Human keratinocytes acquire cellular cytotoxicity under UV-B irradiation: implication of granzyme B and perforin*

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Running Title: Granzyme B and perforin induction by UV-B in keratinocytes

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Ultraviolet (UV) radiation from the sun is widely considered as a major cause of human skin photoaging and skin cancer. Granzyme B (GrB) and perforin (PFN) are two proteins contained in granules and implicated in one of the mechanisms by which cytotoxic lymphocytes and natural killer cells exert their cytotoxicity against virus-infected, alloreactive, or transformed cells. The distribution of GrB and PFN in the skin has received little attention. However, Berthou and co-workers described that, whereas freshly isolated epidermal cells did not express GrB or PFN, keratinocyte growth to confluence was associated to GrB and PFN mRNA and protein synthesis. In this work, we have then investigated the possible role of UV-B on GrB and PFN expression in keratinocytes. We found that UV-B induces GrB and PFN expression in these cells through a redox-, epidermal growth factor receptor (EGFR) -, and MAPK-dependent signalling. Furthermore under UV-irradiation, keratinocytes acquire a significant cytotoxicity GrB and PFN dependent, towards a variety of cellular targets including transformed T-lymphocytes, melanocytes and keratinocytes. This phenomenon may have important functional consequences in the regulation of skin inflammatory response and in the emergence of cancer skin.

Human skin, unlike all other organs, is continuously and directly exposed to environmental influences. Ultraviolet (UV) radiation from the sun is among the most ubiquitous damaging environmental factors from which human skin must protect itself. UV radiation in sunlight is divided into three regions depending on wavelength, short-wave UV-C (200–280 nm), mid-wave UV-B (280–320 nm) and long-wave UV-A (320–400 nm). UV-C has the highest energy and, hence, is the most biologically damaging region of UV radiation. However, UV-C in solar radiation is filtered out by ozone layer of the Earth's atmosphere, and therefore, its role in human pathogenesis is minimal. Both UV-B, and to a lesser extent, UV-A radiation are responsible as causative factors for various skin disorders including photoaging and skin cancer (1-3). UV radiation in particular UV-B from sunlight is known to alter cellular function via DNA damage (4), generation of ROS (5), and the resultant alterations in a large variety of signaling events (6,7). UV-B is therefore one of the most important external stimuli that affect skin by inducing immuno-suppression, cancer, premature skin aging, inflammation and cell death (2,3,8,9).

The granule secretory pathway is one of the mechanisms by which cytotoxic lymphocytes (CTLs) and natural killer (NK) cells exert their cytotoxicity against virus-infected, alloreactive, or transformed cells. The contact between effector cells and aberrant target cells induces exocytosis from the CTL or the NK cells, of granules that contain the potential cytolytic effector molecules (10). This mechanism depends on the actions of several constituents of the secreted granules that contain the pore-forming molecule perforin (PFN) together with a variety of granule-associated enzymes (such as granzymes and granulysin) (11). Among of them, granzyme B (GrB) is the main effector...
when dealing with target-cell death (12). Although the precise molecular and cellular pathways through which PFN and GrB cooperate to bring about cell death are still highly controversial, experiments indicate an absolute dependence on PFN for all granule-mediated cell death (13). In vivo, this is illustrated by the fact that targeted mutation of the PFN gene in mice results in profound immunosuppression and altered skin and tumour allograft rejections (14). By contrast, deficiency of GrB has a severely depressed ability to cause target cell lysis (12). Previous studies have described that GrB and PFN are not strictly distributed in lymphoid cells. Indeed, GrB and PFN are contained in other hematopoietic cells, such as normal and leukemic myeloid cells (15), as well as non-hematopoietic cells, such as chondrocytes of articular cartilage (16) and cells of the reproductive system, including spermatogenic cells (17), granulosa cells of human ovary (18) or placental trophoblasts (17).

The distribution of GrB and PFN in the skin has received little attention except in the context of immune skin disorders, such as in allergy, psoriasis, vitiligo and lichen planus, or eventually in graft-versus-host disease related skin injury. These situations are often associated with dermal or epidermal infiltration by GrB- and PFN-producing activated T cells. However, Berthou and co-workers described that, whereas freshly isolated epidermal cells did not express GrB or PFN, keratinocyte growth to confluence was associated to GrB and PFN mRNA and protein synthesis (19). In this study, the authors proposed that these proteins could be involved in epidermal homeostasis by preventing invasion by pathogens or migration of inflammatory cells (19). The role of GrB and PFN in keratinocytes has not deserved further attention. This is surprising based on the importance of PFN-dependent cytolysis not only in a large variety of bacterial and viral infections but also in tumour surveillance (for review, see (20)). This is also surprising based on the role of GrB, which is not simply implied in cytolysis, but also exerts, as an extracellular enzyme, PFN-independent important functions in extracellular matrix remodeling as well as in cell adhesion and motility (21).

In this study, we have investigated the possible role of UV-B on GrB and PFN expression in keratinocytes. We found that UV-B induces GrB and PFN expression in these cells through a redox-, epidermal growth factor receptor (EGFR) - and MAPK-dependent signalling. Furthermore under UV-irradiation, keratinocytes acquire a significant cytotoxicity GrB and PFN dependent, towards a variety of cellular targets including transformed T-lymphocytes, melanocytes and keratinocytes.

**EXPERIMENTAL PROCEDURES**

*Cell culture and chemicals* - Immortalized human HaCaT keratinocytes, provided by CERPER (Pierre-Fabre, France), were cultured in Dulbecco’s modified Eagle’s medium at 37°C in 5 % CO₂. Culture medium was supplemented with 10 % heat-inactivated foetal calf serum (FCS), glutamine (2 mM), streptomycin (100 µg/mL), penicillin (200 U/mL) (all these reagents were purchased from Eurobio, les Ulis, France). Normal human primary keratinocytes (HK) were kindly provided by Pr. Hovnanian (Toulouse, France). HK were isolated from skin biopsies as previously described (22) and were expanded on a feeder layer of lethally irradiated 3T3-J2 mouse fibroblasts in keratinocyte-growth medium, following the method described by Rheinwald and Green (23). K562 and Jurkat cell lines were obtained from the ATCC (Rockville, MD, USA). K562 cell line was stably transfected with pEGFP/P1-9 plasmid, kindly provided by Dr. P.I. Bird (Monash University, Victoria, Australia), using Lipofoctamine™ 2000 (Invitrogen, Cergy Pontoise, France) according to manufacturer’s recommendations. Selection of transfected cells was done with 500 µg/ml geneticin. Melanoma cell line (KAL) was established by Cohen-Knafo et al. (24). K562, Jurkat and KAL cells were grown in RPMI 1640 containing 10 % FCS, glutamine (2 mM), streptomycin (100 µg/mL), and penicillin (200 U/mL).

The UV-B irradiation source was a fluorescent lamp that emitted an energy peak at 310 nm. The emitted dose was calculated using a UV-B radiometer photodetector. UV-B irradiation was performed in cells incubated in phosphate-buffered saline (PBS).

*Drugs and reagents* - The rabbit polyclonal antibodies (Abs) against perforin and JNK (used at 1/500) were purchased from Santa Cruz Biotechnology (Tebu, France). The rabbit polyclonal anti- MAPKAPK-2 and anti- phospho MAPKAPK-2 Thr222 Abs (used at 1/1000) were purchased from Cell Signaling Technology (St Quentin en Yvelines, France). The mouse
monoclonal anti-GrB (clone GrB-7; used at 1/200), anti-β-actin (used at 1/1000), anti-phospho-JNK (used at 1/1000) and anti-GFP (used at 1/500) Abs were purchased, respectively, from Euromedex (Souffelweyersheim, France), NeoMarkers (Interchim, Montluçon, France), Cell Signaling Technology and Santa Cruz Biotechnology. Affinity-purified secondary Abs were purchased from Jackson ImmunoResearch Laboratories Inc. (Beckman Coulter Company, Marseille, France).

3,4-Dichloroisocoumarin (DCIC) was purchased from ICN Biomedical (Aurora, OH) and dissolved in dimethyl sulfoxide (DMSO). SB203580, SP600125, and PD98059 were purchased from VWR International (Fontenay sous Bois, France).

Antisense oligonucleotides and controls directed against GrB have been designed and manufactured by Biognostik (Euromedex) (15). Other products were purchased from Sigma (Saint Quentin-Fallavier, France).

**Western blot analysis** - Cells were washed with cold PBS and lysed, after scrapping in lysis buffer (30 mM Tris HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 % NP40, 10 % glycerol, 1 mM Na3VO4, 10 mM NaF, 1 mM PMSF, 10 mM β-glycerophosphate, 10 µg/mL leupeptin and 10 µg/mL aprotinin) for 5 min on ice, followed by centrifugation at 13000 rpm for 5 min at 4°C. For each lysate, 30 µg of total protein was boiled for 5 min at 95°C in the presence of 5 % β-mercaptoethanol.

Proteins were separated in a 10 % SDS-PAGE, transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, Saclay, France). Nonspecific binding to the membrane was blocked for 1 h at room temperature with 10% non-fat milk in PBS containing 0.1% Tween 20 (PBST). Membranes were incubated overnight at 4 °C with specific primary antibody diluted at an appropriate concentration in PBST containing 1% non-fat milk. Membranes were then washed three times at room temperature and bound immunoglobulins were detected with HRP-conjugated secondary Ab for 30 min at room temperature in 1% non-fat milk dissolved in PBST. Membranes were then washed with PBST and bound Abs were detected by the enhanced chemiluminescence system ECL kit (Amersham Pharmacia Biotech).

**Real-time quantitative polymerase chain reaction (PCR)** - Total cellular RNA was extracted with TRIzol®. The cDNA was synthesized using random hexamers and OligodT from 4 µg of mRNA and performed with the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen™ Life Technologies, Cergy Pontoise, France). Forward and reverse primers were designed following Guilloton et al. for GrB (25) and following Simon and al. for PFN (26) (Table 1). Real-time PCR was performed using an iCycler thermal cycler (Applied Biosystems 7000 Real-Time PCR System, Courtaboeuf, France) according to the manufacturer’s instructions. Reactions were performed with 0.3 µM of primers. Nucleotides, Taq DNA polymerase, and buffer were included in SYBR Green JumpStart™ Taq ReadyMix™ for quantitative PCR. cDNA amplification consisted of one cycle at 95°C for 1 min 30 s, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. The threshold cycle (Ct) values were determined by iCycler software (ABI Prism 7000) and the quantification data were analyzed following the ∆∆Ct method using S14 as reference. We have checked that PCR efficiency (E) of the amplification was similar whatever the primers and we calculated the relative amount (RA): RA=(1+E)−∆∆Ct.

Electrophoresis of PCR products on a 2 % agarose gel was undertaken when no gene expression was observed in non-treated cells.

**Confocal laser scanning microscopy analysis** - Cells were fixed with 4 % (w/v) paraformaldehyde (10 min at room temperature (RT)), washed twice with PBS, and permeabilized using 100 % methanol (2 min at RT). Coverslips were washed with PBS, saturated with 20 % FCS-PBS for 20 min at RT and incubated with anti-GrB and/or anti-PFN Abs, both at a 1/20 dilution in 1 % FCS-PBS, followed by FITC-conjugated goat anti-rabbit IgG and/or Cy3 goat anti-mouse IgG (Beckman-Coulters) diluted at 1/100 in 1 % FCS-PBS for 25 min at RT. After washing, the coverslips were sealed and examined with confocal imaging system that was a Zeiss (Oberkochen, Germany) scanning assembly incorporating argon and helium/neon lasers coupled to a Zeiss Axiovert 100 fluorescence microscope.

**Cytotoxicity assays** - The CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) was used to evaluate stress-induced cytotoxicity (15). This assay is a colorimetric alternative to 51Cr-release cytotoxicity assay based on the measurement of lactate dehydrogenase (LDH), a stable cytosolic
enzyme that is released on cell lysis, in much the same way as $^{51}$Cr is released in radioactive assay and that allows discrimination between effectors and targets LDH release (27). Variations on this technology have been reported for measuring natural cytotoxicity and have been demonstrated to be identical (within experimental error) to values determined in parallel $^{51}$Cr-release assays and was performed according to manufacturer recommendations. Briefly, effector cells (HaCaT: $4 \times 10^6$ cells/mL) were irradiated with 0.2 kJ/m$^2$ UV-B, incubated for 16 h, trypsinized, washed, resuspended in RPMI-1640 supplemented with 5 % FCS, incubated for 1 h and mixed with target cells (K562, HaCaT, and HK cells: $1 \times 10^5$ cells/mL) in U-bottom 96-microwell plates (Nunc, Roskilde, Denmark) at various effectors-to-target (E:T) ratio in triplicate. Microplates were spun for 3 min at 200 g and incubated for 4 h at 37°C, 5 % CO$_2$. Supernatant (50 µL) was collected from each well and added to 50 µL reconstituted Substrate Mix for 30 min in the dark at RT. Enzymatic reaction was stopped by adding Stop Solution (50 µL). Counting was realized by recording absorbance at 490 nm. Maximum release (TM) was determined by lysing target cells with 20 µL of Lysis Solution. Spontaneous release (TS) was determined by incubation of target cells in medium in the absence of effectors cells. Effectors spontaneous release (ES) was done with effectors cells alone at the same E:T ratio. Results are expressed as a percentage of cytotoxicity, using the following formula:

$\%$ cytotoxicity $= [(\text{experimental} - \text{ES} - \text{TS}) / (\text{TM} - \text{TS})] \times 100$.

Blocking experiments were performed with 3,4-Dichloroisocoumarin (DCIC) (20 µmol/L), a broadly reactive serine esterase inhibitor, that has been shown to neutralize GrB enzymatic activity (28,29), MgCl$_2$ (1.5 mM)/EGTA (1 mM) that blocks degranulation and prevents PFN polymerization (30), anti-CD95 (Fas/APO-1) blocking Ab (ZB4) (500 ng/mL), or phosphorothioate oligodeoxynucleotides (5 µmol/L) antisense or sense directed against GrB.

Statistics - The Student’s t test was performed to evaluate the statistical significance.

RESULTS

UV-B induced GrB and PFN mRNA expression in keratinocytes

GrB and PFN mRNA expressions in HaCaT cell line were analyzed by real-time quantitative PCR followed by a separation of PCR products in 2 % agarose gel. We first examined whether UV-B irradiation was able to stimulate production of GrB and PFN in HaCaT cells. Cells were irradiated with UV-B at different doses. In untreated non-confluent HaCaT cells, GrB and PFN mRNA were undetectable as previously described (19) (Fig. 1A, B). However, UV-B irradiation induced GrB and PFN mRNA in a dose- and time-dependent manner (Fig. 1A, B). At the dose of 0.2 kJ/m$^2$, cell loss was about 15 % after 48 h (data not shown). Therefore, this dose was used for further experiments.

In HaCaT cells growth at confluence, GrB and PFN mRNA were detectable as previously described (19). However, in these conditions of culture, UV-B irradiation was still able to increase from 3 to 4-fold GrB and PFN expression (Fig. 1C).

In primary normal human keratinocytes (HK) from different donors, UV-B also increased GrB and PFN expression (Fig. 1D). It is interesting to note that, contrarily to HaCaT cells, untreated fresh human keratinocytes do express GrB but not PFN. S14, a ribosomal protein, was used as an internal control as previously described (31,32). Indeed, S14 mRNA expression was found to be invariable, whereas we detected minor but significant changes in β-actin in UV-irradiated HaCaT cells. These results demonstrate that treatment with UV-B irradiation resulted in a significant increase in GrB and PFN gene expression in both HaCaT and fresh human keratinocytes.

UV-B induced GrB and PFN protein expression in keratinocytes

GrB and PFN protein expressions in HaCaT cell line were analyzed by western blot and confocal microscopy. Western blot analysis revealed that treatment with UV-B (0.2 kJ/m$^2$) resulted in an increase in GrB and PFN protein expression as illustrated in Fig. 2A, 16 h after irradiation. Confocal analysis confirmed the lack of GrB or PFN in untreated cells (Fig. 2B). However, after irradiation, PFN was easily detected in the cytoplasm of HaCaT cells whereas immunostaining with anti-GrB showed a more diffuse distribution with a predominant
cytoplasmic distribution associated with a weak but detectable accumulation in the nucleus (Fig. 2B). Merging revealed that, in the cytoplasm, PFN and GrB co-localized at 72.6% (Fig. 2B). This co-localisation was also tested with Cy5 goat anti-mouse IgG and we obtained the same results (data not shown). These data suggested that these two lytic proteins accumulated into granules as they do in cytototoxic granules contained in activated immune effectors.

These results demonstrate that, in keratinocytes, UV-B irradiation induces the expression of GrB and PFN proteins which co-localize in cytoplasm.

Role of MAPK pathway in the induction of GrB and PFN by UV-B irradiation
UV-B irradiation activates a large variety of signaling pathway, including c-Jun NH2 terminal kinases (JNK), extracellular signal-regulated kinases (ERK) and p38 MAPK modules (33-35). To assess the involvement of MAPK activation in HaCaT cells, we examined whether different inhibitors suppressed GrB and/or PFN induction in response to UV-B irradiation.

As shown in Fig. 3A-B, SB203580, a specific and potent chemical inhibitor for p38 MAPK, totally inhibited GrB mRNA (A) and protein (B) expression. In contrast, SP600125, a specific inhibitor for JNK, and PD98059, a specific inhibitor for ERK, did not significantly reduce GrB mRNA and protein induction. Consistent with these results, phosphorylation of MAPKAPK-2, a substrate of p38 MAPK (36), was also observed in HaCaT cells irradiated with UV-B, indicating that UV-B activates p38 MAPK (Fig. 3C). Then, GrB gene stimulation was found to be specifically dependent from p38 MAPK module.

As shown in Fig. 3A-B, SP600125, but not SB203580 or PD98059, inhibited PFN gene stimulation in irradiated HaCaT cells. Consistent with these results, in these cells, UV-B induced SP600125-inhibitable JNK phosphorylation (Fig. 3D). PFN gene stimulation was then found to be specifically dependent from JNK module. Moreover, as expected, co-treatment with SB203580 and SP600125 resulted in total inhibition of both GrB and PFN expression (Fig. 3B).

Altogether, these results show that UV-B irradiation activates GrB and PFN through p38 MAPK and JNK, respectively.

Role of epithelial growth factor receptor in the induction of GrB and PFN by UV-B irradiation
Previous studies have documented that UV-B induced rapid activation of EGFR, and that this event is critical for most components of the UV signaling response, including ERK, PI3K/Akt, p38 MAPK and JNK pathways (34,35,37). Based on the presumed role of p38 MAPK and JNK activation in GrB and PFN induction (see above), we examined whether EGFR activation could play a role in regulating these two proteins. For this reason, we investigated the effect of AG1478, a specific inhibitor of EGFR, on UV-induced GrB and PFN stimulation by UV-B in HaCaT cells. We found that pre-treatment with AG1478 used in conditions which allowed abrogation of p38 MAPK and JNK phosphorylation (Fig. 4A, B), resulted in total inhibition of UV-B induced GrB and PFN gene stimulation (Fig. 4C).

These results suggested that EGFR activation is critical for GrB and PFN induction by UV-B in keratinocytes.

Role of reactive oxygen species in the induction of GrB and PFN by UV-B irradiation
Previous studies have indicated that radical oxygen species (ROS) are also important mediators for UV response. For example, ROS production is involved in UV-induced EGFR activation (38-40) as well as in the coupling between EGFR and MAPK signaling. Therefore, we examined whether ROS could be involved in GrB and PFN regulation upon UV exposure in HaCaT cells. As shown in Fig. 5A and B, pre-treatment with N-acetylcysteine (NAC), a potent anti-oxidant agent, at the dose of 25 mM for 2 h, allowed abrogation of UV-induced p38 MAPK (Fig. 5A) and JNK (Fig. 5B) phosphorylation. Treatment with NAC resulted in total inhibition of GrB and PFN mRNA and protein expression after UV irradiation (Fig. 5C, D). Conversely, treatment with hydrogen peroxide (H2O2) resulted in increased GrB and PFN mRNA (Fig. 5E), thus mimicking the effects of UV-B.

Altogether, these results showed that, upon UV-B stimulation, GrB and PFN gene activation is controlled by a signaling cascade which involves ROS, EGFR, p38 MAPK and JNK.
Cellular cytotoxicity of irradiated HaCaT cells

In the immune system, GrB/PFN system confers to activated T- or NK-cells potent cellular cytotoxicity against infected or transformed cells. Therefore, we hypothesized that UV-B induced GrB/PFN stimulation should also confer to keratinocytes some ability to destroy cells through cytotoxic granules. Cellular toxicity was measured using K562 target cells, this leukaemia cell line being the standard cellular model to investigate GrB/PFN mediated cellular cytotoxicity of innate immune effectors. UV-B-irradiated or untreated HaCaT cells were co-cultured with K562 cell target at an effector: target (E:T) ratio between 80:1 and 10:1. Untreated HaCaT cells displayed no cytotoxicity towards K562 cells. When irradiated at 0.2 kJ/m² and then incubated for 16 h, HaCaT cells exhibited cytotoxicity towards K562 cells in a 4 h cytotoxic assay. Albeit low, compared to NK or T-cells, cellular cytotoxicity was significant with a maximum of 48 % at a ratio of 80:1. To confirm these results, we have also shown that irradiated HaCaT cells displayed no cytotoxicity towards K562 cells when HaCaT cells were pre-treated with both SB203580 and SP600125. To assess the role of the GrB/PFN system in the acquisition by keratinocytes of cellular cytotoxicity, we used pharmacological agents currently used to inhibit either GrB intrinsic cytotoxicity properties (DCIC) or PFN function by limiting its polymerisation (MgCl₂/EGTA). Therefore, irradiated HaCaT cells were pre-incubated with DCIC and MgCl₂/EGTA, and allowed to react with K562 target cells (E:T of 80:1). As depicted in Fig. 6B, pre-treatment with DCIC and MgCl₂/EGTA resulted in the abrogation of irradiated-HaCaT cell cytotoxicity whereas the anti-CD95 (Fas/APO-1) blocking Ab (ZB4), used as control reagents, had no effect (Fig. 6B). The role of GrB in keratinocyte-irradiated cellular cytotoxicity was also investigated by transfecting the serine protease inhibitor PI-9 in K562 target cells. Indeed, PI-9 blocks specifically GrB/PFN mediated cellular cytotoxicity. As a matter of fact this serpin was used to ascertain the role of GrB in immune effector mediated cell lysis. In parallel, exposure to antisense oligonucleotide resulted in an inhibition of GrB induced UV-B expression, whereas exposition to control oligonucleotide did not affect significantly GrB expression.

These results show that UV-B conferred to keratinocytes cellular cytotoxicity through the GrB/PFN system.

DISCUSSION

In this study we have demonstrate for the first time that UV-B irradiation induces GrB and PFN gene and protein expression in keratinocytes. Our data supports a model, in which, in irradiated keratinocytes, oxidative stress and EGFR activation results in the activation of p38 MAPK and JNK pathways, which in turn mediates the up-regulation of functional GrB and PFN proteins. It is important to note that GrB and PFN induction by UV-B was observed not only in transformed HaCaT cell line but also in human normal primary keratinocytes, and in
both non-confluent and confluent cells, suggesting that these events were not dependent from the culture conditions. Moreover, although GrB and PFN accumulated in keratinocytes in a dose-dependent fashion, it is important to note that gene activation was detected for non-toxic doses, suggesting that this event does not correlate with cell death.

UV-B light is known to induce diverse DNA damages, including DNA adducts, DNA strand breaks, DNA cross-links and DNA-protein cross-links. The fact that GrB and PFN up-regulation is a part of the cellular response to DNA damage induced by UV irradiation was not totally unexpected. Indeed, in a previous study, we have described that ionizing radiation or anti-cancer agents may induce GrB and PFN in acute myeloid leukemia (AML) cells through a transcriptional mechanism, and that this confers to cells a potent cellular cytotoxicity capacity, which was abrogated by GrB and PFN inhibitors (15). More recently, we have also reported similar events in AML cells treated by inflammatory cytokines such as TNFα (25). Together with the present study, these findings suggest that, at least in some non-lymphoid tissues, diverse conditions of stress may lead to GrB and PFN induction.

This study shows also for the first time that, upon UV-B irradiation, keratinocytes acquired cytotoxic potential against a variety of cellular skin targets, including keratinocytes, T-lymphocytes and melanoma cells. It should be noted that cell lysis was measured using a non-radioactive cytotoxicity assay, a simpler alternative than the conventional Cr51 release, now widely used (15,25,43,44). It should be pointed out that the killing capacity of irradiated keratinocytes was lower than that usually observed for immune effectors. For example, comparative experiments revealed that, at E:T ratio of 80:1, K562 cell lysis was 80 %, 70 %, and 48 % for IL2-activated peripheral blood lymphocytes, NK cells, and irradiated HaCaT cells, respectively (data not shown). However, due to the keratinocytes density, one can speculate that, in vivo, the acquisition of cellular cytotoxicity could have profound consequences if irradiated keratinocytes are directed against minor cell population such as epidermal lymphocytes or melanocytes.

The fact that, in irradiated keratinocytes, GrB and PFN gene activation correlates with the acquisition of cellular cytotoxicity raised the possibility that the GrB/PFN system contributed to UV-B induced cellular cytotoxicity. Several lines of evidences support this hypothesis. First, the GrB/PFN system is very efficient to induce cell lysis as illustrated by its function in immune effectors such as activated T- or NK cells. Second, the role of the GrB/PFN system in the acquisition of cellular cytotoxicity upon stress has been previously documented in other cellular models (15). Third, the present study shows that both PI-9 expression as well as GrB and PFN inhibitors abolished the lytic function of irradiated keratinocytes. Moreover, we have observed that irradiated HaCaT displayed no lytic ability when separated from target cells by nylon membrane (data not shown). The fact that cell-cell contact is required for cellular cytotoxicity precludes the role of soluble mediators, including those which have been found to be produced after UV exposure, such as TNFα, TGFβ, interferon, and Fas-ligand (45,46). In addition, irradiated keratinocytes were efficient against K562 cells that are known to be highly resistant to TNFα (47) and Fas agonist (48). This observation renders unlikely the contribution of TNFα or Fas-ligand in both their soluble and membrane-bound forms. However, one can speculate that in our model, cell-cell contact requires receptor/ligand interaction such as described for NK cell lysis (49). This point is currently investigated in our laboratory.

Altogether, these results support the fact that, upon UV-B irradiation, not only keratinocytes produced GrB and PFN proteins, but also that the lytic function of these proteins was preserved. The fact that these two lytic proteins co-localize in the cytoplasm of keratinocytes as revealed by confocal microscopy raises the possibility that there are contains in cytotoxic granules like in activated T- or NK cells.

The mechanism by which UV-B up-regulates GrB and PFN has been also examined. UV elicited a number of interconnected signaling pathways, known as the UV response, which includes ROS-dependent activation of PI3K/Akt, p38 MAPK, JNK, ERK, and EGFR (6). By using chemical inhibitors, we describe that GrB induction is dependent from p38 MAPK whereas, for PFN, JNK is most critical. However, ERK or PI3K pathways play no role in this regulation (data not shown). Altogether these results demonstrate that p38 and JNK govern distinctly GrB and PFN expression after UV-irradiation. This observation is rather intriguing and suggests that these two pathways are coordinated and likely under common...
regulators, among them ROS and EGFR play a critical role. Based on previous studies, which have indicated that PFN promoter contains c-fos region (50,51), the role of JNK in PFN regulation can be more complex. Indeed, although p38 MAPK activates CREB (52) and AP1, two putative regulators of GrB gene, other studies also indicate that the p38 MAPK pathway may also influence mRNA stability through a MAPKAPK2-dependent, AU-rich elements-targeted mechanism (53). Thus, it is possible that p38 MAPK acts directly through a CREB- or AP1-mediated transcriptional mechanism, and indirectly at a post-transcriptional level by stabilising GrB mRNA. The significance and the functional consequences of GrB/PFN accumulation on the epidermis homeostasis remain uncertain. Because of the relatively low efficiency of cell lysis capacity towards themselves, it is unlikely that this serves at eliminating DNA damaged keratinocytes in the context of a cell-to-cell killing interaction. In contrast, we can speculate that the acquisition of cellular toxicity by keratinocytes results in the partial destruction of skin-resident immune cells (epidermal T-cell) or in the limitation of cell recruitment in the context of UV-induced acute inflammation. Therefore, it is possible that the GrB/PFN system may be involved in the regulation of skin inflammatory process to maintain epidermal cell defence after UV stress. In our work, we have also show that irradiated keratinocytes results in the destruction of melanoma cells. In vivo, melanocytes are in contact with keratinocytes. We can then hypothesize that DNA damaged melanocytes can be killed by irradiated keratinocytes. The acquisition of this cytotoxicity could then also prevent the emergence of melanoma cells. Alternatively, it is possible that the consequence of GrB or PFN accumulation in irradiated keratinocytes implies other properties of these two proteins, totally distinct from their lytic function. For example, GrB has been shown to cleave proteoglycan (54) as well as major components of the extra-cellular matrix such as vitronectin, fibronectin and laminin (21). Whether or not GrB accumulation in the epidermis may contribute to the alteration of skin extra-cellular composition after chronic UV exposure should be investigated. To conclude, we propose a model in which after UV-B irradiation, both PFN and GrB accumulated under their active forms in cytoplasmic granules of keratinocytes and that conferred to these cells potent cellular cytotoxicity. Although the significance of this intriguing observation remains uncertain, this phenomenon may have important functional consequences in the regulation of skin inflammatory response and in the emergence of cancer skin.

REFERENCES


FOOTNOTES
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1 The abbreviations used are: 3,4-Dichloroisocoumarin (DCIC), acute myeloid leukemia (AML), effectors-to-target (E:T), granzyme B (GrB), hydrogen peroxide (H2O2), lactate dehydrogenase (LDH), normal human primary keratinocytes (HK), N-acetylcysteine (NAC), PBS containing 0.1% Tween 20 (PBST), perforin (PFN), relative amount (RA), room temperature (RT).

FIGURE LEGENDS

Fig. 1. Expression of GrB and PFN mRNA measured by real-time quantitative PCR. (A) HaCaT cells were irradiated at different doses of UV-B and incubated for 16 h before mRNA extraction. (B) HaCaT cells were irradiated at 0.2 kJ/m2 and incubated for different times before mRNA extraction. Electrophoresis of PCR products on a 2% agarose gel was undertaken because no gene expression was observed in non-treated cells. (C) HaCaT cells at confluence were irradiated at 0.2 kJ/m2 and
incubated for 16 h (■). The data were expressed as the relative amounts compared with values from non-treated cells (■). These data are the mean of 3 independent experiments. (D) Primary human keratinocyte (HK) cells were irradiated at 0.2 kJ/m² and incubated for 16 h before mRNA extraction (■). The data for GrB was expressed as the relative amounts compared with values from non-treated cells (■). The data for PFN was showed on a 2 % agarose gel because no PFN expression was observed in non-treated cells. * p < 0.05 and ** p < 0.01. These data are representative of 3 independent experiments.

Fig. 2. Expression of GrB and PFN proteins in HaCaT cells. Cells were irradiated with UV-B (0.2 kJ/m²) and incubated for 16 h before protein extraction (A) or confocal analysis (B). Western blot and confocal analysis was performed using anti-GrB and anti-PFN Abs. β-actin was used as a loading control for western blot (A). These data are representative of 3 independent experiments.

Fig. 3. Role of MAPK pathway in the induction of GrB and PFN by UV-B irradiation. (A-B) HaCaT cells were pre-treated with SB203580 (1 µM), an inhibitor for p38 MAPK, and/or SP600125 (5 µM), a specific inhibitor for JNK, or PD98059 (25 µM), a specific inhibitor of ERK for 1 h. Cells were then irradiated with UV-B (0.2 kJ/m²) and incubated for 16 h before mRNA extraction (A) or confocal analysis (B). (A) Expression of GrB and PFN mRNA was measured by real-time quantitative PCR. Electrophoresis of PCR products on a 2 % agarose gel was undertaken because no gene expression was observed in non-treated cells. (B) Confocal analysis was performed using anti-GrB and anti-PFN Abs. (C-D) HaCaT cells were pre-treated with SB203580 (1 µM) or SP600125 (5 µM) for 1 h. Cells were or not irradiated with UV-B (0.2 kJ/m²) and incubated for 1 h before protein extraction. Expression of phosphorylated MAPKAPK-2 (C) and phospho-JNK (D) was analyzed by western blot. These data are representative of 3 independent experiments.

Fig. 4. Role of EGFR in the induction of GrB and PFN by UV-B irradiation. HaCaT cells were or not pre-treated with AG1478 (10 µM) an inhibitor for EGFR, for 1 h, irradiated with UV-B (0.2 kJ/m²) and incubated for 1 h before protein extraction. Expression of phosphorylated MAPKAPK-2 (A) and phospho-JNK (B) was analyzed by western blot. (A) HaCaT cells were pre-treated with AG1478 (10 µM) for 1 h. Cells were then irradiated with UV-B (0.2 kJ/m²) and incubated for 16 h before mRNA extraction. Expression of GrB and PFN mRNA were measured by real-time quantitative PCR. Electrophoresis of PCR products on a 2 % agarose gel was undertaken because no gene expression was observed in non-treated cells. These data are representative of 3 independent experiments.

Fig. 5. Role of reactive oxygen species in the induction of GrB and PFN by UV-B irradiation. HaCaT cells were or not pre-treated with NAC (25 mM), an inhibitor of oxidative stress, for 2 h, irradiated with UV-B (0.2 kJ/m²) and incubated for 1 h before protein extraction. Expression of phosphorylated MAPKAPK-2 (A) and phospho-JNK (B) was analyzed by western blot. (C-D) HaCaT cells were pre-treated with NAC (25 mM) for 2 h. Cells were then irradiated with UV-B (0.2 kJ/m²) and incubated for 16 h before mRNA extraction (C) or confocal analysis (D). (D) Confocal analysis was performed using anti-GrB and anti-PFN Abs. (E) HaCaT cells were treated with H2O2 at different concentration for 1 h in PBS. Cells were then incubated for 16 h before mRNA extraction. (C-E) Expression of GrB and PFN mRNA was measured by real-time quantitative PCR. Electrophoresis of PCR products on a 2 % agarose gel was undertaken because no gene expression was observed in non-treated cells. These data are representative of 3 independent experiments.

Fig. 6. Cellular cytotoxicity of irradiated HaCaT cells towards K562 cell line. Irradiated HaCaT cells were cultured for 16 h. Cytotoxicity was measured by using a 4 h non-radioactive LDH-release assay at the indicated E:T ratio. (A) Irradiated (▲ or ○) or not (◆ or □) HaCaT cells were pre-treated (gray symbols) or not (black symbols) with SB203580 (1 µM) and SP600125 (5 µM), before co-incubation with K562 cells. (B) Percentage of cytotoxicity of UV-B irradiated HaCaT cells pre-treated or not with MgCl2/EGTA, DCIC or ZB4 Ab, towards K562 cells at an E:T ratio of 80:1. (C) Untreated HaCaT (◆ or □) and irradiated HaCaT (▲ or ○) cells were co-incubated with K562 (black symbols) or GFP-
PI-9 transfected K562 (gray symbols) cells as targets. Expression of GFP-PI-9 proteins in transfected (2) or not (1) K562 cells. Western blot analysis was performed using anti-GFP Ab. β-actin was used as a loading control for western blot. (D) HaCaT cells were incubated with antisense GrB or sense (control) oligonucleotides for 6 h, irradiated (■) or not (□), and cultured for 16 h, and then co-incubated with K562 cells as targets. Cytotoxicity was measured by using a 4 h nonradioactive LDH-release assay at 80:1 E:T ratio. Western blot analysis of antisense or sense oligonucleotides on GrB expression after irradiation. S: sense, AS: antisense. These data are representative of 3 independent experiments. Results are the mean ± standard deviation of three independent experiments performed in triplicate. *P = 0.02.

Fig. 7. Cellular cytotoxicity of irradiated HaCaT cells towards different skin cells. Irradiated HaCaT cells were cultured for 16 h. Cytotoxicity was measured by using a 4 h non-radioactive LDH-release assay at 80:1 ratio. Untreated HaCaT (□) and irradiated HaCaT (■) cells were co-incubated with non irradiated HaCaT (1), HK (2), Jurkat (3) or KAL (4) cells as targets. These data are the mean ± standard deviation of 3 independent experiments performed in triplicate. **p < 0.01.
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Table 1. Oligonucleotide primer sequences for real-time quantitative PCR.
Figure 1

A. UV B (kJ/m²)  

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C. Relative amount  

- **GrB**  
  - **PFN**

D. Relative amount  

- **GrB**
  - UV B (kJ/m²)  
    - 0  
    - 0.2  
  - PFN
  - S14
Figure 2

A. UV-B (kJ/m²)

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Figure 4

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<tr>
<td>AG1478</td>
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<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 5

A. P-MAPKAPK2
   MAPKAPK2
   UV-B  -  +  -  +
   NAC   -  -  -  -

B. P-JNK
   JNK
   UV-B  -  +  -  +
   NAC   -  -  -  -

C. G6b
   PFN
   S14
   UV-B  -  +  -  +
   NAC   -  -  -  -

D. G6b
   PFN
   FFN/G6b
   NT
   NAC
   UV-B
   UV-B NAC
Figure 6

A. % of cytotoxicity

B. % of cytotoxicity

C. % of cytotoxicity

D. % of cytotoxicity
Figure 7
Human keratinocytes acquire cellular cytotoxicity under UV-B irradiation: Implication of granzyme B and perforin
Hélène Hernandez-Pigeon, Christine Jean, Alexandra Charruyer, Marie-José Haure, Matthias Titeux, Laure Tonasso, Anne Quillet-Mary, Caroline Baudouin, Marie Charveron and Guy Laurent

J. Biol. Chem. published online March 8, 2006

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