Effects of UVB on the Synthesis of Complement Proteins by Keratinocytes

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UVB exposure of the skin results in increased production of several cytokines by keratinocytes and infiltration of inflammatory cells. We hypothesized that UVB may increase the expression of complement (C) components and C-regulatory proteins by keratinocytes. In vivo, UVB may upregulate these proteins by direct effects or via cytokines released by keratinocytes or infiltrating inflammatory cells. In vitro, UVB may upregulate these proteins only directly, because of dilution of released cytokines in the medium. To test this, we exposed cultured human keratinocytes to UVB (0–64 J per m²) and monitored C3 and Factor B release in the medium by enzyme-linked immunosorbent assay, and surface expression of decay accelerating factor, membrane cofactor protein, and CD59 by flow cytometry. Keratinocytes produced small amounts of C3 and Factor B, which remained unaffected by UVB. UVB (32 J per m²) caused a transient upregulation of all three C-regulatory proteins. Decay accelerating factor expression was maximal at 48 h (1.81 ± 0.06-fold increase in mean fluorescence intensity over nonexposed cells), membrane cofactor protein at 72 h (2.13 ± 0.09-fold increase in mean fluorescence intensity), and CD59 at 120 h (1.96 ± 0.09-fold increase in mean fluorescence intensity), returning to baseline values within 96, 192, and 192 h, respectively. Exposure to 64 J per m² resulted in significant cell death; cells surviving this dose up to 48 h expressed a higher level of all the three proteins than those surviving 32 J per m².

In conclusion, UVB upregulated membrane cofactor protein, decay accelerating factor, and CD59 on keratinocytes without affecting the constitutive release of C3 and Factor B. Thus, UVB can increase the resistance of keratinocytes against their own C known to be produced excessively in response to cytokines of inflammatory cells that infiltrate the skin following UVB exposure. Key words: complement regulatory molecules/CD59/C3/decay accelerating factor (DAF)/Factor B/keratinocytes/membrane cofactor protein (MCP). J Invest Dermatol 111:683–688, 1998

Human keratinocytes, the major cell type in the epidermis, have been recognized as initiators of inflammation (Barker et al., 1991). They are immunocompetent cells: they act as accessory cells in T cell responses (Chu and Morris, 1997) and synthesize a number of cytokines (Luger and Schwarz, 1990). They also synthesize complement (C) components C3 (Basset-Seguin et al., 1990) and Factor B (Yancey et al., 1992). Recently it has been shown that some of the cytokines produced by keratinocytes and inflammatory cells can differentially enhance the synthesis of C3 (Terui et al., 1997) and Factor B (Pasch et al., unpublished work) by keratinocytes. Keratinocytes are also expected to produce other components of C whose synthesis is also likely to be regulated by cytokines. Therefore, under inflammatory conditions of the epidermis, keratinocytes may be considered a local source of C.

C3 and Factor B produced by keratinocytes are beneficial to the host in that they participate in the first line of defence against invasion by foreign cells such as microbes in the skin. These components can eliminate foreign cells via the alternative pathway of C by generating the C3/C5-convertases (C3b.Bb)/(C3b)n.Bb of the alternative pathway on them (Asghar, 1997). C5-convertase in the presence of C5 through C9, can cause the assembly of the C5b-9 complex, the membrane attack complex, on the cell surface that lyses the foreign cells.

Although C produced by keratinocytes is beneficial in host defence against foreign cells, it potentially can damage autologous keratinocytes as well, through the membrane attack complex assembly on them. Keratinocytes have C-regulatory proteins embedded in their membranes to prevent this damage (Sayama et al., 1991; Dovezenski et al., 1992; Venneker et al., 1994). These include decay accelerating factor (DAF; CD55), membrane cofactor protein (MCP; CD46), and CD59 (Liszewski et al., 1996). These proteins inhibit different steps of C activation on cell surfaces. DAF reversibly interferes with the formation of C3/C5-convertases of both pathways of C and accelerates their decay (Nicholson-Weller and Wang, 1994). MCP acts as a cofactor for the C3 cleaving enzyme, Factor I, and interferes irreversibly with the assembly of C3/C5-convertases of both pathways (Liszewski and Atkinson, 1992). CD59 is incorporated into the membrane attack complex during its assembly on the cell membrane and makes the membrane attack complex noncytolytic (Davies and Lachmann, 1993). Because DAF, MCP, and CD59 are mostly coexpressed, they act synergistically to inhibit C activation on autologous cells to protect them.

Ultraviolet (UV) light exposure of the skin induces dramatic changes in vivo, among them the release of a large number of cytokines from keratinocytes, including IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, TNF-α,
GM-CSF, M-CSF, and MCAF (Schwarz et al., 1994). Some of these cytokines can attract other cell types, such as monocytes and T cells. As a result inflammatory cells infiltrate the epidermis after UVB exposure (Hawk et al., 1988; Di Nuzzo et al., 1996). These cells secrete cytokines, some of which are not secreted by keratinocytes, e.g., T cells can secrete IL-2 and IFN-γ (Keho, 1989; Swain, 1991). Thus, UVB exposure results in accumulation in the skin of different cytokines, which have profound effects on cellular immune responses.

From the foregoing and from other studies showing suppression of delayed hypersensitivity it is clear that UVB exposure exerts important effects on cellular immune reactions (Granstein, 1990); however, the effects of UVB exposure on humoral immune system, including the synthesis of C components, has not yet been studied. We argued that UVB exposure of the skin may increase synthesis of C components by keratinocytes either directly or indirectly via some cytokines released by keratinocytes or by inflammatory cells infiltrating the skin in response to UVB. If synthesis of components of C is increased after UVB exposure, increased levels of C can damage keratinocytes. Therefore, a mechanism(s) must exist to protect keratinocytes from C, excessively produced by them, in response to UVB. This mechanism could be the increased expression of C-regulatory proteins on keratinocyte, either directly by UVB or indirectly via some cytokines released by keratinocytes or by inflammatory cells infiltrated following exposure to UVB. We investigated whether direct UVB exposure of cultured human keratinocytes can increase (i) the constitutive release of C3 and Factor B and (ii) the surface expression of DAF, MCP, and CD59.

**MATERIALS AND METHODS**

**Keratinocyte culture** Human keratinocytes were isolated by incubation of foreskin with thermolysin (0.50 mg per ml, Sigma, St. Louis, MO) at 4°C for 16 h and subsequent trypsinization (0.025%) for 5 min at 37°C. Trypsin (Sigma) was then neutralized by an excess volume of heat inactivated fetal calf serum (GibcoBRL, Breda, The Netherlands). Cells were separated from debris by filtering through a nylon mesh, centrifuged, and resuspended in keratinocyte serum free medium (GibcoBRL) supplemented with penicillin/streptomycin (100 IU per ml, 100 µg per ml; GibcoBRL). The keratinocytes were plated onto 100 mm plastic Petri dishes at a density of 400,000 cells per Petri dish and were incubated at 37°C in humidified, 5% CO2, tissue culture incubator. Cells were separated from debris by incubation of 100 µl PBS containing 2% bovine serum albumin (Sigma) in dimethylsulfoxide (Merck, Hohenbrunn, Germany)-citrate buffer for 10 min. Reaction was stopped with 100 µl H2SO4 (2M). Optical density (OD) was measured at 450 nm. The detection limit of this ELISA was 1 ng per ml of C3.

Factor B was assayed by a previously described sandwich ELISA (Mitani et al., 1991) with several modifications. Briefly, wells were coated overnight at 4°C with 3 µg polyclonal goat anti-human Factor B IgG (ATAB, Stillwater, MN) per ml in carbonate buffer. After thorough washing with PBS/Tween-80 (0.05%), wells were blocked with PBS/milk powder (2%) (Nutricia, Zoetermeer, The Netherlands) for 1 h at room temperature, and then washed again. Wells were then incubated for 2 h at 37°C with 200 µl PBS containing 1% bovine serum albumin. Washing was repeated and wells were incubated with 100 µl of sample, diluted in the same buffer that was used for blocking. Plates were incubated for 1 h at 37°C. After washing, the wells were incubated with biotinylated goat anti-human Factor B IgG (1.25 µg per ml) for 1 h at 37°C. After washing, the wells were incubated for another hour at 37°C with peroxidase conjugated poly streptavidin (1:1000; DAKO, Glostrup, Denmark). The wells were then repeatedly washed. Incubation of the wells with the peroxidase substrate, termination of the reaction, and measurement of absorbance was carried out as described for C3. The detection limit of this ELISA was 100 pg per ml.

For IL-8 ELISA, wells were coated with 1 µg monoclonal mouse anti-human IL-8 (Biosource, Breda, The Netherlands) per ml in 100 µl carbonate buffer (pH 9.6) overnight at 4°C. After thorough washing with Tween-80 (0.05%) samples, the wells were blocked for 1 h at 37°C with 200 µl PBS containing 1% bovine serum albumin. Washing was repeated and wells were incubated with 100 µl of sample, diluted in the same buffer that was used for blocking. Plates were incubated for 2 h at 37°C. The wells were then washed and incubated with 100 µl biotinylated mouse anti-human IL-8 (0.1 µg per ml) (Biosource) for 1 h at room temperature. After washing, the wells were incubated for another hour at 37°C with peroxidase conjugated poly streptavidin (1:1000; DAKO, Glostrup, Denmark). The wells were then repeatedly washed. Incubation of the wells with the peroxidase substrate, termination of the reaction, and measurement of OD was carried out as described for C3. The detection limit of this ELISA was 5 pg per ml.

Standard curves for C3 and Factor B ELISA were made using human complement calibrator CA1 (ATAB). Standard curve for IL-8 ELISA was made using IL-8 Calibrator (Biosource).

**Flow cytometry** Keratinocytes were detached with the trypsin/EDTA solution for 3–5 min. Trypsin was inactivated by fetal calf serum and detached cells were washed and resuspended in fluorescence-activated cell sorter (FACS) buffer ( PBS/2% fetal calf serum, 0.1% sodium azide). Approximately 105 cells were incubated with specific monoclonal antibodies to MCP (clone J4–48, CLB, Amsterdam, The Netherlands), DAF (clone BRIC 110, CLB) or CD59 (1FS) (Okada et al., 1989) or isotype control (Becton Dickinson, San Jose, CA) for 30 min at 4°C. Cells were washed twice and incubated for 30 min with fluorescein-conjugated F(ab)2 fragments of goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Hereafter, cells were washed twice with FACS buffer. Propidium iodide (Sigma) was added to identify dead cells and 20,000 cells were analyzed immediately by FACSCAN® (Becton Dickinson). Dead cells were excluded.

The detachment of keratinocytes with trypsin (0.025%/EDTA (1.5 mM) at 37°C for 3–5 min did not cause degradation of any of the three C-regulatory proteins, as expression of these molecules on cells detached by this procedure was the same as on those detached with EDTA (2 mM) alone at 4°C for 30 min. The former procedure was selected because it did not cause loss of cell viability, whereas the latter procedure caused a high degree of loss of cell viability. Mean fluorescence intensity (MFI) was calculated with WinMDI software. MFI was corrected for isotype MFI and values of cells not exposed to UVB were considered significant.

**RESULTS** Increasing doses of UVB cause progressive loss of cell viability. In order to study the effect of UVB exposure on the expression of C and C-regulatory proteins by keratinocytes, suitable
UVB exposure has no influence on the synthesis of C3 and Factor B by human keratinocytes. Subconfluent keratinocyte cultures were exposed to UVB, Supernatants were harvested at 72 h and concentrations of C3 and Factor B determined by ELISA. IL-8 was included as a positive control. The figure shows C3, Factor B, and IL-8 protein concentrations in supernatants of three independent experiments. In each experiment three wells were exposed to each dose and the contents of each well were analyzed in quadruplicate. Mean values and SD are shown.

UVB exposure has no influence on the synthesis of C3 and Factor B by keratinocytes. To see if UVB influences the constitutive production of C3 and Factor B, keratinocytes were exposed to increasing doses of UVB (4–32 J per m²). Non-exposed cells served as controls. Both exposed and nonexposed cells were cultured for 72 h and supernatants were collected. The concentrations of C3, Factor B, and IL-8 were measured by ELISA in the supernatants.

In supernatants harvested from cultures of nonexposed keratinocytes C3, Factor B, and IL-8 were found to be present in low concentrations. In supernatants of cultures of keratinocytes exposed to increasing doses of UVB, concentrations of C3 and Factor B remained low but the concentration of IL-8 was significantly increased (Fig. 2). IL-8 production was highest at 32 J per m². The maximal IL-8 level in the supernatant was 609 pg per ml. To exert biologic activity much higher concentrations are required (Schroder et al., 1987). These results show that UVB activates keratinocytes, as could be concluded from the IL-8 response studies with a moderate dose of up to 32 J per m² were carried out at 48 h of culture after irradiation for DAF and at 72 h of culture after irradiation for MCP and CD59. In the case of the

Cultured keratinocytes express DAF, MCP, and CD59. Previous immunohistochemical studies in our laboratory have shown that DAF, MCP, and CD59 were present in situ on several structures of human skin, including keratinocytes (Vennekens et al., 1994). Flow cytometric analysis of cultured keratinocytes in this study confirmed that all three proteins were expressed on these cells (Fig. 3).

A single dose of UVB causes a prolonged increase in the surface expression of DAF, MCP, and CD59. When cultured keratinocytes were exposed to UVB (32 J per m²) and surface expression of the C-regulatory proteins was analyzed at several time points (0–192 h), expression of all the three proteins was found to be transiently increased (Fig. 4). DAF expression increased rapidly, reaching its maximum at 48 h after UVB exposure (1.81 ± 0.06-fold increase in MFI over nonexposed cells). Thereafter, DAF expression decreased rapidly, returning to control values within 96 h. MCP expression increased, reaching its maximum at 72 h (2.13 ± 0.09-fold increase in MFI). Expression of MCP gradually returned to control values in 192 h. CD59 expression increased rather slowly, reaching a plateau at 72–120 h (1.74 ± 0.07-fold and 1.96 ± 0.09-fold increase in MFI, respectively). Expression of CD59 returned to almost baseline values in 192 h.

Increasing doses of UVB cause a dose-dependent increase in surface expression of C-regulatory proteins. Time-response studies described above showed that with 32 J per m², surface expression of DAF was maximal at 48 h of culture after irradiation and surface expression of MCP and CD59 was maximal at 72 h. Therefore, dose–response studies with a moderate dose of up to 32 J per m² were carried out at 48 h of culture after irradiation for DAF and at 72 h of culture after irradiation for MCP and CD59. In the case of the
with 32 J per m² were carried out at time points of maximal expression of the skin (Hawk and Fukuyama, 1972; Morison, 1983). In some diseases of unknown etiology, such as photoallergy, polymorphous light eruptions, solar urticaria, solar eczema, and actinic reticuloid, the disease is exacerbated by light but the role of C remains unknown. In none of these diseases is the origin of C in the skin known. And, in none of these diseases, except the aforementioned autoimmune diseases, has the effect of UVB on the development of C deposits been studied. In spite of these gaps in knowledge, studies on the effects of UV exposure on the expression of C and C-regulatory proteins by keratinocytes isolated from normal individuals and from patients has not been carried out.

We argued that UVB exposure can probably increase the constitutive production of C components by keratinocytes. In evolutionary terms, this may be to compensate for local immunosuppression induced by UVB. We also argued that UVB exposure can probably increase the constitutive production of C components by keratinocytes. In evolutionary terms, this may be to compensate for local immunosuppression induced by UVB.

A high dose of UVB results in a high surface expression of DAF, MCP, and CD59. Further studies were aimed at comparing the effects of moderate (32 J per m²) and high doses of UVB (64 J per M²) on the expression of all three C-regulatory proteins. The studies with 32 J per m² were carried out at time points of maximal expression of the C-regulatory molecules (48 h for DAF; 72 h for MCP and CD59; see above) and the studies with 64 J per m² were carried out at 48 h of culture because of the low degree of cell survival at 72 h (see above). The results showed that expression of all three C-regulatory proteins was higher with 64 J per m² than with 32 J per m², although the difference in expression of MCP did not reach significant levels (p = 0.18) (Fig 5).

**DISCUSSION**

UVB radiation is the mid-range portion (290–320 nm) of the ultraviolet radiation spectrum (200–400 nm). UVB is present in biologically significant amounts at the Earth’s surface and exhibits pleomorphic radiation spectrum (200–400 nm). UVB is present in biologically significant amounts at the Earth’s surface and exhibits pleomorphic radiation spectrum (200–400 nm). UVB is present in biologically significant amounts at the Earth’s surface and exhibits pleomorphic radiation spectrum (200–400 nm). UVB is present in biologically significant amounts at the Earth’s surface and exhibits pleomorphic radiation spectrum (200–400 nm). UVB is present in biologically significant amounts at the Earth’s surface and exhibits pleomorphic radiation spectrum (200–400 nm). UVB is present in biologically significant amounts at the Earth’s surface and exhibits pleomorphic radiation spectrum (200–400 nm). UVB is present in biologically significant amounts at the Earth’s surface and exhibits pleomorphic radiation spectrum (200–400 nm). UVB is present in biologically significant amounts at the Earth’s surface and exhibits pleomorphic radiation spectrum (200–400 nm). UVB is present in biologically significant amounts at the Earth’s surface and exhibits pleomorphic radiation spectrum (200–400 nm). UVB is present in biologically significant amounts at the Earth’s surface and exhibits pleomorphic radiation spectrum (200–400 nm). UVB is present in biologically significant amounts at the Earth’s surface and exhibits pleomorphic radiation spectrum (200–400 nm).

Table 1. Effect of increasing doses of UVB on the expression of MCP, DAF, and CD59 by keratinocytes

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<th>Dose (J per m²)</th>
<th>Mean fluorescence intensity (% of untreated cells)</th>
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<td>8</td>
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<td>16</td>
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Subconfluent cultures were exposed to various doses of UVB. After 48 h (DAF) or 72 h (MCP and CD59) membrane expression of C-regulatory proteins on 20,000 cells was determined by FACS analysis. MFI values of unexposed keratinocytes were taken as 100%. All values represent mean ± SD of six experiments; *p < 0.05, **p < 0.01, ***p < 0.001.

**Figure 4.** A single dose of UVB causes prolonged increase in the surface expression of DAF, MCP, and CD59. Subconfluent keratinocyte cultures were exposed to 32 J per m² and expression of DAF, MCP, and CD59 was monitored by flow cytometer at the indicated time points. MFI, corrected for isotype control, is shown. Data represent one of two independent experiments. In each experiment three wells were exposed to UVB for each time point and cells in each well were analyzed in duplicate. Mean values and SD are shown.

**Figure 5.** Increasing doses of UVB cause a progressive increase in surface expression of DAF, MCP, and CD59. Subconfluent keratinocyte cultures were exposed to a moderate dose (32 J per m²) and a high dose (64 J per M²) of UVB. Expression of DAF, MCP, and CD59 was analyzed by flow cytometer at the time points at which a maximum increase in upregulation was seen in previous experiments: 48 h for DAF and 72 h for MCP and CD59 with 32 J per m² and at 48 h for all three proteins at 64 J per M² (see Fig 4). Single parameter histograms for DAF, MCP, and CD59 expression are shown. Thin lines show the expression of DAF, MCP, and CD59 on unexposed keratinocytes and bold lines show that on UVB-exposed keratinocytes. Broken lines show the expression obtained with isotype controls. The data are representative of at least two independent experiments carried out in triplicate. MFI after exposure to 64 J per M² was significantly higher for DAF (p < 0.01) and CD59 (p < 0.001), but not for MCP (p = 0.18), than after exposure to 32 J per m².
from their own C. We raised the possibility that UVB can upregulate the synthesis of both C and C-regulatory proteins, either directly or indirectly via cytokines released by keratinocytes or infiltrated inflammatory cells.

We observed that keratinocytes constitutively produced small amounts of C3 and Factor B. This was in agreement with previous observations (Basset-Seguin et al., 1990; Yancey et al., 1992). Exposure to UVB caused stimulation of keratinocytes, as evidenced by the production of IL-8, but did not increase production of C3 and Factor B (Fig. 2). This proved that direct stimulation of keratinocytes by UVB could not induce an increase in production of C3 and Factor B. Thus, we raised the question why those C3 and Factor B upregulating cytokines that are released from keratinocytes upon UVB exposure do not increase the production of C3 and Factor B in our system. One of the possible explanations is that they may be released in the medium in concentrations not high enough to stimulate the keratinocytes. The concentrations of TNF-α needed to upregulate C3 in our system were 50–1000 U per ml. But the concentrations of TNF-α in culture medium of UVB-exposed keratinocytes (at 72 h) was found to be very low (<1 U per ml; data not shown here). The same appears to be true for IL-8, whose effects on the expression of C3, Factor B, and C-regulatory proteins are not known. The level of IL-8 in the culture medium of UVB-exposed keratinocytes was always lower than 610 pg per ml (Fig 2). Biologically effective IL-8 concentrations vary from 5 to 50 ng per ml (Schorer et al., 1987). These results indicate that cytokines released from keratinocytes in culture medium after UVB exposure probably become too diluted to be effective. It remains to be seen if cytokines released from keratinocytes upon UVB exposure can upregulate C3 and Factor B in vivo.

DAF, MCP, and CD59 are present on keratinocytes in the human skin as seen in situ in previous immunohistochemical studies (Dovezenski et al., 1992; Venneker et al., 1994). This study shows surface expression of DAF, MCP, and CD59 on cultured keratinocytes. The coexpression of DAF, MCP, and CD59 on human keratinocytes suggests that these molecules collectively offer a high degree of protection to keratinocytes against C attack. Indeed, human keratinocytes (Norriss et al., 1985) and a keratinocyte-derived squamous carcinoma cell line, SCC-1-2F (Whitlow and Klein, 1997), is remarkably resistant to complement mediated lysis. In addition, cell death is not a prominent feature in diseases like pemphigus in which strong C attack on keratinocytes occurs in vivo. Keratinocytes are strongly protected against autologous C. Keratinocytes do need strong protection against C attack under physiologic conditions, because the epidermis is under a continuous threat of exposure to microbes and other agents that can activate C and cause bystander C attack on keratinocytes.

This study also shows that the expression of DAF, MCP, and CD59 on keratinocytes is increased for several days by UVB exposure in a dose-dependent manner. Because these molecules act synergistically, increases in all three implies that UVB exposure results in a prolonged and a high degree of protection against autologous C. We did not try to correlate increased expression with increased protection against C attack because increased expression of DAF, MCP, and CD59 in response to UVB exposure could be accompanied by increased or decreased expression of other C-regulatory proteins, such as complement receptor 1, C1q receptor, and homologous restriction factor. Possible changes in expression of these latter C-regulatory proteins would have made results difficult to interpret. For example, an increase in the expression of CD59 in the EA.hy926 cell line caused by inducers of protein kinase C and protein kinase A could not be correlated with the increase in resistance to C mediated lysis (Meri et al., 1993).

The increased expression of DAF, MCP, and CD59 seen in vitro could have been due either to a direct effect of UVB on keratinocytes or to an indirect effect in response to mediators released by keratinocytes. The latter possibility appears to be less likely because the concentrations of cytokines released from UVB-exposed keratinocytes into the culture medium do not appear to be high enough to be able to upregulate C-regulatory proteins significantly.

If these findings may be extrapolated to the in vivo situation, keratinocytes in UVB-exposed areas may be better protected against C-mediated lysis than keratinocytes in nonexposed areas. The situation in vivo, however, may differ from the situation in vitro in which infiltrating cells may also influence the expression of C-regulatory proteins through the release of their cytokines.

UVB is known to activate protein kinase C in keratinocytes (Matsui et al., 1996). Increase in expression of DAF, MCP, and CD59 by activators of protein kinase C, phorbol myristate acetate, and calcium ionophore A23187, and an activator of protein kinase A, butyryl-cAMP, has been shown in our laboratory (data not presented here). Protein kinase C and protein kinase A signaling may perhaps be involved in the upregulation of DAF, MCP, and CD59 by UVB. In conclusion, this study demonstrates that cultured human keratinocytes constitutively release low amounts of C3 and Factor B, which remain unaffected by UVB. They also express DAF, MCP, and CD59. Expression of these C-regulatory proteins is increased by UVB. Because UVB may increase C production in vivo through cytokines of keratinocytes and infiltrated cells, this increase in C-regulatory proteins may be important for protecting keratinocytes from becoming bystander victims of C during UVB-mediated inflammation.

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REFERENCES


