CSF production.**

Conclusion **: UVB induced GM-CSF production seemed to be mainly mediated by UVB induced IL-1α but these results suggest that UVB may also induce GM-CSF production through an IL-1α independent pathway. (Ann Dermatol 9:(4):246~252, 1997).**

Key Words : Ultraviolet B, IL-1α, GM-CSF, RT-PCR

Epidermal keratinocytes are capable of producing a wide variety of cytokines and growth factors that may act as mediators in an immune response and play an important role in proliferation and repair[1-4]. The production of epidermal cytokines may be induced by bacterial products, by other proinflammatory cytokines, or by physical alterations[5]. The most relevant physical injury to human skin is ultraviolet(UV) radiation. Recent studies suggest that UVB radiation stimulates the production of IL-1α, IL-3, IL-6, TNF, and GM-CSF in keratinocytes[4-10]. However, little is known about the molecular mechanisms of how UV radiation regulates cytokine production in human keratinocytes. GM-CSF is a survival and growth factor for hematopoietic cells and also a growth factor for epithelial cells[8] but the functional significance of GM-CSF production by keratinocytes remains to be elucidated. Prior studies have revealed that UVB increases the synthesis of IL-1α and GM-CSF in keratinocytes. Upregulation of GM-CSF mRNA by UVB is reported to be mediated by IL-1α [10]. The purposes of this study were to evaluate the effects of UVB on the kinetic profile of IL-1α and GM-CSF mRNA expression and to observe the blocking effects of anti-IL-1α antibody on GM-CSF production by UVB using HaCaT cell line. HaCaT cells are spontaneously transformed human keratinocytes that have the characteristics of basal epidermal keratinocytes. This line can be used as an in vitro model for highly proliferative epidermis, as well as a model for cells defective in ter-

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minal differentiation[11,12]

MATERIALS AND METHODS

Culture of HaCaT cells

Human HaCaT keratinocytes(kindly provided by Dr. N. E. Fusenig, DKFZ, Heidelberg, Germany)[11], spontaneously transformed human keratinocytes, were cultured in DMEM supplemented with 10% fetal bovine serum, 1mM sodium pyruvate, 50μg/ml streptomycin, and 50μg/ml of penicillin at 37℃ in 5% CO_2.

IL-1α antibody

Recombinant rabbit anti-human IL-1α antibody was obtained from Endogen (Cambridge, MA, USA).

UV irradiation of cell cultures

Subconfluent cultures of HaCaT cells were irradiated through PBS(2ml/dish). The UVB light source was a Waldmann UV-800(Waldmann Co., Germany) which used fluorescent lamps(Philips type TL 20W/12) that emitted 2.5 mW/cm^2 of UV light between 285 and 350nm(peak 310-315nm) at a distance of 25cm. Energy was measured with Waldmann UV meter(model no, 585100: Waldmann Co., Germany).

RNA standards for quantitative PCR

To quantify various cytokine RNA by PCR, plasmid pHCQ1 and pHCQ2(Fig. 1) which encode RNA standards were constructed as described previously[13]. To generate standard RNA, plasmids were linearlized with HindIII(Promega, Madison, WI) and transcribed in vitro using T7 RNA polymerase under conditions recommended by suppliers(Stratagene, La Jolla, CA). In vitro transcription of pHCQ1 and pHCQ2 yielded an 840nt and a 413nt RNA product, respectively.

Oligonucleotide primers for PCR amplification

Sequences of the oligonucleotide sense and antisense primers for PCR amplification of the IL-1α were 5'-GTCTCTGAATCAGAAATCCTTCTATC-3', and 5'-CATGTCAAATTTCACTGCTTCATCC-3', respectively. The size of the predicted PCR products from target RNA and standard RNA were 420bp and 530bp, respectively

Sequences of primers for GM-CSF were 5'-ACACTGCTGAGATGAATGAAACAGTAG-3' and 5'-TGGACTGGCTCCCAGCAGTCAAAGGGGATG-3'. The size of the predicted PCR products from target and synthetic RNA were 286bp and 337bp, respectively. Primers were purchased from Korea Biotech (Dae Jeon, Korea).

Detection of GM-CSF and IL-1α mRNA

GM-CSF and IL-1α mRNA were assessed by RT-PCR[13]. Briefly, at various time points following UVB exposure, cellular RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform method as previously described[15]. Known quantities of standard RNA molecules were mixed with total cellular RNA and reverse transcribed at 37℃ for 60min in 20 μl buffer containing 10mM Tris(pH8.3), 50mM KCl, 5mM $MgCl_2$, 1mM each of dATP, dCTP, dGTP, and dTTP, 20u RNasin ribonucelase inhibitor, 0.1μg oligo(dT)15, and 50u M-MLV reverse transcriptase (Promega, Madison, WI). Reactions were stopped by heat inactivation for 10min at 95℃ and chilled on ice. cDNA products were subsequenctly amplified by PCR in 50μl of 10mM Tris(pH8.3), 50mM KCl, 2mM MgCl2, 200μM each of dATP, dCTP, dGTP, and dTTP, in the presence of 25pmol each of 5' and 3' primer. Hot start PCR was used to increase specificity of amplification. The temperature profile of the amplification consisted of 35 cycles of 1 min denaturation at 95℃ and 2.5 min annealing and extension at 60℃ . Negative controls were carried out by omitting RNA for the cDNA synthesis and specific PCR amplification.

Quantitation of PCR products

PCR products were electrophoresed in 2% NuSieve agarose gels and bands were visualized by ethidium bromide staining. Photographs of the gels were taken with a Polaroid 667 film after which band intensities were quantified using a BioRad GS-670 imaging densitometer(BioRad Instruments, Hercules, CA). In this method, a point is determined where the starting number of standard RNA transcripts is equal to the starting number of cellular target mRNA transcripts. To determine this point, the ratios of the band intensities of the PCR products from the standard RNA and target RNA(i.e., ratio standard RNA/target RNA band instensity) are plotted against the starting number of standard RNA molecules using a double logarithmic

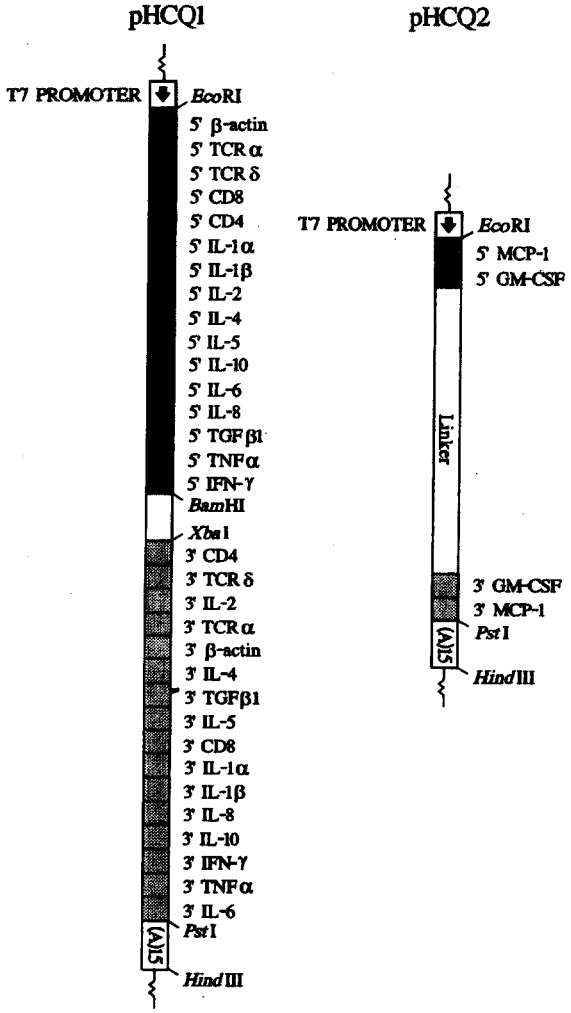

Fig. 1. Structure of plasmids pHCQ1 and pHCQ2 used for generation of synthetic cRNA.

scale(Fig. 2).

ELISA

GM-CSF proteins in cell-free supernatant were measured by enzyme linked immunosorbent assays(ELISAs). The detection limits of these kits are < 2pg/ml of human GM-CSF(Endogen, Cambridge, MA, USA).

RESULTS

IL-1α mRNA expression in HaCaT cells

Recently, we demonstrated that UVB irradiation upregulated IL-1α and the kinetic profile of IL-1α mRNA expression showed a biphasic re-

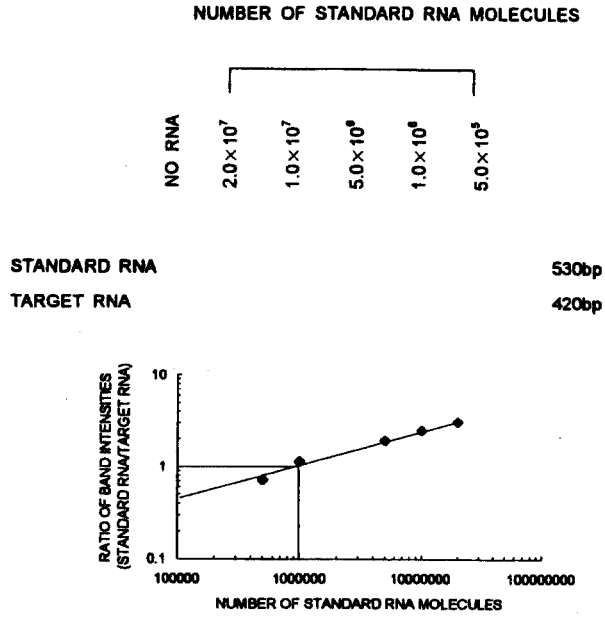

Fig. 2. Quantification of RNA molecules by RT-PCR. Varying numbers of standard RNA molecules were co-reverse transcribed with a constant amount of cellular RNA. After reverse transcription, the cDNA reaction mixture was amplified using IL-1α specific primers. PCR products were electrophoresed and bands were visualized by ethidium bromide staining.

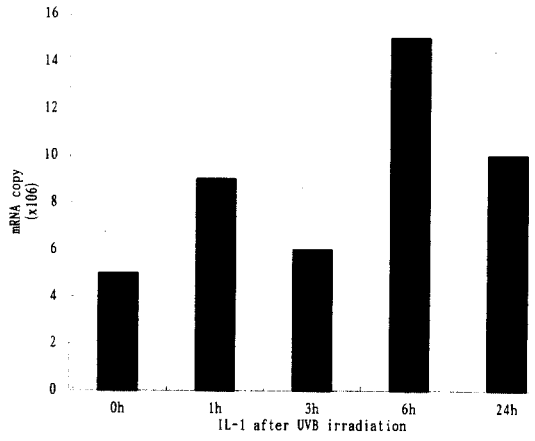

Fig. 3. RT-PCR analysis of IL-1α mRNA expression after UVB irradiation.
HaCaT cells were exposed to 20mJ/cm² of UVB light, and total RNA was isolated at various time intervals after UVB exposure. Oneμg of total RNA was reverse-transcribed with varying numbers of standard RNA molecules.

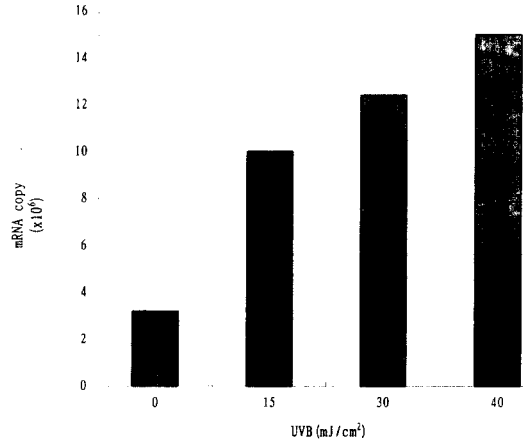

Fig. 4. HaCaT cells were exposed to increasing doses of UVB(0-40mJ/cm^2) light and total RNA was isolated 6h after UVB exposure. Oneμg of total RNA was reverse-transcribed with varing numbers of standard RNA molecules.

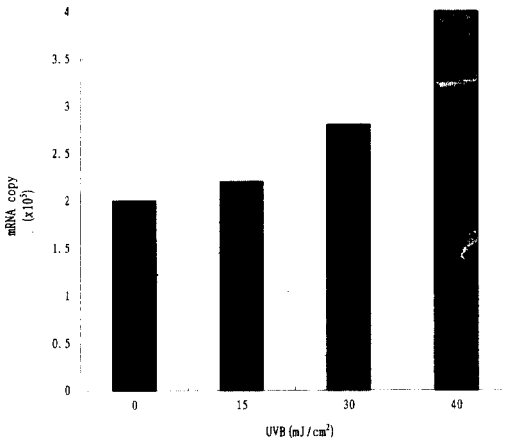

Fig. 5. RT-PCR analysis of GM-CSF mRNA expression after UVB irradiation. HaCaT cells were exposed to increasing doses of UVB(0-40mJ/cm^2), and total RNA was isolated 1h after UVB exposure. One μg of total RNA was reverse-transcribed with varing numbers of standard RNA molecules.

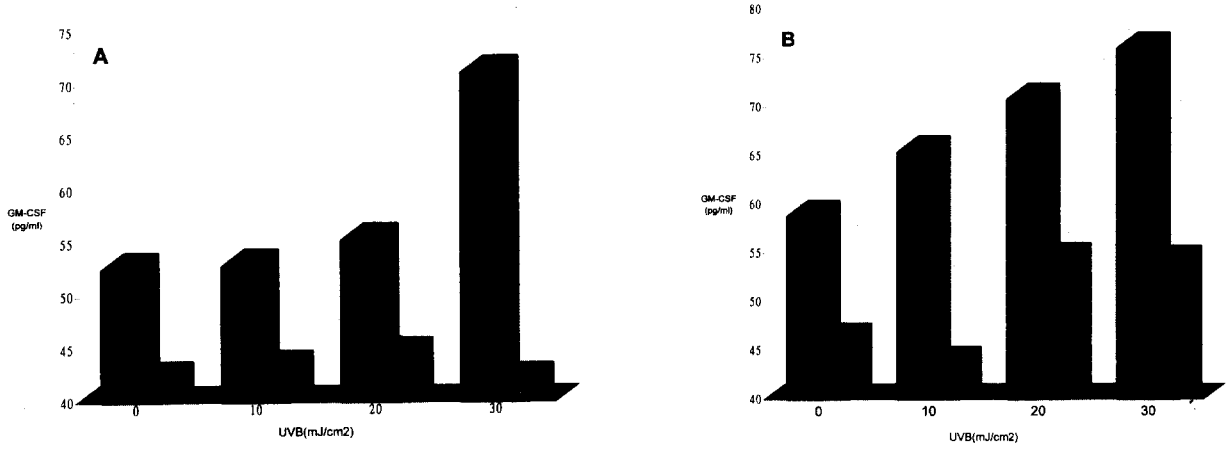

Fig 6. Blocking effects of anti-IL-1α antibody on UVB mediated GM-CSF production.
GM-CSF protein levels were measured by ELISA. A) At 48h after UVA irradiation, the quantity of GM-CSF secreted approximately paralleled the UV doses. When anti-human IL-1α antibody(20μg/ml) was added to irradiated cultured cells, the production of GM-CSF was completely suppressed. B) At 24h after UVA irradiation, secretion of GM-CSF from cultured HaCaT cells also increased according to the UV doses. But the addition of an antibody partially suppress the production of GM-CSF especillay with high UV doses(20-30mJ/cm^2).

sponse after UVB radiation in normal human keratinocytes using Northern blot analysis[6]. In this experiment, expression of mRNA was evaluated by RT-PCR analysis. By the UVB(20mJ/cm^2) irradiation, the response pattern of IL-1α mRNA was also biphasic(Fig. 3). Cultured epithelial cells were irradiated with various doses of UVB(0-40mJ/cm^2) and recovered with sham irradiated samples at the same time. mRNA of IL-1α was compared with that of sham irradiated sample. The IL-1α mRNA increased as early as 1h after UV irradiation, and then decreased at 3h after the irradiation. Thereafter, the responses of IL-1α mRNA was upregulated with a second peak at 6h after the UVB irradiation. These results were the same as those observed in our experiment using UVB irradiated normal human keratinocytes[9]. Thus UVB has a significant effect on the expression of IL-1α in Ha-

CaT cells. The IL-1α mRNA was also increased even at 24h after UVB irradiation. As shown in Fig. 4, UVB irradiation significantly upregulated IL-1α mRNA from the doses of 15-20mJ/cm^2 at 6h after UVB irradiation. High doses of UVB (40mJ/cm^2) increased the mRNA level up to five times that of the basal level at 6h(Fig. 4). Unirradiated HaCaT cells expressed more than 3×10^6 IL-1α mRNA transcript/μg cellular RNA.

GM-CSF mRNA expression in HaCaT cells

Expression of GM-CSF was also evaluated by RT-PCR after UV irradiation. Each set of samples, taken at various time points after UV irradiation, includes a sham irradiated sample as a control. The GM-CSF mRNA increased as early as 1h after UVB irradiation(Fig. 5). As shown in Fig. 5, the expression of GM-CSF by unirradiated HaCaT cells was between 1.5×10^5 and 2.3×10^5 transcript/μg cellular RNA. High doses of UVB(40mJ/cm^2) increased mRNA levels for GM-CSF up to twice that of the basal level at 1h. And, GM-CSF mRNA expression returned to the basal level at 6h and even up to 24h after UVB irradiation.

Anti-IL-1α antibody partially inhibit GM-CSF production

The human GM-CSF protein levels in cell-free supernatant was measured with a commercially available ELISA kit. Fig. 6 reflect the total amount of GM-CSF released in a 24h and 48h period following UV exposure. As shown in Fig. 6, GM-CSF secretion by HaCaT cells increased following UV irradiation. The quantity of GM-CSF secreted approximately paralleled the UV doses. Because it is reported that IL-1α can induce the production of GM-CSF in keratinocytes[10], we designed experiments to investigate whether the UVB radiation induced GM-CSF expression is mainly mediated by IL-1α. To determine the effect of anti-human IL-1α antibody on GM-CSF production, we added anti-human IL-1α neutralizing antibody and measured human GM-CSF protein levels in cell-free supernatants. The antibody was added to the medium immediately after UVB irradiation, and the cells were incubated for an additional 24h to 48h. At 24h and 48h after UVB irradiation, cell-free culture supernatants were collected for ELISA analysis. The addition of anti-IL-1α antibody(20μg/ml) decreased the GM-CSF in all tested conditions. As shown in Fig. 6a, the addition of recombinant anti-IL-1α antibody(20 μg/ml) can completely suppress the effect of UV-induced GM-CSF augmentation when supernatant was collected at 48h after UV irradiation. However, the addition of an antibody can not completely suppress the effects of UVB when supernatant was collected at 24h after UV irradiation especially in the case of UVB dose greater than 20mJ/cm^2(Fig 6b). In a control experiment, pro-immune rabbit serum did not show any effects on GM-CSF produciton.

DISCUSSION

In parallel with examining the effects of UV on GM-CSF secretion by ELISA, studies were undertaken to examine the effect of UV irradiation on IL-1α and GM-CSF mRNA expression. The advent of the polymerase chain reaction(PCR) has brought with it the ability to amplify and detect rare mRNA transcripts that has been virtually undetectable using Northern techniques. For more reliable quantification, competitive RT-PCR procedures have been developed, where a second template similar to the target template serves as a stringent internal control at all stages of PCR[13]. In this experiment, we used competitive RT-PCR to quantify IL-1α and GM-CSF mRNA in HaCaT cell after UVB irradiation.

Recently, we have shown biphasic upregulation of IL-1α by UVB irradiation[6] using Northern blot analysis. Here, we showed again the biphasic upregulation of IL-1α by UVB irradiation using competitive RT-PCR analysis. The IL-1α mRNA show double peaks at 1h and 6h. Our results show that unirradiated HaCaT cells expressed more than 3×10^6 IL-1 mRNA transcripts per μg of cellular RNA. High dose of UVB(40mJ/cm^2) increased IL-1α mRNA level up to 5 times that of the basal level at 6h. In the present study, we have shown that IL-1α can be readily quantified by the competitive RT-PCR procedure. This procedure was capable of reliably discriminating between two fold differences in IL-1α RNA copy numbers.

Several hypotheses for the mechanism(s) behind the induction of GM-CSF(granulocyte macrophage colony-stimulating factor), synthesis and release have been proposed. Nozaki et al(1991) reported that GM-CSF mRNA expression increased at 32h

after UV irradiation and demonstrated that UVB irradiation-induced augmentation of GM-CSF is mediated by UV-induced IL-1α[10]. Because anti-IL-1α antibodies can completely inhibit UV-augmented GM-CSF mRNA expression in their experiment, they suggest that UVB induced GM-CSF production is mediated by UVB-induced IL-1α. In order to study the role of IL-1α in UV induced augmentation of GM-CSF, we measured the production of GM-CSF by HaCaT cells and the blocking effects of anti-human IL-1α antibody. The release of GM-CSF in response to UV was observed within 24h in a dose reponse manner. We also demonstrated that the addition of anti-IL-1α antibodies can completely inhibit UV-augmented GM-CSF secretion when GM-CSF was measured at 48h after UV irradiation. These results suggest that delayed augmentation of GM-CSF secretion by UVB is mainly mediated by IL-1α and augmentation effects can be completely blocked by anti-IL-α antibody.

However, Gallo et al(1991) demonstrated that mRNA of GM-CSF increase within 1h of UV exposure[7]. We also observed inceased expression of GM-CSF 1h after UVB irradiation. Because expression of IL-1α and GM-CSF increase as early as 1h after UV irradiation, early increase in expression of GM-CSF does not seem to be mediated by IL-1α. To assess the effect of IL-1α in early UV-induced augmentation of GM-CSF, we measured GM-CSF levels at 24h after UV irradiation. Contrary to the findings at 48h, the addition of anti-IL-1α antibody failed completely to inhibit the UV-augmentation effect although the level of GM-CSF is lower than that of UVB treated samples. These results suggest that UV may have an early and direct effect on the cellular mechanism of gene expression of GM-CSF which is not related to an IL-1α mediated pathway.

UV irradiation may trigger cutaneous inflammatory responses by directly inducing epidermal keratinocytes to elaborate specific cytokines, such as IL-1 and IL-6[15]. Our findings suggest induction of the GM-CSF by UV is a complex process that may not be partly mediated by IL-1α.

Acknowledgements

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