UVA Induces Granzyme B in Human Keratinocytes through MIF

IMPLICATION IN EXTRACELLULAR MATRIX REMODELING

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In a previous study, we have described that UBV induces granzyme B (GrB) in human keratinocyte cells, and that confers potent cellular cytotoxicity against various cellular models, including immune cells (Hernandez-Pigeon, H., Jean, C., Charruyer, A., Haure, M. J., Titeux, M., Tonasso, L., Quillet-Mary, A., Baudouin, C., Charveron, M., and Laurent, G. (2006) J. Biol. Chem. 281, 13525–13532). Herein, we have found that, in contrast to UBV, UVA failed to enhance cellular keratinocyte cytotoxicity but was still able to trigger GrB production. We show that GrB is accumulated through a p38 MAPK-dependent transcriptional mechanism stimulated by redox-dependent migration inhibitor factor release. Moreover, GrB purified from UVA-treated cellular extracts was found to degrade fibronectin in vitro. Treatment with antisense oligonucleotide directed against GrB resulted in the inhibition of UVA-induced cell detachment and cell death and facilitated cell migration through fibronectin and vitronectin matrix upon UVA exposure. Altogether, these results suggest another function for GrB in the context of the UV response. Indeed, combined with our previous study, it appears that, whereas this enzyme mediates keratinocyte cellular cytotoxicity following UVB irradiation, GrB supports the capacity of keratinocyte to degrade extracellular matrix components following UVA irradiation. UV-mediated GrB production may thus have important consequences in photoaging and photocarcinogenesis.

UVA is a major contributor to a vast variety of skin lesions following prolonged exposure to solar radiation culminating in photoaging or photocarcinogenesis. In this context, extracellular matrix degradation is an important event responsible for wrinkle formation, reduced recoil capacity, and blister formation, all hallmarks of photoaging. UVA-induced matrix degradation is generally considered as the result of the production of various matrix metalloproteinases (MMP) not only by dermal fibroblasts but also by keratinocytes. MMP display substrate specificity for dermal (collagens I, III, and V, proteoglycans, and elastin) and basement membrane compounds (collagens IV and VII, proteoglycans, laminin, and fibronectin). The effects of MMP are counteracted by tissue inhibitor of MMP1. The fact that UV also triggers extracellular matrix component biosynthesis renders even more complex this balance. For example, as evidenced in the hairless mouse model, fibronectin accumulates upon prolonged UV exposure (1). However, the increase of this important extracellular matrix component reflects the net balance between stimulated biosynthesis (2) and an enhanced degradation through specific MMP, such as MMP2 and MMP9, but not MMP1 (3, 4).

Granzyme B (GrB) is a serine protease contained in cytotoxic granules inside activated immune cells. Upon release from these cells, GrB induces target cell lysis in combination with perforin, another protein component of the cytotoxic granules. Although the role of GrB in non-lymphoid cells has received little attention, one previous study has described that significant amount of catalytically active GrB accumulated into keratinocytes when these cells were plated at high density, suggesting that, at least in some stress conditions, keratinocytes may produce this enzyme (5). Moreover, we have recently shown that UBV induces GrB expression in keratinocytes, and this confers to the cells potent cellular cytotoxicity against a wide variety of cellular models, including immune cells (6). It is surprising that this observation has attracted no further attention in the field of photoaging. Indeed, previous studies dealing with the role of GrB in the destruction of cartilage in the context of rheumatoid arthritis have already described that GrB degrades glycosaminoglycans (7), and particularly aggregan (8), which is distributed in the skin. More recently, GrB has been identified as a central player for extracellular matrix remodeling based on

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4 The abbreviations used are: MMP, matrix metalloproteinase; Ab, antibody; GrB, granzyme B; MIF, macrophage migration inhibitor factor; NAC, N-acetylcysteine; PBS, phosphate-buffered saline; rGrB, recombinant granzyme B; rMIF, recombinant MIF; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; MAPK, mitogen-activated protein kinase; MAPKAPK, MAPK-activated protein kinase; ROS, radical oxygen species; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; CREB, cAMP-responsive element-binding protein.
its potent proteolytic capacity for vitronectin, laminin, and fibronectin (9, 10).

Based on these findings and considerations, we speculated that UVA irradiation could trigger GrB production in skin cells, and that this event contributes to the degradation of fibronectin in combination with MMP. Moreover, based on a previous study that showed MMP production upon UVA stimulation is mediated by the release of macrophage migration inhibitory factor (MIF) (11), we also investigated whether MIF could be involved in UVA-mediated GrB production.

This study shows that, indeed, UVA irradiation induces the production of catalytically active GrB through MIF, UVA-induced GrB was able to degrade fibronectin, and consequently GrB influences cell detachment, cell death, and cell migration.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Chemicals**—Immortalized human HaCaT keratinocytes, provided by CERPER (Pierre-Fabre, France), were cultured in DMEM at 37 °C in 5% CO2. Culture medium was supplemented with 10% FCS, glutamine (2 mM), streptomycin (100 μg/ml), penicillin (200 units/ml) (all these reagents were purchased from Eurobio, les Ulis, France). Normal human skin was obtained immediately after surgery for breast reduction.

The UVA irradiation source was a fluorescent lamp (Uvitec, Cambridge) that emitted an energy peak at 340 nm. The emitted dose was calculated using a UVA radiometer photodetector (Vilber Lourmat, Marne-la-vallée, France). UVA irradiation was performed in cells incubated in phosphate-buffered saline (PBS) at 4 °C.

**Drugs and Reagents**—The rabbit polyclonal antibody (Ab) against fibronectin (used at 1/1000) was purchased from Sigma-Aldrich. The rabbit polyclonal anti-MAPKAPK-2 and anti-phospho MAPKAPK-2 Thr-222 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) and anti-β-actin (used at 1/1000) Abs were purchased, respectively, from Euromedex (Souffelweyersheim, France) and NeoMarkers (Interchim, Montluçon, France). Affinity-purified secondary Abs were purchased from Jackson ImmunoResearch Laboratories Inc. (Beckman Coulter Company, Marseille, France).

SB203580, SP600125, and PD98059 were purchased from VWR International (Fontenay sous Bois, France). Recombinant human MIF (rMIF) and human MIF Ab were obtained from R&D Systems (Lille, France). Controls and antisense oligonucleotides directed against GrB have been designed and manufactured by Biognostik (Euromedex, Mundolsheim, France) (6, 12). Human purified plasma fibronectin was purchased from Invitrogen. Human recombinant GrB protein was purchased from Coger (Paris, France). Other products were purchased from Sigma-Aldrich.

**Western Blot Analysis**—Western blot analysis was performed as already described (6).

**Real-time Quantitative PCR**—Real-time quantitative PCR analysis was performed as already described (6). Forward and reverse primers are described in Table 1.

**Confocal Laser Scanning Microscopy Analysis**—Confocal analysis was performed as already described (6). Briefly, cells were permeabilized using 100% methanol (2 min at room temperature) and incubated with anti-GrB Ab at 1/20 dilution in 1% FCS-PBS, followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Beckman-Coulters) diluted at 1/100 in 1% FCS-PBS for 25 min at room temperature.

**Immunohistochemistry**—Several 4-μm punch biopsies were taken from normal human skin. Irradiated samples and non-irradiated samples were placed in PBS. Irradiated skin samples received 150 kJ/m² UVA delivered by a blue lamp bulb UVA lamp in the BioSun System (Vilber Lourmat). After irradiation, skin biopsies were incubated in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin at 37 °C in humidified air with 5% CO2 during 24 h. All the biopsies were snap frozen in liquid nitrogen in tissue embedding (Tissue-Tek®, Sakura Finetek, Torrance, CA), and 5-μm-thick sections were prepared in cryostat, air dried, and fixed in acetone for 10 min at −20 °C. Sections were first rehydrated in PBS, and endogenous peroxidases were inactivated. Tissue sections were incubated for 15 min with Protein Block (DakoCytomation, Carpinteria, CA), and then for 1 h with 1/100 GrB primary antibody (goat anti-GrB, Santa Cruz Biotechnology). Negative control was obtained without GrB primary antibody. Sections were rinsed in PBS and revealed with DAKO LSAB+ kit peroxidase and diaminobenzidine (DakoCytomation) according to the manufacturer’s instructions. Sections were observed using light microscopy (Zeiss, Oberkochen, Germany).

**Human MIF Immunoassay**—For the quantitative determination of MIF concentrations in cell culture supernatants, we used the human MIF immunoassay kit (Quantikine, R&D Systems). The assay was performed according to the manufacturer’s instructions.

**Assessment of Fibronectin Cleavage by GrB**—HaCaT cells were irradiated or not at 100 kJ/m², and GrB was immunoprecipitated as previously described (6). For assessment of cleavage of fibronectin, wells of a 24-well tray were coated with 500 ng of fibronectin, blocked with bovine serum albumin, and incubated with GrB immunoprecipitated or with human recombinant GrB protein (20 ng). After 24 h of incubation, Laemmli sample buffer was added, complexes were scrapped, and cleavage of

**TABLE 1**

| Oligonucleotide primer sequences for real-time quantitative PCR |
|---------------------------------------------------------------|
| Target gene | Forward primer (5' → 3') | Reverse primer (5' → 3') |
|-------------|---------------------------|-------------------------|
| GranzymeB   | TGCCTGCGCTGGAGCCTCTCCT    | CTTCCAGTCGTAACATGGA     |
| MMP1        | CAGATTTCTTGCTCAAGTCCA     | AGGTTCTCGAGGAGTCAGACA   |
| MMP2        | TGACCAAGATAATACCCTGAGACC  | GCAAGACCAAAAGCCATCA7C   |
| MMP9        | GCGGAATTTGGGCAACGGCTOTA   | GACCCGGCTTGTATAACCCCA   |
| S14         | ATCAAACTCCGGCCACAGAGA     | GTGCTGTCAAGAGGGAAGGGG   |
fibronectin was visualized by Western blot analysis using anti-fibronectin Ab.

Cell Death Assays—HaCaT cells were plated onto 96-well tissue culture tray coated with 2 μg of fibronectin (8 × 10^3/well) and allowed to adhere overnight. Cells were pretreated with sense or antisense GrB oligonucleotides for 6 h at 37 °C prior to exposing cells at 100 or 150 kJ/m^2 UVA. Cells were then incubated at 37 °C for 24 h in DMEM 0.2% FCS to inhibit cell proliferation. For quantification of the remaining survival cells, cells were incubated with 100 μl of 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 1 h. Deposited formazan crystals were solubilized by addition of 100 μl of Me₃SO (DMSO) and detected at Abs 595 nm.

Cell Migration and Invasion Assays—For invasion assays, 10^5 HaCaT cells were plated in DMEM containing 10% FCS into Falcon™ HTS Fluoroblok™ 24-Multiwell Insert System (6.5-mm diameter, 3-μm pore size, BD Biosciences) that had been precoated with 2 μg of fibronectin. Cells were allowed to adhere for 24 h and were washed, pre-treated or not with GrB antisense or sense oligonucleotides (5 μM) for 6 h, irradiated at 100 or 150 kJ/m^2 UVA, marked with 5 μM CellTracker Green CMFDA (5-Chloromethyl fluorescein diacetate) (Invitrogen) for 1 h at 37 °C, and the medium was replaced with 300 μl of DMEM containing 0.2% FCS to inhibit cell proliferation. Cells were allowed to migrate toward 10% FCS for 24 h, after which cells on the underside of the membrane were detected by fluorescence of CMFDA (Abs 490 nm/Em 520 nm).

**RESULTS**

Induction of GrB Expression by UVA in HaCaT Cells—HaCaT cells were treated with UVA at different doses and incubated for 16 h (Fig. 1A) or were irradiated at 100 kJ/m^2 and were incubated for different times (Fig. 1B). GrB mRNA expression was analyzed by real-time quantitative PCR followed by a separation of PCR products in 2% agarose gel. In non-irradiated non-confluent HaCaT cells, GrB mRNA was undetectable as previously described (5, 6), whereas UVA irradiation induced time and dose-dependent increase in GrB mRNA with a maximum of 100 kJ/m^2 above which we detected toxicity (Fig. 1, A and B). In HaCaT cells grown at confluence, GrB mRNA was detectable as previously described (5, 6). In these conditions of culture, UVA irradiation was still able to induce a 3-fold increase in GrB gene expression (data not shown). Immunoblot revealed GrB protein in HaCaT cells following UVA stimulation (Fig. 1C) and confocal microscopy showed that GrB accumulated into cytoplasmic vesicles (Fig. 1D). Interestingly, treatment with UVA results in an increase in mRNA of MMP1 in HaCaT cells as previously described in dermal fibroblasts (11), whereas mRNA levels of MMP2 and MMP9 were unchanged (Fig. 1E). This response profile was different from that we observed in human dermal fibroblasts, which displayed increased expression of each of these MMP, whereas GrB was unchanged (data not shown).

Induction of GrB Expression by UVA in Human Skin—Normal human skin from different donors was irradiated at 150 kJ/m^2 UVA. This dose was used due to the thickness of the skin. In these conditions, as shown by immunohistochemistry for a representative donor, UVA also stimulated GrB protein expres-
sion (Fig. 2). As also illustrated in Fig. 2, GrB accumulated into the epidermal compartment and remained undetectable in dermis. This result was in agreement with the lack of GrB induction in primary fibroblast cultures. Altogether, these results suggest that UVA irradiation activates GrB at the mRNA level in human keratinocytes.

Role of MIF in the Induction of GrB by UVA Irradiation— Based on a previous study, which showed that not only did treatment with UVA result in the release of MIF from dermal fibroblasts but also that MIF played a central role in the production of MMP1 by these cells (11), we investigated whether similar mechanism could operate in keratinocytes cells. We found that UVA induced a time-dependent release of MIF in HaCaT culture medium as measured by enzyme-linked immunosorbent assay with a maximum for 100 kJ/m2 at 24 h (Fig. 3A). The kinetics and magnitude of MIF production in keratinocytes were similar to those we observed in primary fibroblasts (data not shown). We were unable to detect MIF gene activation or modification in cellular MIF protein content (data not shown) suggesting that, in keratinocytes, UVA acts on MIF transport rather than on gene or protein regulation.

The role of MIF in GrB induction was then evaluated. As shown in Fig. 3B, treatment with human rMIF resulted in GrB mRNA expression in HaCaT cells, thus mimicking the effect of UVA, whereas pre-treatment of HaCaT cells with antibody directed against MIF, completely inhibited GrB induction by UVA irradiation (Fig. 3B). These results demonstrated that MIF plays a critical role in GrB gene activation by UVA.

In the abovementioned study (11), the authors have not investigated the signaling events that control MIF production. Based on the important role of radical oxygen species (ROS) in the UVA response, we hypothesized that ROS could play a role in the release of MIF and, subsequently, in GrB induction. As a matter of fact, pre-treatment with N-acetylcysteine (NAC), a potent anti-oxidant agent, prevented the release of MIF (Fig. 3C), whereas treatment with H2O2 resulted in MIF secretion, thus mimicking the effect of UVA (Fig. 3D). These results demonstrate that ROS plays a central role in UVA-induced MIF release from keratinocytes.

Because, on one hand, ROS regulated MIF, and, on the other hand, MIF is essential for GrB induction, one could easily speculate that ROS are also essential for UVA-mediated GrB regulation. Indeed, treatment with H2O2 resulted in GrB gene activation (Fig. 3E), whereas NAC inhibited UVA-induced GrB activation, both at the mRNA (Fig. 3F) and at the protein level (Fig. 3G). Altogether, our study argues for a model in which UVA-induced ROS production mediates the release of MIF, which, in turn, induces GrB gene activation and protein accumulation.

FIGURE 3. Role of MIF in the induction of GrB by UVA irradiation. A, HaCaT cells were irradiated with UVA (100 kJ/m2) and incubated for different times before enzyme-linked immunosorbent assay analysis. B, HaCaT cells were treated with rMIF (200 ng/ml) and incubated for 16 h before mRNA analysis. Cells were also pre-treated with anti-MIF Ab (used at 1/100) for 1 h, irradiated with UVA (100 kJ/m2), and incubated for 16 h before mRNA extraction. C, HaCaT cells were pre-treated with NAC (25 mM), an inhibitor of oxidative stress, for 2 h. Cells were then irradiated with UVA (100 kJ/m2) and incubated for 16 h before enzyme-linked immunosorbent assay analysis. Cells were treated with H2O2 at indicated doses for 1 h in PBS and incubated for 16 h before enzyme-linked immunosorbent assay (D) or GrB mRNA analysis (E). HaCaT cells were pre-treated with NAC (25 mM) for 2 h, then irradiated with UVA (100 kJ/m2) and incubated for 16 h before mRNA (F) or confocal (G) analysis. Expression of GrB 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. The values are the mean ± S.E. of three different experiments.
Role of p38 MAPK in MIF- and UVA-induced GrB Activation—In further studies, we investigated the mechanism that links UVA-induced MIF production and UVA-induced GrB activation. We hypothesized that MAPK could play a role in coupling MIF with GrB gene regulation based on three lines of evidence. First, MIF and UVA were found to activate ERK, p38 MAPK, and JNK modules (13, 14). Second, MAPK are candidates for the regulation of AP1 and CREB, two regulatory elements of GrB promoter (15, 16). Third, we have already demonstrated that UVB induced GrB expression via MAPK pathway in keratinocytes (6). In initial experiments, we found that UVA as well MIF stimulates ERK, JNK, and p38 MAPK (data not shown). Then, HaCaT cells were pre-treated with MAPK inhibitors and then ultimately treated with rMIF. Only SB203580, the p38 MAPK inhibitor, totally inhibited GrB mRNA (Fig. 4A) and protein induction (Fig. 4B) by UVA, whereas both JNK and ERK inhibitors had no effect either on GrB mRNA (Fig. 4A) or on protein induction (data not shown). As a control, we show in Fig. 4C that rMIF did activate p38 MAPK as attested by SB203580-inhibitable phosphorylation of MAPKAPK2, a substrate of p38 MAPK.

To confirm the implication of p38 MAPK in UVA-induced GrB activation, we performed similar experiments in HaCaT cells irradiated with UVA. As expected from the above results, we found that UVA triggered p38 MAPK, and that this stimulation was essential for GrB gene activation (Fig. 5A) and protein accumulation (Fig. 5B). As a control, we show in Fig. 5C that UVA, like MIF, activated p38 MAPK as attested by SB203580-inhibitable phosphorylation of MAPKAPK2. Altogether, these results suggest a model in which UVA induces the production of MIF which, in turn, activates GrB through p38 MAPK.

Effect of GrB on Fibronectin Cleavage following UVA Irradiation—GrB has been shown to cleave extracellular matrix proteins like fibronectin (9, 10). To confirm that GrB induced by UVA irradiation is able to degrade this extracellular matrix protein, we irradiated HaCaT cells at 100 kJ/m², and 24 h after irradiation, GrB protein was immunoprecipitated from cell extracts. In irradiated cells, GrB was totally retained in immunoprecipitates, because it was undetectable in the supernatants (Fig. 6A). These immunoprecipitates were then incubated for 24 h with 500 ng of plated purified fibronectin. As illustrated in Fig. 6B, we observed that anti-GrB immunoextracts from UVA-irradiated cells contained less fibronectin than those from non-irradiated cells, suggesting that GrB was able to degrade fibronectin.
Motility.

migration. Finally, these results illustrate the potency of GrB to important intrinsic potential of UVA to trigger keratinocyte detachment and survival in the context of the UVA response. These results confirm the role of GrB as an important player in cell motility with a magnitude amplification of ~10-fold. Moreover, treatment with rGrB abrogated the migration capacity of keratinocytes depleted in endogenous GrB by antisense (Fig. 7C). Similar results were obtained with vitronectin (data not shown). Moreover, pre-treatment with anti-MIF antibody resulted in enhanced cell migration after UVA exposure, thus mimicking GrB depletion (data not shown). Altogether, these results confirm the role of GrB as an important player in cell detachment and survival in the context of the UVA response. Moreover, as an unexpected result, our study unmasked an important intrinsic potential of UVA to trigger keratinocyte migration. Finally, these results illustrate the potency of GrB to counteract the tremendous stimulatory effect of UVA on cell motility.

DISCUSSION

In this work we have shown for the first time that, in keratinocytes, UVA irradiation induces GrB gene and protein expression. Our data support a model in which UVA induces a redox-dependent release of MIF, which, in turn, activates the production of active GrB through p38 MAPK. GrB accumulation was observed in immortalized HaCaT cells and normal human skin from different donors, suggesting that this event is not related to a specific cellular model or culture conditions. However, among skin cells, GrB induction upon UVA stimulation seems specific to keratinocytes, because it was not...
observed in primary dermal fibroblasts. In this perspective, it is also interesting to note that UVA-irradiated keratinocytes produced simultaneously GrB and MMP1, the former (see below), but not the latter (3, 4), being able to cleave fibronectin, whereas fibroblasts produced MMP2 and MMP9, which are active against fibronectin, but not GrB. This result confirms that, upon UVA irradiation, keratinocytes and fibroblasts cooperate for producing various proteases with complementary properties for extracellular matrix remodeling.

The fact that GrB up-regulation is part of the cellular response to stress induced by UVA irradiation was not totally unexpected. Indeed, not only GrB was detected in confluent keratinocytes (5), but also we have recently described GrB production in keratinocytes following UVB irradiation (6) and, in non-epithelial cells following genotoxic stress (12). We have also reported similar events in cells treated by inflammatory cytokines such as TNFα (17). Altogether, these results suggest that, at least in a number of tissues, GrB induction may be part of a general response to cellular stress.

The mechanism by which UVA up-regulates GrB has been also examined. Our study provides strong evidences that MIF is a central player in UVA-mediated GrB regulation. MIF, a pituitary-derived hormone, is ubiquitously expressed in various tissues, including the skin. The role of MIF in the UVA response has been previously documented in dermal fibroblasts. In these cells, Watanabe and co-workers (11) have reported that UVA stimulates MIF production and that the event is critical for the production of MMP1, the principal fibroblast-derived secreted proteinase capable of degrading native fibrillar collagens of types I, II, III, and VII (3, 4). Combined with our results, MIF appears as an important mediator for the production of extracellular matrix-remodeling factors, such as at least MMP1 and GrB, but also, perhaps, other proteases. Clinical evidences of increased MIF expression in chronic inflammatory states or in skin diseases suggest that MIF may also operate in other conditions than in the context of UVA irradiation (for a review, see Ref. 18). Whether or not, in these conditions, MIF accumulation may result in GrB induction with similar functional consequences needs further information.

The mechanism by which MIF stimulates GrB was also examined. UV irradiation induces various but coordinated signaling pathways, known as the UV response, which include ROS-dependent activation of phosphatidylinositol 3-kinase/Akt, p38 MAPK, JNK, and ERK (19). By using chemical inhibitors, we describe that GrB induction by MIF (and by UVA) is dependent upon p38 MAPK. However, ERK, JNK, or phosphatidylinositol 3-kinase pathways play no role in this regulation. The link between p38 MAPK activation and GrB has not been investigated. However, it should be noted that GrB promoter contains binding sites for AP1, CAMP-responsive element-binding protein (CREB), core-binding factor, and Ikaros transcription factors (15, 16). Moreover, p38 MAPK can also activate CREB (20) and AP1 transcription factors (21, 22). Finally, some findings indicate that the p38 MAPK pathway contributes also to stabilization of mRNAs through an MAPKAPK2-dependent and AU-rich element-targeted mechanism (23). Based on these findings, one could speculate that p38 MAPK acts directly through a CREB- or AP1-mediated transcriptional mechanism, and/or indirectly at a post-transcriptional level by stabilizing GrB mRNA.

The significance and the functional consequences of GrB accumulation on the epidermis homeostasis following UVA irradiation were unknown. In this study, we have first provided direct evidences that GrB synthesized upon UVA irradiation was able to degrade purified fibronectin. This finding is in agreement with a recent study that elegantly shows that purified or recombinant GrB is able to cleave laminin, vitronectin, and fibronectin both in vitro and in cell culture (9). The specificity of this enzyme for extracellular matrix components has also been described by adding purified GrB to smooth muscle cell culture (10). Therefore, our study raises the possibility that, following UVA, increased fibronectin synthesis and subsequent accumulation (2) are counteracted by GrB production and fibronectin cleavage and that any disturbances of this balance may result in potentially severe alterations of the composition of extracellular protein components.

In the two studies mentioned above (9, 10), it has been shown that GrB-mediated degradation of extracellular matrix structure resulted in the inhibition of cell spreading, cell detachment, and cell death via anoikis. Our study shows that UVA-induced GrB production also influences cell detachment and cell death in keratinocytes culture. Moreover, we provide direct evidences that GrB efficiently counteracts the stimulatory effect of UVA on cell motility. These findings suggest that GrB is an important factor for eliminating damaged cells through cell detachment following UVA irradiation. If this is the case, one could speculate that deficiency in GrB production should result in both cell death inhibition and cell migration facilitation, two effects that may have serious consequences in terms of skin organization and carcinogenesis. It is important to note that human GrB displays polymorphism with significant differences in terms of catalytic capacities (24). It is therefore possible that the GrB allele profile influences long term UVA effects such as photoaging and photocarcinogenesis.

Finally, it is interesting to note that, in contrast to UVB, UVA failed to confer cellular cytotoxicity capacity to keratinocytes (data not shown). The mechanism that underlies this difference is unclear. Indeed, as we have described for UVB (6), UVA also stimulates not only the production of GrB (this study) but also the accumulation of perforin in HaCaT cells (data not shown). These observations raise the possibility that UVB, but not UVA, irradiation activates mechanism resulting in cell-to-cell recognition and interaction required for target cell lysis. Combining our two studies, it appears that GrB could confer to keratinocyte the ability to degrade extracellular matrix components or the capacity to eliminate non-epithelial surrounding epidermal cells, depending on the UV source.

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REFERENCES

1. Schwartz, E. (1988) J. Invest. Dermatol. 91, 158–161
2. Boyer, B., Fourtanier, A., Kern, P., and Labat-Robert, J. (1992) J. Photochem. Photobiol. B 14, 247–259
3. McCawley, L. J., and Matrisian, L. M. (2001) Curr. Opin. Cell Biol. 13, 534–540
4. Werb, Z. (1997) Cell 91, 439–442
5. Berthou, C., Michel, L., Soulie, A., Jean-Louis, F., Flageul, B., Dubertret, L., Sigaux, F., Zhang, Y., and Sasportes, M. (1997) J. Immunol. 159, 5293–5300
6. Hernandez-Pigeon, H., Jean, C., Charruyer, A., Haure, M. J., Titeux, M., Tonasso, L., Quillet-Mary, A., Baudouin, C., Charveron, M., and Laurent, G. (2006) J. Biol. Chem. 281, 13525–13532
7. Ronday, H. K., van der Laan, W. H., Tak, P. P., de Roos, J. A., Bank, R. A., TeKoppele, J. M., Frollich, C. J., Hack, C. E., Hogendoorn, P. C., Breedveld, F. C., and Verheijen, J. H. (2001) Rheumatology (Oxford) 40, 55–61
8. Frollich, C. J., Zhang, X., Turbou, J., Hudig, D., Winkler, U., and Hanna, W. L. (1993) J. Immunol. 151, 7161–7171
9. Buzza, M. S., Zamurs, L., Sun, J., Bird, C. H., Smith, A. I., Trapani, J. A., Frollich, C. J., Nice, E. C., and Bird, P. I. (2005) J. Biol. Chem. 280, 23549–23558
10. Choy, J. C., Hung, V. H., Hunter, A. L., Cheung, P. K., Motyka, B., Goping, I. S., Sawchuk, T., Bleakley, R. C., Podor, T. I., McManus, B. M., and Granville, D. J. (2004) Arterioscler. Thromb. Vasc. Biol. 24, 2245–2250
11. Watanabe, H., Shimizu, T., Nishihira, J., Abe, R., Nakayama, T., Taniguchi, M., Sabe, H., Ishibashi, T., and Shimizu, H. (2004) J. Biol. Chem. 279, 1676–1683

12. Bruno, A. P., Lautier, D., d’Orgeix, A. T., Laurent, G., and Quillet-Mary, A. (2000) Blood 96, 1914–1920
13. Santos, L. L., Lacey, D., Yang, Y., Lee, M., and Morand, E. F. (2004) J. Rheumatol. 31, 1038–1043
14. Silvers, A. L., Bachelor, M. A., and Bowden, G. T. (2003) Neoplasia 5, 319–329
15. Babichuk, C. K., Duggan, B. L., and Bleackley, R. C. (1996) J. Biol. Chem. 271, 16485–16493
16. Wargnier, A., Legros-Maida, S., Bosselut, R., Bourge, J. F., Lafaurie, C., Ghysdael, C. J., Sasportes, M., and Paul, P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6930–6934
17. Guilloton, F., de Thonel, A., Jean, C., Demur, C., Mansat-De Mas, V., Laurent, G., and Quillet-Mary, A. (2005) Leukemia 19, 2206–2214
18. Shimizu, T. (2005) J. Dermatol. Sci. 37, 65–73
19. Bowden, G. T. (2004) Nat. Rev. Cancer 4, 23–35
20. Butler, M. P., Hanly, J. A., and Moynagh, P. N. (2005) J. Biol. Chem. 280, 27759–27768
21. Chen, R., Lim, J. H., Jono, H., Gu, X. X., Kim, Y. S., Bashaum, C. B., Murphy, T. F., and Li, J. D. (2004) Biochem. Biophys. Res. Commun. 324, 1087–1094
22. Efimova, T., LaCelle, P., Welter, J. F., and Eckert, R. L. (1998) J. Biol. Chem. 273, 24387–24395
23. Winzen, R., Kracht, M., Ritter, B., Wilhelm, A., Chen, C. Y., Shyu, A. B., Muller, M., Gaestel, M., Resch, K., and Holtmann, H. (1999) EMBO J. 18, 4969–4980
24. McIlroy, D., Cartron, P. F., Tuffery, P., Dudoit, Y., Samri, A., Autran, B., Vallette, F. M., Debre, P., and Theodorou, I. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 2562–2567