The Flavonoid Luteolin Increases the Resistance of Normal, but Not Malignant Keratinocytes, Against UVB-Induced Apoptosis

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Adequate protection of skin against the carcinogenic effects of UVB irradiation is essential. Flavonoids may have a conspicuous role in cancer prevention because of their antioxidant, anti-inflammatory, and growth-inhibitory effects. Therefore, we tested the effects of the flavone luteolin (LUT) on selected parameters of the sunburn response in normal human keratinocytes, exposed to physiological doses of UVB. LUT attenuated UVB-induced cell death through delay and inhibition of intrinsic apoptotic signaling. Moreover, LUT not only predominantly affected the mitochondrial apoptosis pathway through its antioxidant capacity, but also changed the balance of Bcl2 (B-cell leukemia/lymphoma 2)-family members. Furthermore, LUT had inhibitory effects on the UVB-induced release of the inflammatory mediators, IL-1α and prostaglandin-E_2. Using different cell lines derived from squamous cell carcinomas, we showed that LUT did not increase the resistance of malignant keratinocytes to UVB. Our data suggest that LUT inhibits different aspects of the sunburn response, which results ultimately in an increased survival of normal keratinocytes, whereas the sensitivity of malignant cells to UVB remain unchanged. Hence, LUT might have value in new photoprotective applications or improve existing ones.

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INTRODUCTION

Flavonoids, part of the group of the polyphenols, are exclusively produced in plants through the phenylpropanoid pathway to help plants to cope with stress; for instance, UV irradiation and oxidative stress (Harborne and Williams, 2000; Julsing *et al.*, 2006). These natural compounds show strong antioxidant effects and some also show other biochemical effects in human cells, such as enzyme inhibition, anti-inflammatory, and anticarcinogenic capacities (Nijveldt *et al.*, 2001). These characteristics make flavonoids potent candidates for photoprotective applications (Morquio *et al.*, 2005; Afaq and Mukhtar, 2006; Verschooten *et al.*, 2006a).

Luteolin (LUT or 3′,4′,5,7-tetrahydroxyflavone), which is structurally categorized under the flavones, is known as a very good antioxidant (Cai *et al.*, 1997). This can be ascribed to its highly hydroxylated structure, which contains the known structural antioxidant features of flavonoids (Rice-Evans *et al.*, 1996). Furthermore, LUT has been shown to have anti-inflammatory effects (Gutierrez-Venegas *et al.*, 2006) and even anticancer activity through interference with several signaling pathways, such as mitogen-activated protein kinase, NF-κB, PI3K/AKT (phosphatidylinositol-3-kinase/acutely transforming retrovirus AKT8 in rodent T cell lymphoma) (Lin *et al.*, 2008), and the specific induction of apoptosis (Ramos, 2007). The topical application of *Achyrocline satureioides* and perilla leaf extract, both containing a significant amount of LUT, inhibited UV-induced reactive oxygen species (ROS) formation in rabbits (Morquio *et al.*, 2005) and 12-O-tetradecanoylphorbol-3-acetate (TPA)-induced tumor formation in mice (Ueda *et al.*, 2003), respectively. A very recent study, in which a LUT-rich *Reseda* extract was applied to human skin before UVB irradiation, showed the potential of LUT in the prevention of UVB-induced erythema (Casetti *et al.*, 2009).

Our skin, as the biggest organ covering our body, is equipped with several structural and biochemical features to

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Abbreviations: $\Delta\psi_m$, mitochondrial membrane potential; COX-2, cyclooxygenase-2; CPD, cyclobutane pyrimidine dimer; LUT, luteolin or 3′,4′,5,7-tetrahydroxyflavone; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NHK, normal human keratinocyte; PARP, poly(adenosine diphosphate-ribose) polymerase; PGE_2, prostaglandin-E_2; ROS, reactive oxygen species; SCC, squamous cell carcinoma

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protect our inner body against several environmental stresses. The exposure of the skin to solar UV on earth (containing UVB and UVA radiation) has both short-term and long-term deleterious wavelength-dependent effects on skin. In contrast to tanning and sunburn, which occur shortly after UVB exposure, long term UVB effects include photoaging and photocarcinogenesis. Although UVB is still considered the most important risk factor for squamous cell carcinoma (SCC) of the skin, the contribution of the more abundant UVA component of sunlight is gaining more and more support (Mouret *et al.*, 2006). UVB is thousand times more effective than UVA in inducing a sunburn response (de Gruijl, 2002), which is in fact an acute inflammatory response of the skin, visible as erythema. At the microscopic level, it is characterized by an inflammatory infiltrate, vasodilatation, formation of sunburn cells, and depletion of Langerhans cells. Several proinflammatory cytokines (e.g., IL-1α) secreted by keratinocytes or other immune competent cells, as well as vasoactive mediators (e.g., prostaglandin E2 (PGE_2)), are important mediators of the sunburn response (Rhodes *et al.*, 2009).

UVB irradiation damages skin cells through direct and indirect mechanisms. Direct absorption of UVB by DNA results in the formation of pyrimidine dimers. However, other aromatic chromophores also undergo structural changes after UVB photon absorption. This can result in the formation of aggressive radicals and ROS, which in turn can damage proteins, lipids, and DNA as an indirect consequence of UVB irradiation (Jin *et al.*, 2007).

Upon perseverance of the cellular damage, UVB elicits a fail-safe mechanism evidenced by the formation of sunburn cells, representing keratinocytes undergoing apoptosis to ensure the disposal of the damaged, cancer-prone cells (Claerhout *et al.*, 2006). Sunburn cells are morphologically characterized by cell shrinkage, formation of pyknotic nuclei with condensed chromatin, and finally membrane blebbing and the formation of apoptotic bodies. In their final stage, they are engulfed by surrounding phagocytotic cells, minimizing a possible inflammatory response (Van Laethem *et al.*, 2005). The formation of sunburn cells is regulated through several signaling cascades arising from DNA damage, membrane receptor clustering, and ROS formation, converging into the permeabilization of the outer mitochondria membrane, and ultimately resulting in the activation of caspases and apoptotic cell death (Zimmermann *et al.*, 2001). Apoptosis functions in healthy epidermis in a well-balanced way to remove irreversibly damaged cells without killing healthy proliferating cells, which may abrogate the barrier function of the epidermis. However, in malignant cells apoptosis is often deregulated, allowing clonal expansion of damaged cells. In the protection of skin against the harmful effects of UVB, one has to be careful not to stimulate the survival of damaged cells, nor to protect cells with a deranged genetic background.

Although LUT is known as an excellent antioxidant (Lopez-Lazaro, 2009), and it reduces UVB-induced erythema in human skin (Casetti *et al.*, 2009), the effects of LUT on the molecular aspects of the sunburn response in human skin cells have not been investigated yet. In this study, we investigated the effect of LUT on apoptosis and other selected parameters of the sunburn response in normal human keratinocytes (NHKs) as well as in malignant keratinocytes exposed to a physiological UVB dose. We found that LUT was very efficient in inhibiting the sunburn response in NHK, as it interfered with the intrinsic apoptosis pathway and with the release of proinflammatory mediators after UVB irradiation. On the contrary, LUT did not affect UVB-induced apoptosis in malignant keratinocytes, thus showing a high degree of specificity in its sunburn protection mechanism of action.

RESULTS

LUT increases cell survival in NHKs after UVB irradiation

NHKs were treated 24 hours before and immediately after apoptotic doses of UVB irradiation with LUT. This low, nontoxic dose of LUT significantly increased the resistance to UVB irradiation, as shown by a higher retention of metabolic activity (Figure 1a) and by significantly higher cell viability (Figure 1b) until 72 hours after irradiation. Light microscopic pictures, shown in Figure 1c, illustrate the decreased sensitivity of LUT-treated cells to a UVB dose that is lethal to the control cells. Note a slight change in the morphology of LUT-treated NHKs (Figure 1c). In the presence of LUT, NHKs appear to grow slower and more in clusters in comparison to untreated or vehicle-treated cells. We could confirm the growth-inhibitory effect by plotting the proliferation curve of long-time exposure of NHK to LUT (Supplementary Figure S1a online). Both morphological changes and growth inhibition suggest the induction of differentiation. Indeed, LUT-treated cells showed an increase of involucrin, a late differentiation marker, at the protein level (Supplementary Figure S1b online).

LUT delays the apoptotic response of human keratinocytes

UVB irradiation induced an apoptotic phenotype in NHKs within 16 hours, including caspase 3 activation and subsequent cleavage of poly(adenosine diphosphate-ribose) polymerase (PARP) (Figure 2a). Treatment with LUT before and immediately after irradiation with UVB inhibited and delayed UVB-induced cleavage of caspase 3 and PARP (Figure 2a). Nevertheless, a fraction of LUT-treated cells did finally undergo apoptosis at 48 hours after irradiation, which indicates that the apoptotic machinery is not completely suppressed by LUT. These data are further confirmed and quantified by flow cytometric analysis of propidium iodide-stained NHKs (Figure 2b), showing that LUT delays DNA fragmentation and thus decreases the fraction of ''SubG1'' cells at all tested time points after irradiation as also shown by the visualization of pyknotic nuclei by Hoechst 33342 staining (Figure 2c and d).

As direct DNA damage is one of the triggers leading to apoptosis, we checked the effect of LUT on the induction and repair of cyclobutane pyrimidine dimers (CPDs) after a low and high UVB dose. In cells irradiated with nonlethal UVB doses, a part (27.5%) of the CPDs were removed within 24 hours. Exactly the same pattern of induction and repair was observed in the LUT-treated cells (Figure 2e), indicating

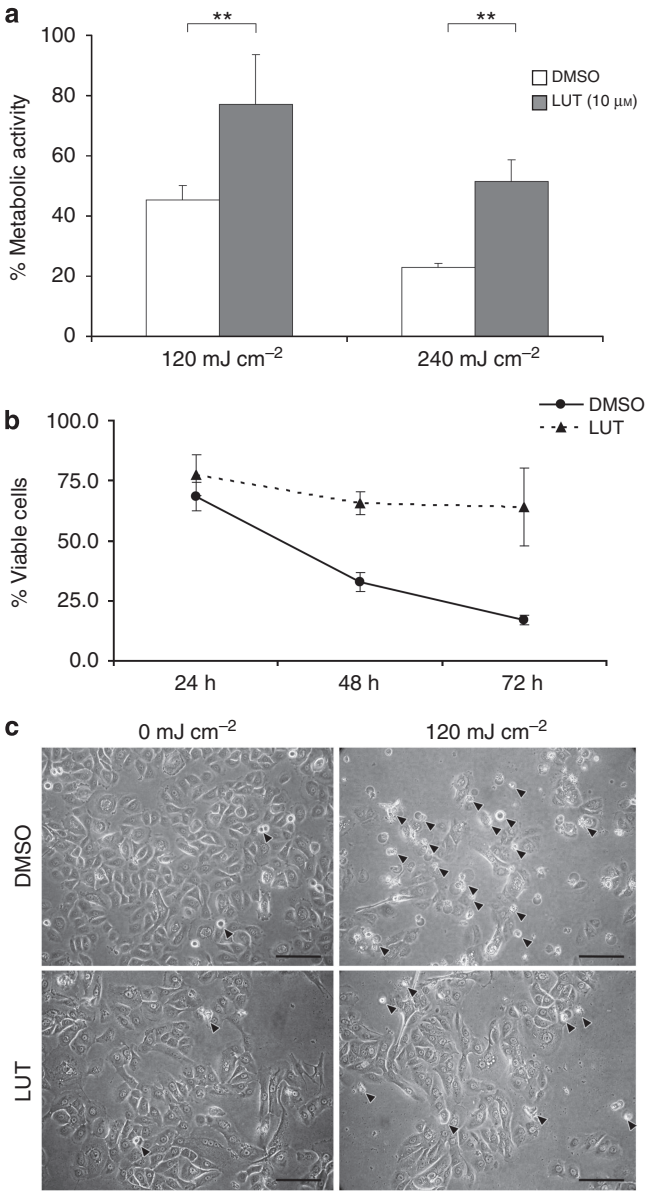

Figure 1. Luteolin (LUT) increases the survival of normal human keratinocytes (NHKs) after UVB irradiation. (**a**) MTT assay of NHKs grown in the presence or absence of LUT (10 μM) 24 hours after irradiation with 120 or 240 mJ cm^{-2} of UVB. The graph represents relative values of metabolic activity by normalization to unirradiated controls (at least 8 wells/conditions, graph of three experiments; $^{**}P<0.001$). (**b**) The viability of NHKs treated or not with LUT was obtained using Trypan blue analysis on three successive days after UVB irradiation (120 mJ cm^{-2}) (experiment performed twice in duplicate). (**c**) Morphologic pictures of NHKs, grown in the presence of vehicle alone or LUT, 24 hours after sham or UVB irradiation (scale bar = 100 μm; arrows point to death cells).

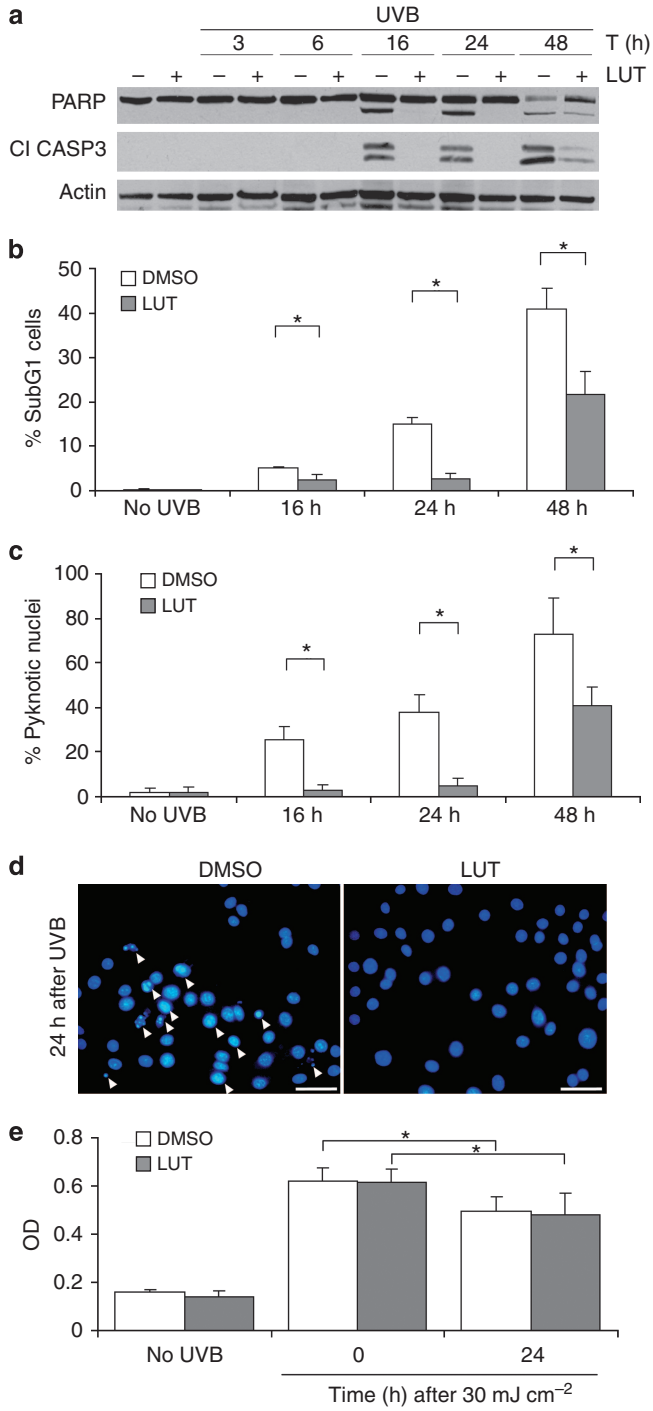

Figure 2. Luteolin (LUT) inhibits and delays UVB-induced apoptosis. (**a**) Western blot analysis of normal human keratinocytes (NHKs) treated with LUT or DMSO and/or UVB (120 mJ cm^{-2}) at different time points after irradiation. (**b**) Flow cytometry analysis of propidium iodide (PI)-stained NHKs at indicated time points (representative of three independent experiments, $n=3$, $P<0.05$). (**c**) NHK, DMSO- or LUT-treated, irradiated or not (120 mJ cm^{-2}), were stained with Hoechst33342 and visualized. Pyknotic cell percentage was calculated by counting at least 10 fields of each condition mentioned ($P<0.001$). (**d**) Representative pictures of Hoechst33342-stained cells 24 hours after UVB irradiation (scale bar = 50 μm; arrows point to cell with condensed nuclei). (**e**) Immunodetection of cyclobutane pyrimidine dimers (CPDs) in genomic DNA of cells irradiated or not with 30 mJ cm^{-2} UVB and harvested immediately or 24 hours after irradiation (representative experiment, $P<0.05$).

that the repair capacity is preserved but that LUT does not induce further repair nor reduces initial DNA damage. In cells exposed to a high UVB dose (240 mJ cm^{-2}) resulting in apoptosis, no repair was observed in both untreated and LUT-treated cells (Supplementary Figure S2 online). These data indicate that the effect of LUT on apoptosis is mediated through interference with indirect damage.

LUT influences apoptosis, at least partially, through modulating the mitochondrial apoptotic pathway

In concordance with the antioxidant properties of LUT (Cai *et al.*, 1997), UVB-induced ROS, as measured by the shift in 2′,7′-dichlorofluorescin diacetate (DCFDA) fluorescence, were reduced in LUT-treated cells (Figure 3a). As ROS-mediated pathways in UVB-treated NHKs are crucially involved in mitochondrial apoptosis, we evaluated the effect

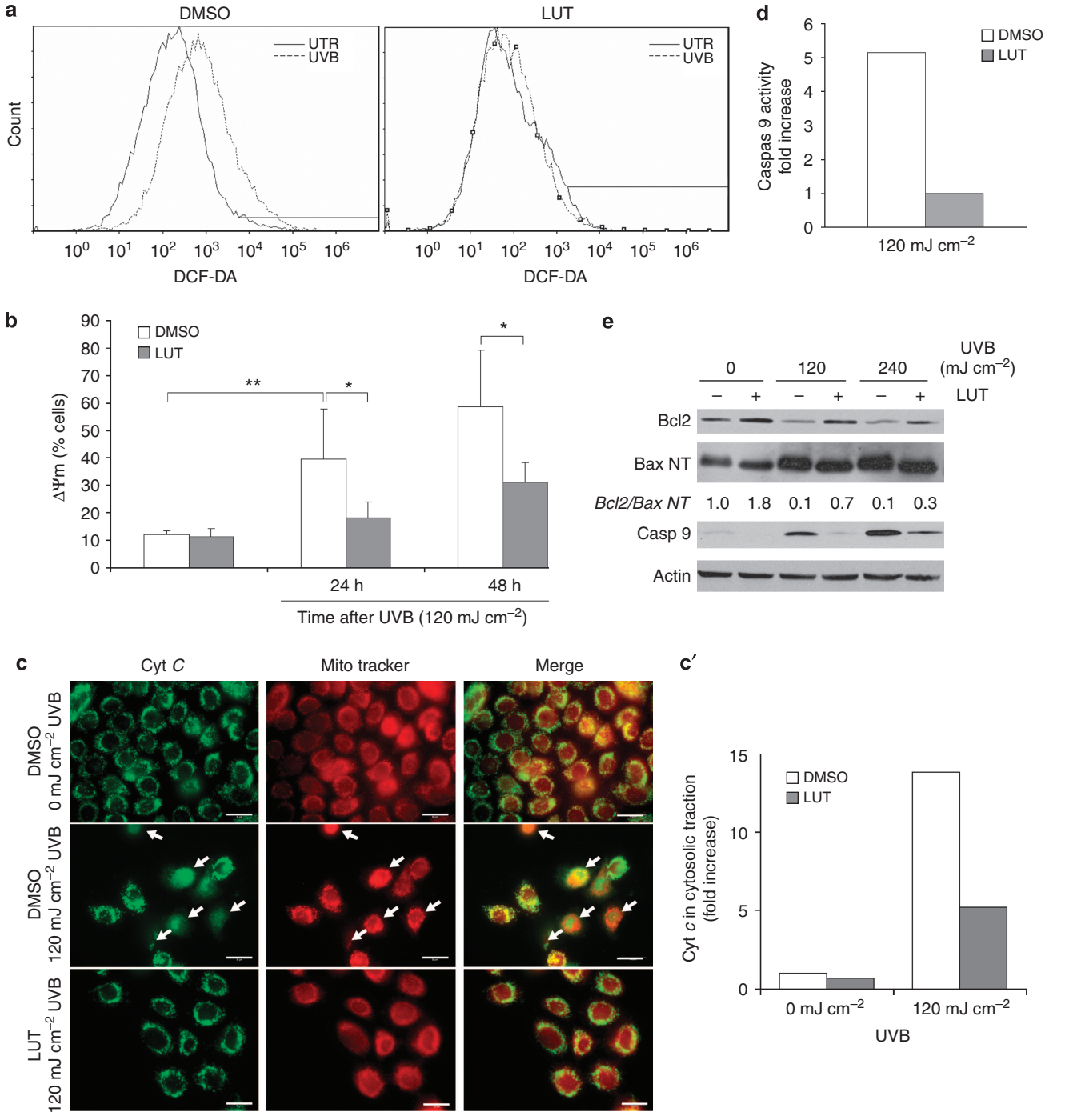

Figure 3. Luteolin (LUT) inhibits UVB-induced mitochondrial effects. (**a**) DCFDA-H_2 stained normal human keratinocytes (NHKs), treated or not with LUT, were analyzed using a flow cytometer. Reactive oxygen species (ROS) was measured 16 hours after irradiation as the increase in fluorescence intensity (representative experiment, $n=3$). (**b**) Percentage of cells with changed mitochondrial membrane potential ($\Delta\psi_m$) was measured by staining NHK cells with $DiOC_6$ followed by flow cytometric analysis ($n=3$, representative experiment). (**c**) Fluorescence microscopy using Mitotracker Red and anti-cytochrome *c* antibody 16 hours after treatment (scale bar $=20\,\mu m$; arrows indicate cells with cytochrome *c* release). (**c′**) Quantification of western blot analysis (using *UnScanIt)* of cytosolic lysates of NHKs 16 hours after irradiation. (**d**) The fold increase in activity of caspase 9, 16 hours after UVB irradiation. (**e**) Western blot analysis of lysates of LUT- and/or UVB-treated cells (quantification using *UnScanIt*).

of LUT on reduction of the mitochondrial membrane potential ($\Delta\psi_m$) and mitochondrial outer membrane permeabilization. Figure 3b clearly shows that the reduction of $\Delta\psi_m$ after UVB was inhibited by LUT until 24 hours after UVB, and, to a lesser extent, until 48 hours after irradiation. These results are consistent with our observations showing inhibition but not a complete block of apoptosis (Figure 2).

Upon mitochondrial outer membrane permeabilization, cytochrome *c* is released from the mitochondria into the cytosol, and triggers the activation of caspase 9 through the formation of the apoptosome (Kroemer *et al.*, 2007). Immunofluorescent staining of NHKs (Figure 3c) and western blot analysis (Figure 3c′) showed that LUT inhibited the release of cytochrome *c* 16 hours after UVB irradiation (as colocalization between cytochrome *c* and the mitochondria was observed in nearly all LUT-treated cells (Figure 3c)), and the subsequent activation of caspase 9 (Figure 3d) was inhibited.

Because mitochondrial outer membrane permeabilization is primarily regulated by Bcl2 (B-cell leukemia/lymphoma 2)-family members, we next evaluated whether LUT affected the intracellular ratio between antiapoptotic (i.e., Bcl2) and proapoptotic (i.e., Bax) family members at the protein level. NHKs treated with LUT, for at least 24 hours, showed higher levels of Bcl2, whereas the level of active Bax (detected using BaxNT antibody) was not influenced (Figure 3e). As expected, UVB irradiation of control NHKs resulted in an increased pool of active Bax, whereas Bcl2 levels slightly decreased. Addition of LUT partially prevented UVB-mediated Bax activation and concomitantly maintained Bcl2 levels higher, thus resulting in a higher Bcl2/active Bax ratio in agreement with the protective effects on $\Delta\psi_m$ and on cytochrome *c* release.

LUT diminishes the release of important inflammatory mediators upon UVB irradiation

UVB irradiation of skin *in vivo* is followed by the known sunburn reaction, which is mediated by the release and action of proinflammatory cytokines, vasoactive mediators, and adhesion molecules. The inducible form of the cyclooxygenase enzyme (COX), COX-2, is known to be the major enzyme responsible for the UVB-induced prostaglandin synthesis, predominantly of PGE_2. Although we could not detect an inhibitory effect of LUT on the COX-2 protein level after UVB (Supplementary Figure S3a online), LUT was able to inhibit the release of PGE_2 upon UVB irradiation (Figure 4a). In control conditions, we detected increasing amounts of PGE_2 in the supernatant from 3 hours after-irradiation. In LUT-treated keratinocytes, the initially lower PGE_2 release did not increase significantly until at least 24 hours after UVB treatment with 120 mJ cm^{-2} (Figure 4a). We also measured the release of IL-1α because it is an important cytokine in the UVB response of NHKs. Similarly, we could detect an inhibitory effect of LUT on the release of this cytokine in response to UVB irradiation (120 mJ cm^{-2}) until at least 24 hours after UVB (Figure 4b).

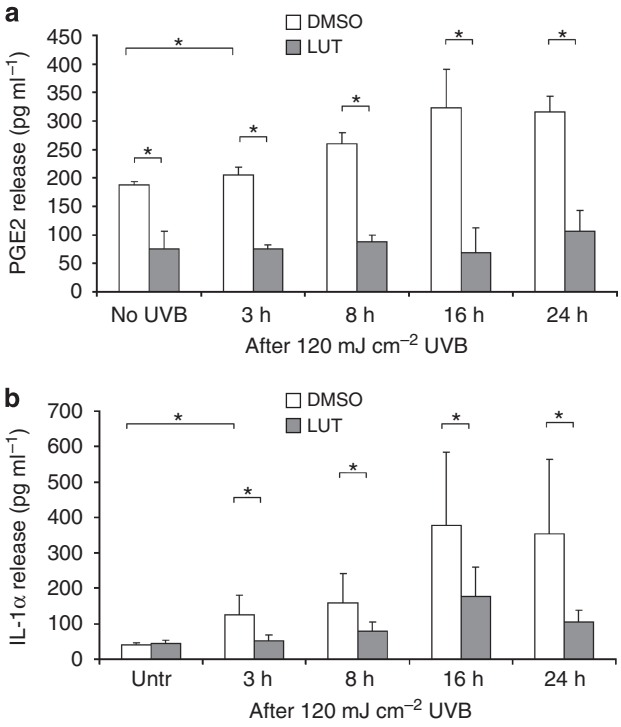

Figure 4. Effect of luteolin (LUT) on the inflammatory UVB response. (**a, b**) Release of prostaglandin-E_2 (PGE_2) (**a**) and IL-1α (**b**) out of irradiated keratinocytes, treated or not with LUT (5 μM), was determined by ELISA of the supernatant (values, normalized to mean of control condition, from three independent experiments with two samples per condition; *$P<0.05$).

LUT does not influence the survival of UVB-exposed skin cancer cells

Unlike the stimulatory effect of LUT on apoptosis observed in different cancer cell lines (Lopez-Lazaro, 2009), the above results indicate an inhibitory effect on apoptosis in normal, human keratinocytes irradiated with UVB. However, UV protection of premalignant or malignant skin cells by LUT should be avoided, as these cells might give rise to skin cancer. Therefore, we tested whether LUT influences the survival of skin cancer cells after UVB exposure. We used MET1 cells, derived from a primary cutaneous SCC (Proby *et al.*, 2000), and exposed them to LUT and to apoptotic doses of UVB as we did with the NHKs. The morphological analysis (Figure 5a) showed a similar fraction of detached and death cells in vehicle- and LUT-treated cells.

In addition, using Trypan blue viability assay (Figure 5b) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Figure 5c), we, respectively, found a comparable amount of viable cells and of metabolic activity in DMSO and LUT condition. To exclude a protective effect of LUT on UVB-induced apoptosis in cancer cells, we analyzed the fraction of subG1 cells and indeed found no reduction of apoptotic cell death in the LUT-treated samples (Figure 5d). To rule out a cell-specific effect, we examined the cleavage of caspase 3 and PARP as markers of apoptotic signaling after UVB and/or LUT treatment in different SCC cell lines, namely, MET1-, MET4- (Proby *et al.*, 2000),

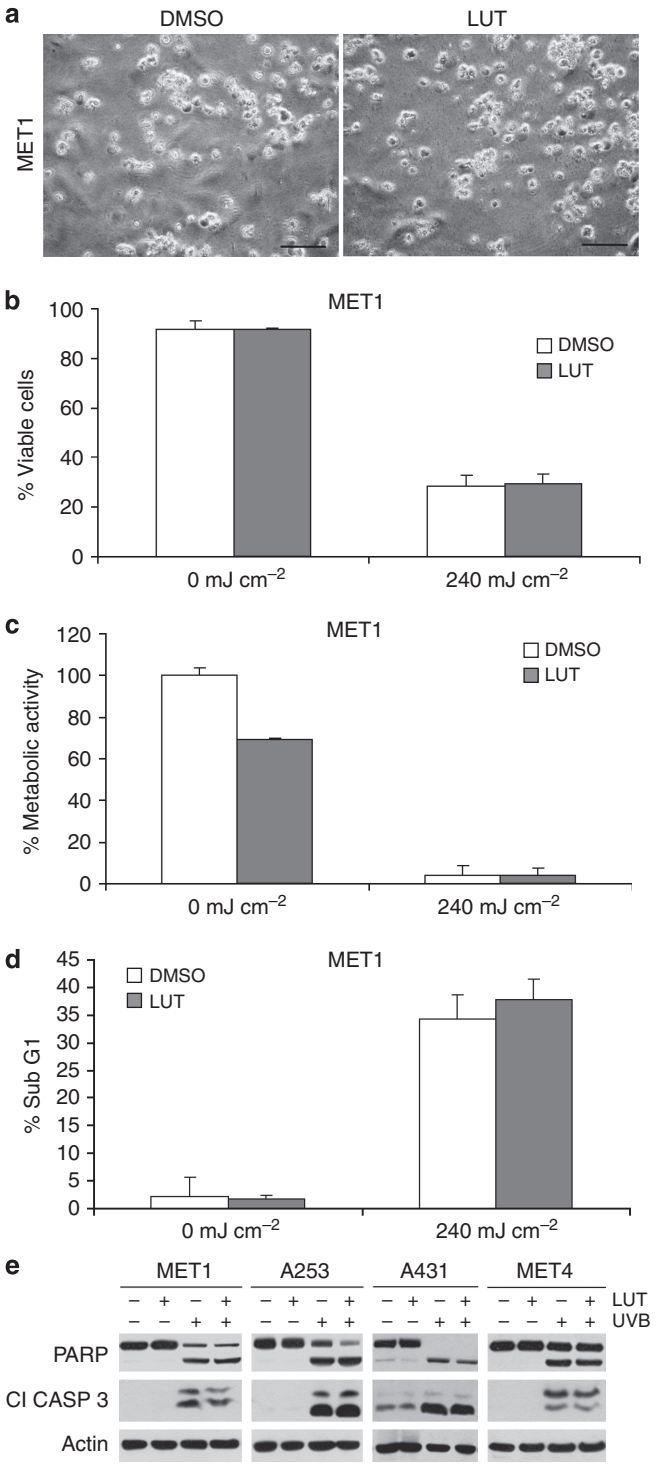

Figure 5. Effect of luteolin (LUT) on the sensitivity of malignant skin cells to UVB. (**a**) Light microscopic pictures of LUT- and/or UVB-treated (240 mJ cm^{-2}) MET1 cells 24 hours after irradiation (scale bar = 100 μm). (**b**) Trypan blue exclusion assay of MET1 cells treated with LUT and/or UVB (average values shown). (**c**) MTT assay of MET1 cells treated with LUT- and/or UVB (representative experiment, $n = 16$). (**d**) Flow cytometric analysis of propidium iodide (PI)-stained MET1 cells treated or not with LUT and UVB (240 mJ cm^{-2}). SubG1 cell fraction reflects the percentage of cells with defragmented DNA (representative experiment out of 3, $n = 3$). (**e**) Squamous cell carcinoma (SCC) cells were exposed to LUT and/or UVB. Cell lysates were analyzed by western blot using specific antibodies. Actin was used as a loading control.

A431-, and A253-SCC cells. Figure 5e shows that LUT did not inhibit UVB-induced apoptosis in all SCC-derived cells.

To exclude the effect of the medium used for SCC cell lines, we repeated a subset of experiments in NHKs grown in the same medium as the SCC cells. The western blot for apoptotic markers shown in Supplementary Figure S4 online confirmed that the protective effect of LUT against UVB-induced cell death in NHKs was independent of the medium used.

All together, these results indicate that although LUT did protect NHKs significantly against UVB-mediated sunburn responses, it did not increase the resistance of malignant cells to UVB.

DISCUSSION

In this study, we show that LUT increases the survival of NHK upon UVB irradiation through inhibition/suppression of the mitochondrial intrinsic apoptotic pathway. However, LUT does not inhibit UVB-induced apoptosis in malignant keratinocytes. LUT also reduces UVB-induced release of PGE_2 and IL1-α in keratinocytes, indicating a protection against the UVB-induced sunburn response.

Previous work indicated that ROS-mediated cascades are required to engage the mitochondrial pathways of UVB-induced apoptosis in NHKs (Van Laethem *et al.*, 2006). Although the structure of the flavonoid 3,′4′,5,7,-tetrahydroxyflavone or LUT is extremely suitable for antioxidant effects, these effects are concentration and cell-type dependent. We expected LUT, as a good antioxidant, to inhibit apoptosis mainly through inhibition of the mitochondrial pathway. Indeed, after UVB irradiation, we could show a LUT-dependent inhibition of different characteristics of the mitochondrial apoptosis pathway, including ROS production, loss of $\Delta\psi_m$, and subsequent release of cytochrome *c* from the mitochondria into the cytosol. Besides the direct ROS scavenging effect, LUT also increased the level of Bcl2 protein in NHKs. In contrast to control cells, these high Bcl2 levels were maintained after UVB irradiation, thereby changing the Bcl2/active Bax balance in favor of the antiapoptotic Bcl2 member. A similar effect has been observed in keratinocytes treated with epigallocathecin-3-gallate (Chung *et al.*, 2003).

The apoptotic response of normal skin cells can be observed as a final escape mechanism to cope with damaged DNA. Namely, unrepaired photodimers (primarily CPDs), because of excessive UVB irradiation, lead to programmed cell death predominantly under regulation of p53 (Verschooten *et al.*, 2006b). Therefore, it remains to be established whether the prosurvival effect of LUT through suppression of UVB-induced mitochondrial apoptosis is beneficial for the cell in the long term. Although LUT delays the induction of cell death, allowing the cells more time for specific repair of photodimers through nucleotide excision repair, we could not detect a significant reduction of CPDs because of LUT treatment in UVB-irradiated NHKs. Thus, we cannot provide evidence that the cells surviving UVB irradiation at late time points are entirely damage free. Therefore, we recommend the use of DNA-repair stimulating agents or

enzymes in the case that LUT might be used for photoprotective application.

However, LUT does not completely inhibit apoptotic signaling, but rather delays cell death by apoptosis. Hence, apoptotic cell death is still possible at later time points for cells in which damage is beyond repair. Moreover, as LUT also induced growth inhibition and differentiation, indicated by the induction of the late differentiation marker, involucrin, eventually surviving cells with damaged DNA might not be able to give rise to tumor formation. Further investigation of the UV-induced DNA damage in LUT-treated skin equivalents or organ cultures might reveal whether differentiation and consequently the thickening of the *Stratum Corneum* decreases damage in the proliferating basal layer of the epidermis.

Excessive exposure of skin to solar irradiation leads to a sunburn reaction, which is mediated by proinflammatory cytokines (e.g., IL-1α) and vasoactive mediators (e.g., PGE_2), secreted at least partially by epidermal keratinocytes (Grone, 2002). Our results, obtained with specific ELISAs for the main proinflammatory and vasoactive mediators, namely IL-1α and PGE_2, showed a strong inhibition by LUT treatment of the UVB-induced release in the supernatant of cultured NHKs. The anti-inflammatory effects of LUT can be explained as an indirect consequence of its ROS scavenging capacity. Although the inducible COX-2 enzyme is responsible for PGE_2 synthesis upon UVB irradiation, COX-2 protein level was not decreased in LUT-treated keratinocytes after UVB (Supplementary Figure S3a online). This indicates that LUT is able to block COX-2 activity (PGE_2 release), whereas it does not affect COX-2 protein level, consistent with a previous structural–activity relationship analysis of LUT (Rosenkranz and Thampatty, 2003). The expression of IL-1α and other proinflammatory cytokines, chemokines, and enzymes is primarily regulated through NF-κB and activator protein-1. The study of the anti-inflammatory capacity of LUT after LPS treatment in different cell types *in vivo* and *in vitro* revealed LUT-dependent inhibition of both pathways (Gutierrez-Venegas *et al.*, 2006). PGE_2 release specifically, and also a proinflammatory microenvironment in general, are not only related to the sunburn response, but are also believed to contribute to epithelial tumor formation and progression. Hence, anti-inflammatory drugs are of great interest in the prevention and treatment of epithelial skin cancers (Mueller, 2006).

The differential effect of LUT on UVB response in normal versus malignant keratinocytes suggests that the effect of LUT on the survival of cells after UVB irradiation depends on the malignancy of the cells themselves. Malignant cells often contain a higher background of ROS because of their upregulated metabolism (Ralph *et al.*, 2010). In hepatoma cells, e.g., the anticancer activity of LUT is mediated through a further increase of intracellular ROS, which ultimately leads to cell death (Yoo *et al.*, 2009). Important pathways, especially those related to survival and apoptosis, are deregulated in cancer cells (Claerhout *et al.*, 2006). The SCC cell lines used in this study show abnormalities in the PI3K/AKT pathway (Claerhout *et al.*, 2010) that might influence the effects of LUT treatment (Lin *et al.*, 2008). Finally, as suggested above, induction of differentiation might increase the resistance of normal human keratinocytes to UVB (Hsu *et al.*, 2005). Cancer cells, by definition, lose the capacity to differentiate, which may also partially explain the opposite effect of LUT on normal versus malignant cells.

All together, LUT might have potential value in photoprotective applications, as it inhibits different components of the sunburn response, leading to selective increased survival of NHKs, without affecting the sensitivity of malignant keratinocytes to UVB. Further *in vivo* investigation is required to confirm the photoprotective effect of LUT in human skin.

MATERIALS AND METHODS

Reagents and antibodies

Luteolin (Sigma, St Louis, MO) was dissolved in DMSO and kept protected from light. We purchased the PARP, the Bcl2, the COX-2, and the cytochrome *c* antibody from BD Biosciences (Erembodegem, Belgium), the involucrin antibody from Santa Cruz (Heidelberg, Germany), and the BaxNT antibody from Upstate Biotechnology (Lake Placid, NY). Antibodies against the cleaved form of caspase 3 and caspase 9 and the secondary horseradish peroxidase-labeled goat anti-mouse and goat anti-rabbit antibodies were obtained from Cell Signaling Technology (Beverly, MA). The primary antibody against actin (JLA20) was purchased from Developmental Studies Hybridoma Bank at the University of Iowa. Alexa Fluor goat anti-rabbit 488, MitoTrackerRed CMXRos, Hoechst 33342, $DiOC_6$, and H_2DCFDA were purchased from Molecular Probes (Eugene, OR).

Cell culture and UVB irradiation

Primary human keratinocytes were isolated from foreskins of donors (<5 years), as previously described (Gilchrest, 1983; Claerhout *et al.*, 2007), pooled to circumvent interindividual differences, and grown in specific keratinocyte serum-free medium (Gibco-Invitrogen, Merelbeke, Belgium), supplemented with bovine pituitary extract (50 μg ml^{-1}) and human recombinant epidermal growth factor (5 ng ml^{-1}). The procedure has been approved by the ethical committee of the University of Leuven. MET1 and MET4 keratinocyte cell lines, respectively, derived from a primary epidermal tumor and from metastatic SCC within left axillary lymph nodes from the same patient (Proby *et al.*, 2000), were grown in DMEM plus HAMS F12 (Invitrogen) medium containing 10% fetal calf serum, hydrocortisone, mouse epidermal growth factor, and antibiotics. A253 and A431 cells, derived from a head and neck, and a vulvar SCC, respectively, were grown in DMEM enriched with 10% fetal calf serum and antibiotics. All cells were grown in incubator at 37 °C in 5% CO_2.

UVB irradiation has been described in previous studies (Claerhout *et al.*, 2007). In brief, cells were irradiated at subconfluence (70–80%) with Philips (Anderlecht, Belgium) TL20W12 tubes through a thin layer of phosphate-buffered saline and through the lid of the dishes. Output was measured using an IL700 radiometer (with an SED240/UVB-1 filter) (International light, Newburyport, MA).

Viability assays

Metabolic activity was assessed using MTT (Sigma). After 1 hour of incubation with MTT (1 mg ml^{-1}), the formation of the formazan dissolved in DMSO was measured spectrophotometrically.

In addition, cell viability was assessed using the Trypan blue exclusion assay, analyzed using a Vi-cell XR cell viability analyzer (Beckman Coulter, Fullerton, CA) or Countess Cell Counter (Invitrogen).

Western blot analysis

At indicated time points after treatment, cell lysates were made as reported in previous studies (Claerhout *et al.*, 2007). After determination of the protein concentration using the BCA Protein Assay Reagent (Pierce Chemical Company, Rockford, IL) according to the manufacturer's protocol, samples containing equal amounts of protein (20–50 μg) were prepared and processed on the NuPAGE Novex system (4–12% Bis-Tris; Invitrogen) and transferred to Hybond-C Super membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Protein bands were visualized using enhanced chemiluminescence as described by the supplier (Amersham Pharmacia Biotech).

Hoechst staining

Nuclear changes were visualized by staining the cells for 10 minutes with the Hoechst 33342 dye (3 μM in phosphate-buffered saline), followed by examination under a fluorescence microscope.

Detection of DNA fragmentation

Cells were trypsinized, fixed in ethanol, and stained with propidium iodide. SubG1 fraction was analyzed using FACS Calibur (BD Biosciences) (Van Laethem *et al.*, 2006).

Immunodetection of CPDs

Genomic DNA of trypsinized cells was purified using a genomic DNA extraction kit (Qiagen, Crawley, UK). Polyvinylchloride 96-well flat-bottomed multiwell plates, precoated with 0.003% protamine sulfate, were coated with equal amounts (200 ng) of sample DNA. Using specific anti-CPD antibody and the tetramethylbenzidine ELISA detection kit (Bio-Rad, Hercules, CA), CPDs were quantitated spectrometrically.

Analysis of mitochondrial outer membrane permeabilization and ROS production

Cells were incubated with 3,3′-dihexyloxacarboxyanine iodide ($DiOC_6$; 30 nM) or 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; 10 μM) at 37 °C for 30 minutes, harvested by trypsinization, and kept at 4 °C. $\Delta\psi_m$ or ROS production was analyzed using FACS Calibur (Van Laethem *et al.*, 2006).

Analysis of cytochrome *c* release

The cells were prepared for the immunofluorescence analysis as described before (Van Laethem *et al.*, 2006), using MitoTrackerRed CMXRos (50 nm) and secondary fluorescent Alexa 488 anti-mouse antibody (Molecular Probes). After mounting with 4,6-diamidino-2-phenylindole vectashield mounting medium, analysis was performed with an Olympus (Aartselaar, Belgium) cell imaging station. Cellular fractionation was performed using a digitonin buffer (0.01% digitonine and phosphatase inhibitors) and verified using anti-cytochroomoxigenase antibody. Both fractions were processed for western blot as described above.

Measurement of caspase activity

Caspase 9 Glo assay (Promega, Madison, WI) was performed according to the manufacturer's protocol. Readout was performed using a VICTOR^3 Multilabel counter and Wallac software (Perkin Elmer, Waltham, MA) 60 minutes after addition of the substrate.

Measurement of PGE_2 and IL-1α release by ELISA

The concentrations of PGE_2 and IL-1α in the culture medium were measured using the IL-1α/IL-1F1 (KGE004) and PGE2 ELISA assay kit (DY200) (R&D Systems, Abingdon, UK) according to the manufacturer's instructions.

Statistical analysis

The data were expressed as means ± SD. Statistical analysis was performed using Student's *t*-test (two tailed). The criterion for statistical significance was taken as $P<0.05$ unless stated otherwise.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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