DOI:10.1111/j.1600-0625.2008.00819.x
www.blackwellpublishing.com/EXD

Original Article

Endogenous survivin modulates survival and proliferation in UVB-treated human keratinocytes

Katiuscia Dallaglio[1]*, Elisabetta Palazzo[1]*, Alessandra Marconi[1], Marc Dumas[2], Francesca Truzzi[1], Roberta Lotti[1], Frederic Bontè[2] and Carlo Pincelli[1]

[1]Institute of Dermatology, School of Biosciences and Biotechnologies, University of Modena and Reggio Emilia, Modena, Italy;
[2]LVMH Recherche, St. Jean de Braye, France
Correspondence: Carlo Pincelli, Institute of Dermatology, University of Modena and Reggio Emilia, Via del Pozzo 71, 41100 Modena, Italy, Tel.: +39 (059) 4222931, Fax: +39 (059) 4224271, e-mail: carlo.pincelli@unimore.it
**These authors contributed equally to this work.*

Accepted for publication 27 October 2008

Abstract: Survivin is a bi-functional member of inhibitor of apoptosis protein family, as it is able to both inhibit apoptosis and to regulate cell cycle. We investigated the role of survivin in human keratinocytes under normal conditions and during UVB irradiation. Survivin siRNA decreases proliferation and induces apoptosis in human keratinocytes, in a mode consistent with the mitotic catastrophe. Low doses UVB increase survivin expression at earlier times, while high doses down-regulate survivin level. Low doses UVB induce cell cycle arrest in G2/M, while high doses UVB cause apoptosis. Moreover, overexpression of survivin protects keratinocytes from UVB-induced apoptosis, and silencing of survivin renders keratinocytes more susceptible to UVB-induced cell death. Finally, survivin siRNA increases UVB-induced reduction of cell proliferation. Taken together, these results indicate that survivin plays a critical role in epidermal homeostasis in normal conditions and during UVB exposure, with possible implication in skin carcinogenesis.

Key words: apoptosis – G2/M arrest – keratinocytes – survivin – UVB

Please cite this paper as: Endogenous survivin modulates survival and proliferation in UVB-treated human keratinocytes. Experimental Dermatology 2009; 18: 464–471.

Introduction

Human epidermis is a highly regenerative tissue characterized by a fine balance between keratinocyte proliferation and apoptosis, which is responsible for epidermal homeostasis (1). Keratinocytes are the most numerous cells of the epidermis and are constantly exposed to external insults. UV radiation constitutes the most harmful agent, inducing DNA damage in human keratinocytes that respond by arresting the cell cycle and by activating the nucleotide excision repair programme. When this process fails, cells can be either eliminated via apoptosis or acquire mutations leading to the development of skin cancer (2,3).

Survivin belongs to the inhibitor of apoptosis proteins (IAP) family, which also comprises c-IAP1, c-IAP2, XIAP, NAIP, apollon, ML-IAP/livin and ILP-2 (4). While IAP members are structurally similar, survivin has unique structure and properties. It lacks a zing finger domain, but it contains a BIR (Baculovirus IAP Repeat) domain, followed by a long α-helical region important for its targeting to the mitotic structures (5). Survivin exerts a dual function by both inhibiting cell death and regulating cell cycle (6). Survivin acts either alone or in complex with XIAP to inhibit caspase-9, thus protecting cells from apoptosis (7). Survivin property of modulating cell cycle is still partially understood. It appears that survivin maintains the correct asset of cells during their mitotic division. Indeed, survivin inhibition leads to multinucleated cell formation and cell cycle arrest (8). It has recently been shown that the chromosomal passenger complex proteins Aurora-B kinase, Borealin, INCENP and survivin form a conserved complex and regulate mitotic events (9).

A large body of literature supports the concept that survivin is only expressed in cancer and foetal tissues while it is almost absent in differentiated adult tissues (10). By contrast, survivin is expressed in normal human epidermis, and in particular in a subpopulation of basal keratinocytes (11). More specifically, survivin is almost exclusively expressed in keratinocyte stem cells (KSC), whereas it is only slightly present in transit amplifying (TA) cells. Pro-apoptotic survivin isoforms are predominantly expressed in TA cells as opposed to anti-apoptotic variants that are detected only in KSC. Wild-type anti-apoptotic survivin is down-regulated, whereas pro-apoptotic survivin 2alpha is up-regulated during anoikis in human keratinocytes. Finally, survivin is mainly expressed at the nuclear level in KSC, which proliferate significantly better than TA cells, which, in turn, express mostly cytosolic survivin (12). In substance, this background

points to an important role of survivin in epidermal homeostasis. To further study the functions of survivin in human epidermis, we have silenced survivin mRNA and evaluated the role of this protein with regard to apoptosis and cell cycle under normal conditions or after UVB irradiation.

We present evidence that UVB modulate survivin expression in keratinocytes. Moreover, UVB cause cell cycle arrest or apoptosis, depending on the dose. Silencing survivin reduces proliferation and induces apoptosis in keratinocytes. In addition, inhibiting survivin renders keratinocytes more prone to UVB-induced apoptosis.

Methods

Cell culture

The spontaneously transformed keratinocyte line HaCaT was kindly provided by Dr N. Fusenig (DKFZ Heidelberg) and cultured in Dulbecco's modified Eagle's medium (DMEM; Biochrom AG) plus 10% foetal calf serum (FCS) as described (13).

Synchronization procedure

HaCaT cells were synchronized by cultivation at high density in the absence of serum. After 1 day from cell confluence, the medium was replaced by serum-free medium for 5 days. The synchronized cells were trypsinized and were then seeded into culture plate in DMEM plus 10% FCS for the experiments.

siRNA transfection of HaCaT cells

About 100 000 cells/well were plated on six-well plates in penicillin/streptomycin free medium. Twenty-four hours later, cells were transfected with 25 nM survivin-siRNA (Dharmacon Inc, Lafayette, CO, USA) or water as mock control, combined with Lipofectamine 2000 and Opti-MEM (both from Invitrogen Corporation, Carlsbad, CA) as datasheet suggests. Cells were transfected twice and used for MTT assay, ^{3}H-thymidine incorporation, flow cytometry and Western blotting. Transfection was controlled by Western blotting using anti-survivin antibody at 24, 48 up to 120 h, as described below.

Infection of HaCaT cells

A total of $16 \times 10^3/cm^2$ HaCaT cells were plated and 24 h later, infected twice with conditioned medium containing retrovirus for EGFP (Enhanced Green Fluorescent Protein) or survivin–EGFP (codified by pcz-CFG5.1–EGFP or pcz-CFG5.1-Survivin-EGFP retroviral vector a kind gift from Achim Temme, Technische Universitat Dresden, Germany) and polybrene to a final concentration of 0.8 μg/ml. Then, culture medium was changed, cells were trypsinized and the number of EGFP-positive cells was controlled by flow cytometry. Only if the infection efficiency was at least 80%, HaCaT cells were used for the experiments. Then cells were UVB irradiated (25 mJ/cm^2). Survivin overexpression was controlled 24 h after irradiation by Western blotting, as described below.

UVB irradiation

UVB radiation (290–320 nm) was delivered with a battery of four lamps (TL 20 W/12 RS UV-B Philips Medical). For UVB irradiation, the dishes or 96-well tissue culture plates were placed without covers under lamps. Before irradiation, cells were washed once with phosphate-buffered saline (PBS) and irradiated in the presence of PBS. HaCaT cells were sham or UVB irradiated at subconfluence and analysed 8, 12, 18 and 24 h later. We selected UVB doses of 5, 50 and 75 mJ/cm^2 and times of exposure were 1 min 12 s, 5 min 22 s, 12 min respectively, as measured with an UV-Radiometer (Goldilux smart; Oriel Instruments, Stratford, CT, USA).

Western blotting analysis

HaCaT cells were washed twice with PBS and lysed on ice in lysis buffer pH 7.4 (10 mM Tris, 1% sodium deoxycholate, 1 mM Na_3VO_4) containing protease inhibitors, as described previously (14). Twenty micrograms of total protein was analysed under reducing conditions for survivin, cyclin B, caspase-8 and caspase-3 on 18%, 10% and 12% polyacrylamide gels, respectively, and blotted onto nitrocellulose membranes. To verify equal loading of total proteins in all lanes, the membranes were stained with Ponceau Red. The blots were blocked for 2 h in TBS blocking buffer (pH 7.6 with 0.1% Tween-20 and 5% non-fat dry milk) for caspase-3 and PBS blocking buffer (pH 7.4 with 0.05% Tween-20 and 5% non-fat dry milk) for survivin, cyclin B and caspase-8. The membranes were incubated overnight at 4°C with anti-survivin rabbit polyclonal (1:1000; Abcam, Cambridge, UK), anti-cyclin B mouse monoclonal (1:1000; BD Biosciences Pharmingen, San Diego, CA, USA), anti-caspase-8 mouse monoclonal (1:100; Calbiochem, Merck KgaA, Darmstadt, Germany), anti-caspase-3 rabbit polyclonal (1:1000; Cell Signaling Technology, Danvers, MA, USA), or with anti-β-actin mouse monoclonal antibody (1:5000; Sigma, St Louis, MO, USA) as a control. Then, membranes were washed in TBS/Tween or PBS/Tween, incubated with peroxidase-conjugated goat anti-rabbit antibody or anti-mouse antibody (1:3000; Biorad, Hercules, CA, USA) for 45 min at room temperature, washed, and developed using the ECL chemiluminescent detection system (Amersham Biosciences UK Limited, Little Chalfont Buckinghamshire, UK).

MTT assay

HaCaT cells were plated in 96-well tissue culture plate (4000 cells/well). MTT assay was performed 24, 48 and 120 h after siRNA transfection. For UVB experiments, HaCaT cells were irradiated with 5, 50 and 75 mJ/cm^2 or

HaCaT cells were siRNA transfected and then irradiated with 5 and 50 mJ/cm^2 or HaCaT cells were survivin infected and then irradiated with 25 mJ/cm^2 24 h later. MTT assay was performed 24 h after UVB irradiation. Proliferative cells were detected by incubating with MTT (Sigma-Aldrich) solution at 37°C for 4 h. They were solubilized with DMSO and the formazan dye formation was evaluated by scanning multiwell spectrophotometer at 540 nm. The results are expressed as optical density units or as viability percentage with respect to control. Results are calculated as the mean ± SD of three different experiments. Student's *t*-test was used for comparison of the means.

Cell proliferation assay

HaCaT cells were plated in 96-well tissue culture plate (4000 cells/well). Proliferation assay was performed 24, 48 and 120 h after siRNA transfection. For UVB experiments, HaCaT cells were irradiated with 5, 50 and 75 mJ/cm^2 or HaCaT cells were transfected and then irradiated with 5 and 50 mJ/cm^2 24 h later. Proliferation was performed 24 h after UVB irradiation. ^{3}H-thymidine (1 μCi/well, GE Healthcare, Buckinghamshire, UK) incorporation was performed 12 h before harvesting of the cells and cells were collected at 12, 24, 48 and 120 h after treatment. The incorporated radioactivity was determined by β-counter. Results are calculated as the mean ± SD of three different experiments. Student's *t*-test was used for comparison of the means.

Flow cytometry

After siRNA transfection and UVB irradiation, cells were trypsinized at different time-points and resuspended in hypotonic fluorochrome solution (propidium iodide 10 mg/ml, 0.1% sodium citrate and 0.5% Triton X-100; Sigma) for 15 min. After incubation, cells were analysed using Epics XL flow cytometer (Coulter Electronics Inc, Hialeah, FL, USA). This univariate analysis of PI staining cellular DNA content and deconvolution of the cellular DNA content frequency histograms reveals distribution of cells in three major phases of the cycle (G1 vs S vs G2/M) and makes it possible to detect apoptotic cells with fractional DNA content (SubG1 peak). Results are calculated as the mean ± SD of three different experiments. Student's *t*-test was used for comparison of the means.

Results

Different UVB doses modulate survivin levels, cell cycle and apoptosis in keratinocytes

A correlation between UVB and survivin in the epidermis has been already hypothesized (15). Moreover, survivin exerts its anti-apoptotic function within the intrinsic (mitochondrial) apoptotic pathway that is specifically triggered by UV radiation (7,16). Therefore, we wanted to analyse the effect of UVB on the expression of survivin in human keratinocytes. First, sham irradiated cells show a strong survivin expression, further confirming that this IAP protein is definitely present in adult human keratinocytes (12). At 12 h, 5 mJ/cm^2 UVB induced a strong up-regulation of survivin expression, which tends to decrease at 24 h, going back to control levels. On the other hand, 50 and 75 mJ/cm^2 UVB caused a marked down-regulation of survivin expression both at 12 and 24 h (Fig. 1a).

With this information, we evaluated the effect of the same UVB doses at the same time on keratinocyte proliferation and apoptosis. While at 8 h, all UVB did not affect the cell cycle in synchronized cells (Fig. 1b), at 12 h, 5 mJ/cm^2 UVB determined an increase of cells in G2/M, consistent with the highest expression of survivin that has been shown previously to be up-regulated in G2/M (16). On the other hand, higher doses UVB (50–75 mJ/cm^2) did not affect the number of cells in G2/M, but started to cause cells to undergo apoptosis (subG1 cells) (Fig. 1c). At 18 h, there was a slight increase in G2/M cells upon 5 mJ/cm^2 UVB treatment, and a further decrease in G2/M cells after treatment with higher UVB doses (Fig. 1d). At 24 h, when survivin expression was decreased to the sham level of control, low UVB doses were not sufficient to induce cell cycle arrest in G2/M, and the number of cells in subG1 started to slightly increase. At this time, 50 and 75 mJ/cm^2 UVB, that caused reduction of survivin levels, also induced a marked increase in the number of cells in subG1 (Fig. 1e), suggesting that low amounts of survivin are associated with an higher rate of apoptosis. At 12 h, using 5 mJ/cm^2 UVB, the increase in the number of cells in G2/M is paralleled by the augmented expression of cyclin B (Fig. 1f) (17), suggesting that high levels of survivin are responsible for cell cycle arrest in G2/M. ^{3}H-thymidine incorporation at 12 and 24 h showed that while low-dose UVB slightly decreased keratinocyte proliferation, higher UVB doses completely blocked this function (Fig. 1g). Similarly, an MTT assay demonstrated that cell viability decreased only to some extent in cells treated with 5 mJ/cm^2 UVB, whereas the number of viable keratinocytes almost disappeared with higher doses UVB at 24 h (Fig. 1h). Finally, coherently with the flow cytometry results, only higher doses UVB were capable of activating both caspase-8 and caspase-3 at 24 h (Fig. 1i). Taken together, these results indicate that low dose UVB augment survivin expression and are responsible for the arrest in G2/M. When cells are treated with higher doses, keratinocytes lose survivin and undergo apoptosis.

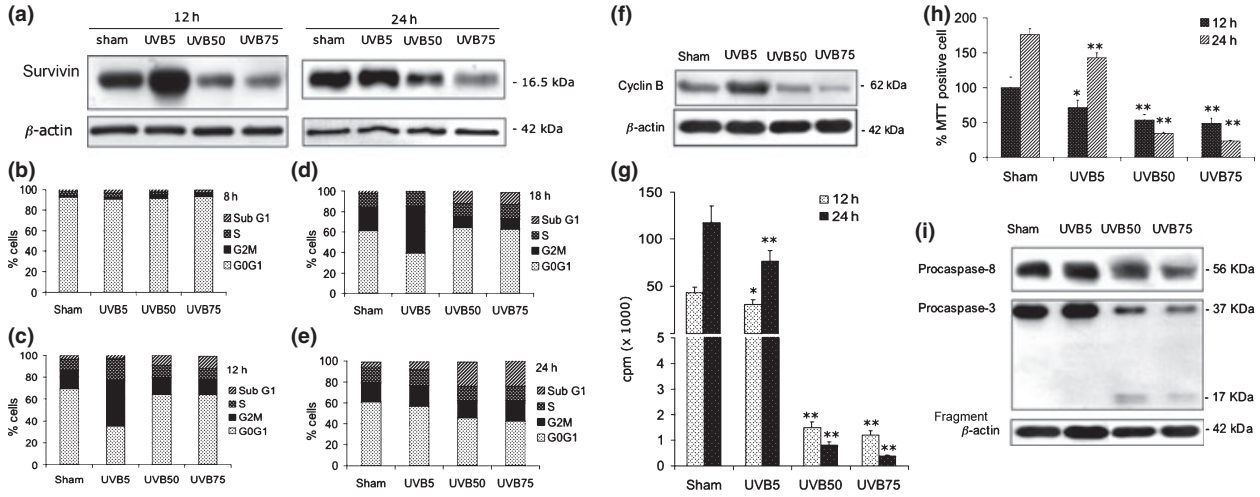

Figure 1. Low doses UVB induce cell cycle arrest while high doses UVB induce apoptosis in keratinocytes. (a) HaCaT cells were sham or UVB (5, 50 and 75 mJ/cm^2) irradiated. 12 and 24 h after irradiation, protein extracts from HaCaT cells were separated on 18% polyacrylamide gel and transferred onto nitrocellulose membrane. Membranes were immunoblotted with anti-survivin antibody. β-actin was used as internal control. (b, c, d, e) HaCaT cells were synchronized by cultivation in serum-free DMEM, as described in Methods. 36 h after the end of synchronization, cells were UVB (5, 50, 75 mJ/cm^2) or sham irradiated. HaCaT cells were trypsinized and stained with 10 mg/ml PI 8, 12, 18 and 24 h later. Cell cycle was analysed by flow cytometry, and one representative experiment is shown. (f) 12 h after sham or UVB irradiation, protein extracts from synchronized cells were separated on 10% polyacrylamide gel and transferred onto nitrocellulose membrane. Membrane was immunoblotted with anti-cyclin B antibody. β-actin was used as internal control. (g) HaCaT cells were seeded in a 96-well tissue culture plate and sham or UVB (5, 50 and 75 mJ/cm^2) irradiated. ^{3}H-thymidine incorporation was determined 12 and 24 h later. (h) HaCaT cells were seeded in a 96-well tissue culture plate and sham or UVB (5, 50 and 75 mJ/cm^2) irradiated. MTT assay was performed 12 and 24 h later. (i) HaCaT cells were sham or UVB (5, 50 and 75 mJ/cm^2) irradiated. 24 h after irradiation, protein extracts were separated on 12% polyacrylamide gels and transferred onto nitrocellulose membrane. Membranes were immunoblotted with anti-procaspase-8 or anti-procaspase-3 antibodies. β-actin was used as internal control. $*P < 0.05$; $**P < 0.01$.

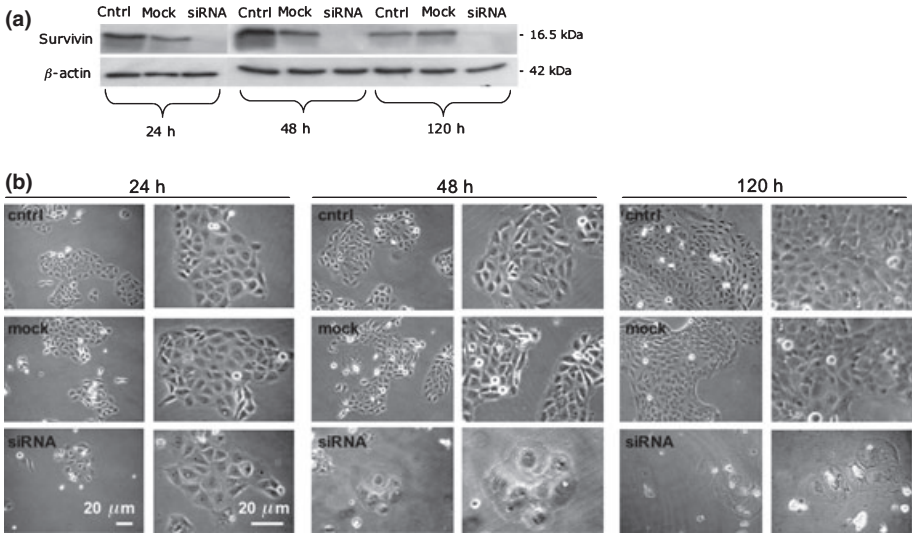

Figure 2. Silencing survivin reduces keratinocyte growth potential. (a) HaCaT cells were transiently transfected with 25 nM of survivin-siRNA. Transfection was controlled by Western blotting using anti-survivin antibody at 24, 48 up to 120 h. β-actin was used as internal control. (b) HaCaT cell cultures 24, 48 and 120 h after survivin-siRNA transfection. Scale bar: 20 μm.

Silencing survivin reduces keratinocyte growth potential

To clarify better the role of survivin in human epidermis, we first evaluated the effect of silencing survivin mRNA under normal conditions. Inhibiting survivin resulted in a complete loss of protein from 24 to 120 h (Fig. 2a). At 24 h, there were no major morphological differences between mock and siRNA transfected cells, while at 48 h,

survivin siRNA-treated keratinocytes lost their capacity of forming large colonies and presented many vacuoles. At 120 h, no colonies were visible, and the cells tend to detach from the plate and round up (Fig. 2b). These pictures clearly suggest that inhibiting survivin markedly affects keratinocyte colony formation.

Silencing survivin reduces proliferation and induces apoptosis in keratinocytes

We next investigated the functions of survivin with regard to keratinocyte proliferation and apoptosis under normal conditions. First, silencing survivin caused a significant and time-dependent decrease in keratinocyte proliferation from 24 to 120 h (Fig. 3a). Inhibition of survivin also induced a significant reduction in cell viability at 24 and 48 h. At 120 h, keratinocytes treated with survivin siRNA were markedly less viable than mock-transfected cells (Fig. 3b). The number of apoptotic cells (subG1) were significantly higher in survivin siRNA-treated cells than in mock cells at 48 and 120 h (Fig. 3c). Apoptosis induced by silencing of survivin was also confirmed by the activation of caspase-3 only in siRNA treated cells (Fig. 3d). In substance, these results indicate that survivin is critical for proliferation as well as a survival factor for human keratinocytes.

Silencing survivin makes keratinocytes more susceptible to UVB-induced damage.

Because UVB modulate the expression of survivin, that in turn influences keratinocyte proliferation and apoptosis, we tried to evaluate the role of survivin during UVB irradiation. We first looked at different phases of the cell cycle by flow cytometry. The addition of 5 mJ/cm^2 UVB to mock cells at 24 h did not change the cell cycle, as previously shown (Fig. 1e). When cells were treated with both survivin siRNA and 5 mJ/cm^2 UVB, cells in subG1 markedly increased ($P < 0.01$). Using 50 mJ/cm^2 UVB, the number of cells in subG1 was significantly higher in siRNA treated cells than in mock keratinocytes ($P < 0.05$) (Fig. 4a). Moreover, viability in 5 mJ/cm^2 UVB-treated keratinocytes was significantly lower in survivin siRNA-transfected cells than in controls. With 50 mJ/cm^2 UVB, the overall viability was markedly reduced, survivin siRNA-treated cells still showing a decreased rate of survival as compared with mock cells (Fig. 4b). Finally, the rate of proliferation upon treatment with 5 mJ/cm^2 UVB was significantly higher in mock than in siRNA-transfected keratinocytes (Fig. 4c). This seems to indicate that survivin influences UVB-induced keratinocyte damage.

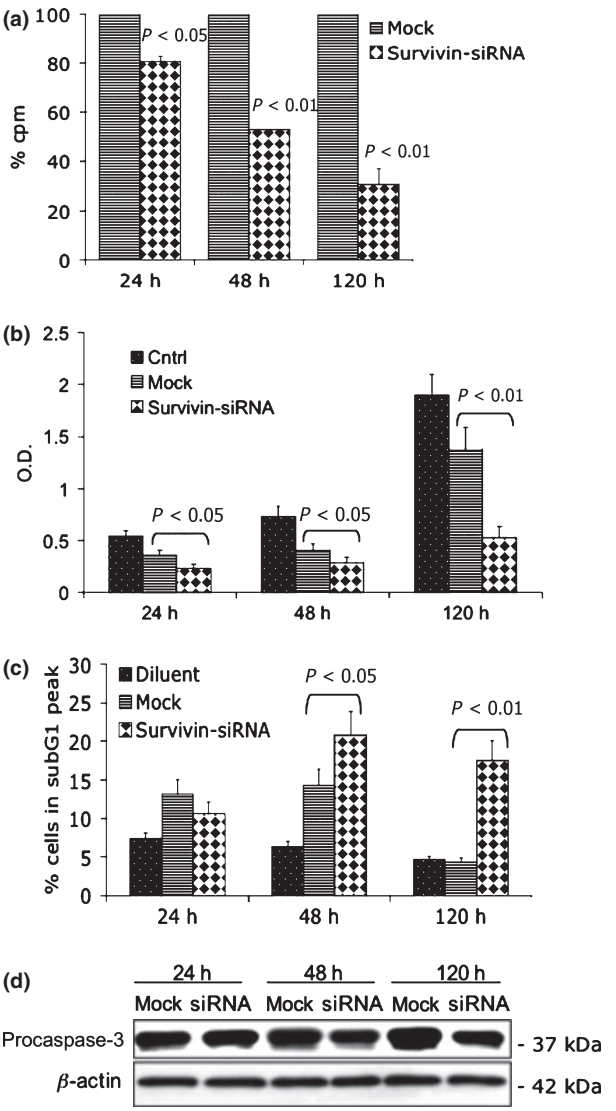

Figure 3. Silencing survivin reduces proliferation and induces apoptosis in keratinocytes. (a) Mock and transfected HaCaT cells were seeded in a 96-well tissue culture plate and cultured up to 120 h after transfection. ^{3}H-thymidine incorporation was determined at different time points. (b) HaCaT cells were seeded in a 96-well tissue culture plate and transiently transfected with 25 nM of survivin-siRNA. MTT assay was performed on control, mock and transfected cells 24, 48 and 120 h later. (c) HaCaT cells were transiently transfected with 25 nM of survivin-siRNA. After 24, 48 and 120 h control, mock and transfected cells were trypsinized and stained with 10 mg/ml PI. Sub G1peak was analysed by flow cytometry. (d) 24, 48 and 120 h after transfection, protein extracts from mock and transfected HaCaT cells were separated on 12% polyacrylamide gel and transferred onto nitrocellulose membrane. Membrane was immunoblotted with anti-procaspase-3 antibody. β-actin was used as internal control.

Survivin overexpression protects keratinocytes from UVB-induced apoptosis

Because survivin protects human keratinocytes from apoptosis and increases the susceptibility of these cells to UVB-induced apoptosis, we reasoned that survivin must protect keratinocytes from cell death. Indeed, infection of keratinocytes with a survivin retroviral vector resulted in cell resistance to UVB-induced protein down-regulation (Fig. 5a).

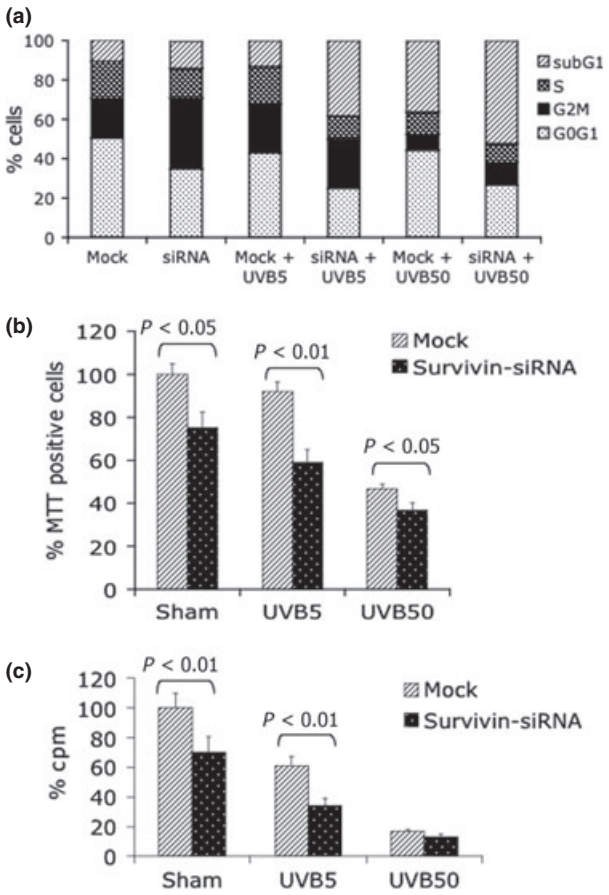

Figure 4. Silencing survivin makes keratinocytes more susceptible to UVB-induced damage. (a) HaCaT cells were transiently transfected with 25 nM of survivin-siRNA. 24 h after transfection, cells were UVB (5 and 50 mJ/cm^2) or sham irradiated. Then, mock and transfected cells were trypsinized and stained with 10 mg/ml PI 24 h later. Cell cycle was analysed by flow cytometry. One representative experiment is shown. (b) HaCaT cells were seeded in a 96-well tissue culture plate and transiently transfected with 25 nM of survivin-siRNA. 24 h after transfection, cells were sham or UVB (5 and 50 mJ/cm^2) irradiated. MTT assay was performed 24 h later. (c) Mock and transfected HaCaT cells were seeded in a 96-well tissue culture plate. 24 h after transfection, cells were sham or UVB (5 and 50 mJ/cm^2) irradiated. ^{3}H-thymidine incorporation was determined 24 h later.

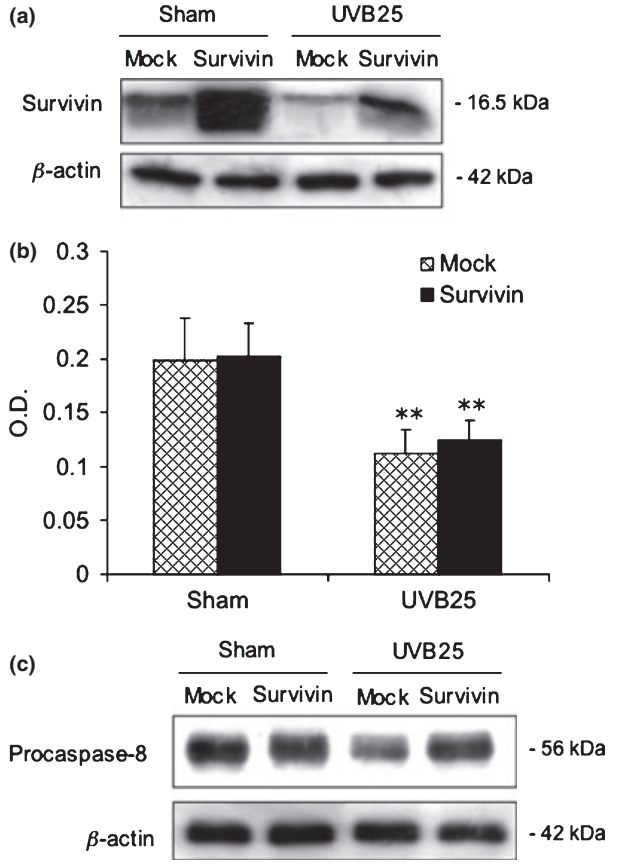

Figure 5. Survivin overexpression protects keratinocytes from UVB-induced apoptosis. (a) HaCaT cells were seeded on Petri dishes and infected with mock or survivin retroviral vector. Then, cells were sham or UVB (25 mJ/cm^2) irradiated. Infection was controlled by Western blotting using anti-survivin antibody at 24 h after irradiation. β-actin was used as internal control. (b) HaCaT cells were seeded in a 96-well tissue culture plate and infected with mock or survivin retroviral vector. Then, cells were sham or UVB (25 mJ/cm^2) irradiated. MTT assay was performed 24 h later. (c) HaCaT cells were seeded on Petri dishes and infected with mock or survivin retroviral vector. Then, cells were sham or UVB (25 mJ/cm^2) irradiated. 24 h after irradiation, protein extracts were separated on 12% polyacrylamide gel and transferred onto nitrocellulose membrane. Membranes were immunoblotted with anti-procaspase-8 antibody. β-actin was used as internal control. *$P < 0.05$; **$P < 0.01$.

Secondly, viability in mock cells was slightly lower than in keratinocytes overexpressing survivin (Fig. 5b). Finally, 25 mJ/cm^2 UVB can activate caspase-8 in mock, but not in keratinocytes overexpressing survivin, definitely indicating that survivin protects keratinocytes from UVB-induced apoptosis.

Discussion

The long-standing misinterpretation of survivin not being expressed in normal adult tissues, but only in tumors has lead most investigators to evaluate the prognostic and therapeutic value of this protein in cancer (18,19) and to overlook survivin function in normal tissue physiology. This holds particularly true for the skin where survivin has been studied mostly in melanoma and non-melanoma skin cancers (20,21). We had previously shown that the basal layer of normal human epidermis expresses survivin protein (11), which was then confirmed by others (22,23). More recently, we have demonstrated that survivin is expressed mostly in KSC (12). This study extends the observation that survivin is important for epidermal self-renewal. Indeed, silencing survivin reduces keratinocyte survival, greatly increases the apoptotic rate and mediates the

activation of caspase-3. The detailed molecular mechanisms by which survivin counteracts apoptosis are not fully understood. At least in cancer, where most of the studies have been performed, the mitochondrial survivin pool is released upon stress stimuli and binds to caspase-9, thus blocking tumor-cell apoptosis (7). This study does not fully address which apoptotic pathway leads to caspase-3 activation following inhibition of survivin, although caspase-8 is activated. Consistently, survivin is down-regulated during anoikis in human keratinocytes (12), while caspase-8 activated extrinsic apoptotic pathway is predominant in this type of cell death (14). On the other hand, survivin is also down-regulated during mitochondrial apoptosis in human keratinocytes (24), and this study clearly shows its involvement in the intrinsic pathway triggered by UVB. This seems to imply that survivin is involved in both apoptotic pathways in human keratinocytes, likely depending on the cell context and the apoptotic stimuli.

Survivin controls mitosis, cell division, cell cycle entry and proliferation (25,26). During these phases, cells could undergo apoptosis. Here, we show that silencing of survivin results in an increased number of keratinocytes in G2/M phase, in agreement with previous observations (6,27). Then, cells progressively exit G2/M phase and enter apoptosis, as shown by the increased number of cells in sub G1 peak. This intersection of mitotic and cytoprotective functions of survivin during cell cycle is widely accepted and is consistent with the mitotic catastrophe (28). In this respect, cell morphology observed in survivin siRNA-treated keratinocytes, characterized by multinucleated giant cells, is similar to the one observed during other examples of mitotic catastrophe induced by inhibition of survivin (29). While a critical issue is whether the underlying mechanism for these functions is quantitatively and/or qualitatively different in normal cells as compared with cancer (30), here, we present evidence that survivin plays a role in cell division in normal human epidermis. Overall, these findings indicate that survivin is critical in epidermal homeostasis. Interestingly, survivin is markedly overexpressed in psoriasis (22), a disease characterized by altered epidermal homeostasis, with keratinocyte hyperproliferation and reduced apoptosis.

Blocking beta1 integrin, a marker of KSC, markedly reduces wild-type survivin levels, while it up-regulates the proapoptotic isoform survivin-2alpha. Moreover, survivin is mostly expressed in the nucleous of highly proliferating KSC *in vitro*, while it is confined to the cytoplasm of TA cells that proliferate to a lesser degree (12). As beta1 integrin and survivin are markers of KSC and both protect them from apoptosis (12,31), it is likely that survivin, like beta1 integrin, is a crucial component of the KSC niche (32). This is consistent with the physiological role of survivin in maintaining normal adult haematopoiesis through regulation of haematopoietic stem cells (33).

As UVB induce both cell cycle arrest and apoptosis, we wanted to dissect the role of survivin during irradiation of human keratinocytes. It was previously shown that ectopic survivin expression counteracts keratinocyte apoptosis in mice (34). Here, we present evidence that constitutive survivin protects human keratinocytes from UVB-induced apoptosis. Indeed, UVB down-regulate survivin expression before caspase-3 activation and silencing survivin augments the rate of UVB-induced apoptosis. Moreover, overexpression of survivin renders keratinocytes more resistant to UVB-induced cell death. This indicates for the first time that survivin expressed in normal adult epidermis can counteract UVB-induced cell death, in agreement with the observation that survivin, likely derived from the mitochondrial pool (28) acts mostly through the 'intrinsic' apoptotic pathway.

Moreover, survivin, at variance with previous reports in mice (34), also interferes with UVB-induced cell cycle arrest. Our results seem to suggest that, as a first effect, survivin up-regulation intervenes in the UVB-induced cell cycle arrest in G2/M. This is also confirmed by the increased UVB-induced reduction of cell proliferation in survivin silenced cells. Subsequently, increasing UVB doses results in marked decrease in survivin levels and apoptosis. Previous work has shown a time-dependent modulation of survivin by UVB, with an initial increase of expression, in agreement with our results, although experiments were performed with only one high dose (35).

Finally, survivin drives clonal expansion of KSC upon UVB irradiation in a mouse model (36), thus favouring skin carcinogenesis. Indeed, upon UVB irradiation, p53-mutant keratinocyte stem cell clones are normally restrained within their stem cell compartment. Chronic UVB exposure drives clonal expansion, and this mechanism allows mutant stem cells to escape from their own stem cell compartment and colonize adjacent compartments (37). We hypothesize that survivin, expressed in KSC, cooperates to drive clonal expansion of mutated p53 and contributes to skin carcinogenesis.

References

1 Fuchs E, Horsley V. More than one way to skin. Genes Dev 2008: **22**: 976–985.
2 D'Errico M, Lemma T, Calcagnile A, Proietti De Santis L, Dogliotti E. Cell type and DNA damage specific response of human skin cells to environmental agents. Mutat Res 2007: **614**: 37–47.
3 Rünger T M, Vergilis I, Sarkar P, DePinho R A, Sharpless N E. How disruption of cell cycle regulating genes might predispose to sun-induced skin cancer. Cell Cycle 2005: **4**: 643–645.
4 Li F. Survivin study: what is the next wave? J Cell Physiol 2003: **197**: 8–29.
5 Altieri D C, Marchisio P C. Survivin apoptosis: an interloper between cell death and cell proliferation in cancer. Lab Invest 1999: **79**: 1327–1333.
6 Li F, Ambrosini G, Chu E Y *et al.* Control of apoptosis and mitotic spindle checkpoint by survivin. Nature 1998: **396**: 580–584.
7 Dohi T, Okada K, Xia F *et al.* An IAP-IAP complex inhibits apoptosis. J Biol Chem 2004: **279**: 34087–34090.
8 Kim K W, Mutter R W, Willey C D *et al.* Inhibition of survivin and aurora B kinase sensitizes mesothelioma cells by enhancing mitotic arrests. Int J Radiat Oncol Biol Phys 2007: **67**: 1519–1525.

9 Lens S M, Vader G, Medema R H. The case for survivin as mitotic regulator. Curr Opin Cell Biol 2006: **18**: 616–622.
10 Ambrosini G, Adida C, Altieri D C. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med 1997: **3**: 917–921.
11 Chiodino C, Cesinaro A M, Ottani D *et al.* Communication: expression of the novel inhibitor of apoptosis survivin in normal and neoplastic skin. J Invest Dermatol 1999: **113**: 415–418.
12 Marconi A, Dallaglio K, Lotti R *et al.* Survivin identifies keratinocyte stem cells and is downregulated by anti-beta1 integrin during anoikis. Stem Cells 2007: **25**: 149–155.
13 Boukamp P, Petrussevska R T, Breitkreutz D, Hornung J, Markham A, Fusenig N E. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 1988: **106**: 761–771.
14 Marconi A, Atzei P, Panza C *et al.* FLICE/caspase-8 activation triggers anoikis induced by beta1-integrin blockade in human keratinocytes. J Cell Sci 2004: **117**(Pt 24): 5815–5823.
15 Aziz M H, Afaq F, Ahmad N. Prevention of ultraviolet-B radiation damage by resveratrol in mouse skin is mediated via modulation in survivin. Photochem Photobiol 2005: **81**: 25–31.
16 Takasawa R, Nakamura H, Mori T, Tanuma S. Differential apoptotic pathways in human keratinocyte HaCaT cells exposed to UVB and UVC. Apoptosis 2005: **10**: 1121–1130.
17 Toyoshima F, Moriguchi T, Wada A, Fukuda M, Nishida E. Nuclear export of cyclin B1 and its possible role in the DNA damage-induced G2 checkpoint. EMBO J 1998: **17**: 2728–2735.
18 Engels K, Knauer S K, Metzler D *et al.* Dynamic intracellular survivin in oral squamous cell carcinoma: underlying molecular mechanism and potential as an early prognostic marker. J Pathol 2007: **211**: 532–540.
19 Konstantinidou A E, Korkolopoulou P, Patsouris E. Apoptotic markers for primary brain tumor prognosis. J Neurooncol 2005: **72**: 151–156.
20 Bowen A R, Hanks A N, Murphy K J, Florell S R, Grossman D. Proliferation, apoptosis, and survivin expression in keratinocytic neoplasms and hyperplasias. Am J Dermatopathol 2004: **26**: 177–181.
21 Piras F, Murtas D, Minerba L *et al.* Nuclear survivin is associated with disease recurrence and poor survival in patients with cutaneous malignant melanoma. Histopathology 2007: **50**: 835–842.
22 Abdou A G, Hanout H M. Evaluation of survivin and NF-kappaB in psoriasis, an immunohistochemical study. J Cutan Pathol 2008: **35**: 445–451.
23 Botchkareva N V, Kahn M, Ahluwalia G, Shander D. Survivin in the human hair follicle. J Invest Dermatol 2007: **127**: 479–482.
24 Chirico F, Fumelli C, Marconi A *et al.* Carboxyfullerenes localize within mitochondria and prevent the UVB-induced intrinsic apoptotic pathway. Exp Dermatol 2007: **16**: 429–436.
25 Knauer S K, Mann W, Stauber R H. Survivin's dual role: an export's view. Cell Cycle 2007: **6**: 518–521.
26 Giodini A, Kallio M J, Wall N R *et al.* Regulation of microtubule stability and mitotic progression by survivin. Cancer Res 2002: **62**: 2462–2467.
27 Zhonghong L, Lianjie L, Changqing Z, Ying H, Yu J, Yan L. The influence of survivin shRNA on the cell cycle and the invasion of SW480 cells of colorectal carcinoma. J Exp Clin Cancer Res 2008: **27**: 20.
28 Altieri D C. Survivin, cancer networks and pathway-directed drug discovery. Nat Rev Cancer 2008: **8**: 61–70.
29 Ngan C Y, Yamamoto H, Takagi A *et al.* Oxaliplatin induces mitotic catastrophe and apoptosis in esophageal cancer cells. Cancer Sci 2008: **99**: 129–139.
30 Li F, Brattain M G. Role of the survivin gene in pathophysiology. Am J Pathol 2006: **169**: 1–11.
31 Tiberio R, Marconi A, Fila C *et al.* Keratinocytes enriched for stem cells are protected from anoikis via an integrin signaling pathway in a Bcl-2 dependent manner. FEBS Lett 2002: **524**: 139–144.
32 Watt F M, Hogan B L. Out of Eden: stem cells and their niches. Science 2000: **287**: 1427–1430.
33 Fukuda S, Pelus L M. Regulation of the inhibitor-of-apoptosis family member survivin in normal cord blood and bone marrow CD34(+) cells by hematopoietic growth factors: implication of survivin expression in normal hematopoiesis. Blood 2001: **98**: 2091–2100.
34 Grossman D, Kim P J, Blanc-Brude O P *et al.* Transgenic expression of survivin in keratinocytes counteracts UVB-induced apoptosis and cooperates with loss of p53. J Clin Invest 2001: **108**: 991–999.
35 Aziz M H, Ghotra A S, Shukla Y, Ahmad N. Ultraviolet-B radiation causes an upregulation of survivin in human keratinocytes and mouse skin. Photochem Photobiol 2004: **80**: 602–608.
36 Zhang W, Hanks A N, Boucher K *et al.* UVB-induced apoptosis drives clonal expansion during skin tumor development. Carcinogenesis 2005: **26**: 249–257.
37 Brash D E, Zhang W, Grossman D, Takeuchi S. Colonization of adjacent stem cell compartments by mutant keratinocytes. Semin Cancer Biol 2005: **15**: 97–102.

