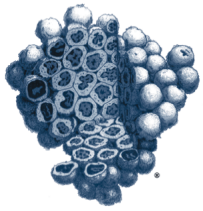

STEM CELLS®

Survivin Identifies Keratinocyte Stem Cells and Is Downregulated by Anti-ß1 Integrin During Anoikis

Alessandra Marconi, Katiuscia Dallaglio, Roberta Lotti, Cristina Vaschieri, Francesca Truzzi, Fabrizio Fantini and Carlo Pincelli

Stem Cells 2007;25;149-155; originally published online Sep 28, 2006;
DOI: 10.1634/stemcells.2006-0165

STEM CELLS®

TISSUE-SPECIFIC STEM CELLS

Survivin Identifies Keratinocyte Stem Cells and Is Downregulated by Anti-β1 Integrin During Anoikis

ALESSANDRA MARCONI, KATIUSCIA DALLAGLIO, ROBERTA LOTTI, CRISTINA VASCHIERI, FRANCESCA TRUZZI, FABRIZIO FANTINI, CARLO PINCELLI

Department of Medicine, Institute of Dermatology, University of Modena and Reggio Emilia, Modena, Italy

Key Words. Survivin • Stem cells • Anoikis • Keratinocytes

ABSTRACT

Survivin belongs to the family of inhibitor of apoptosis proteins and is involved in regulation of cell death as well as cell division. Here, we show that wild-type (WT) survivin is expressed in a subpopulation of basal keratinocytes in normal human skin at the cytoplasmic level. WT survivin is highly expressed in keratinocyte stem cells (KSCs), whereas its mRNA level decreases in transit amplifying (TA) cells and disappears in postmitotic (PM) cells. Likewise, WT survivin protein is expressed in KSCs, almost undetectable in TA cells, and absent in PM cells. Real time polymerase chain reaction demonstrates that the putative antiapoptotic isoforms survivin-2B and survivin-ΔEx3 are expressed at the highest levels in KSCs, whereas they tend to decrease in TA cells and disappear in PM cells. On the contrary, the putative proapoptotic variants of survivin, survivin-3B, and survivin-2α tend to be high in PM and TA cells and are almost absent in KSCs. By confocal microscopy, survivin is predominantly expressed at the nuclear level in KSCs, which proliferate significantly better than TA cells, which, in turn, express mostly cytosolic WT survivin. Blocking β1 integrin signal downregulates WT survivin mRNA and protein expression and induces apoptosis (anoikis) in KSCs. On the other hand, inhibition of β1 integrin upregulates mRNA expression of survivin-2α. Taken together, these results indicate that survivin identifies human KSCs. Expression of nuclear survivin could reflect the different behavior between KSCs in vitro and in vivo, in terms of proliferation. Finally, survivin could be part of the "niche" protection by preventing anoikis in KSCs. STEM CELLS *2007;25:149–155*

INTRODUCTION

The inhibitor of apoptosis proteins (IAPs) are a family of antiapoptotic molecules characterized by one to three BIR (Baculoviral IAP repeats) domains, a COOH-terminal RING finger domain, and a caspase recruitment domain. The IAP family includes X-linked inhibitor-of-apoptosis protein (XIAP), IAP-like protein (ILP2), cIAP1, cIAP2, melanoma inhibitor of apoptosis protein (MLIAP), neuronal apoptosis inhibitory protein (NAIP), Bruce, and Survivin [1]. Survivin is structurally unique among mammalian IAPs, containing only a single BIR domain and lacking the RING finger domain, replaced by an α-helix domain [2]. The survivin gene can produce five different survivin variants, wild-type (WT) survivin, survivin-2B, survivin-ΔEx3 [3], survivin-2α [4], and survivin-3B [5]. It is not completely known yet how these survivin isoforms are regulated and how they function in comparison with WT survivin [1]. Preliminary data seem to indicate that survivin-2B acts as an antagonist to WT survivin and survivin-ΔEx3 function [3]. On the other hand, survivin-3B appears not to be associated with cell division [5], whereas survivin-2α attenuates the antiapoptotic activity of WT survivin [4]. Survivin protects cells from apoptosis both in vitro [6] and in vivo [7] by inhibiting the mitochondrial caspase-9-activated intrinsic apoptotic pathway in association with XIAP [8, 9]. Moreover, unlike other members of the IAP family, survivin regulates cell division: it is expressed at G_2/M in a cell cycle-dependent manner and localizes to components of the mitotic apparatus [10]. The most significant feature of survivin is its strong expression in most human cancers [11]. Whereas fetal tissues contain abundant survivin, most terminally differentiated normal tissues do not [12]. On the other hand, it has been shown that the basal layer of normal human epidermis expresses survivin [13].

A population of keratinocyte stem cells (KSCs) governs the renewal of human epidermis. KSCs generate transient amplifying (TA) cells that divide only a small number of times. TA cells produce daughter cells that are irreversibly committed to terminal differentiation (postmitotic [PM] cells) [14]. KSCs are protected from cell death by a special microenviroment, called niche. In human epidermis, the niche is provided, among other factors, by high levels of β1 integrin, which allows the adhesion to extracellular matrix (ECM) [13], thus preventing differentiation or apoptosis. Alteration of integrin signal is responsible for a particular type of cell death, called anoikis, which is due to cell detachment from ECM [15]. We have shown recently that KSCs are protected from anoikis via an integrin-signaling pathway [16], in that disruption of β1 integrin activates the extrinsic apoptotic pathway [17]. Anoikis plays a crucial role in epithelial homeostasis [16, 18], whereas a defective anoikis allows the survival of squamous cell carcinoma [19, 20].

In this study, we present evidence that KSCs express the highest levels of survivin protein, which is barely detectable in TA cells and absent in PM cells. In particular, survivin is predominantly localized in the nucleus of KSCs and in the cytosol of TA cells. On the contrary, the proapoptotic variant survivin-2α is nearly absent in KSCs, whereas it increases in TA cells and is mostly expressed in PM cells. We also show that

Correspondence: Carlo Pincelli, M.D., Institute of Dermatology, University of Modena and Reggio Emilia, Via del Pozzo, 71, 41100 Modena, Italy. Telephone: +39 059 4222931; Fax: +39 059 4224271; e-mail: carlo@unimo.it Received March 21, 2006; accepted for publication September 20, 2006; first published online in STEM CELLS *EXPRESS* September 28, 2006. 1066-5099/2007/$20.00/0 doi: 10.1634/stemcells.2006-0165

WT survivin is downregulated, whereas the proapoptotic survivin-2α is upregulated during keratinocyte anoikis.

Materials and Methods

Keratinocyte Cultures

Normal human keratinocytes were obtained from foreskin and cultured as described [21]. Keratinocytes were divided into three populations and plated onto plastic dishes, coated for 2 hours at 37°C with type IV collagen (100 μg/ml; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com). They were first allowed to adhere to type IV collagen for 5 minutes, and the nonadherent cells were then transferred to fresh collagen-coated dishes and allowed to attach overnight. Finally, keratinocytes not yet attached after 1 night were plated onto type IV collagen to obtain a third population. The three keratinocyte populations were characterized based on β1 integrin levels and colony-forming efficiency (CFE), as previously described [16]. This allowed us to obtain a population enriched in KSCs, a population consisting of TA cells, and a third population of PM cells. Keratinocytes were then cultured in serum-free medium (KGM; Cambrex Bio Science, Walkersville, MD, http://www.cambrex.com) and used for further experiments.

Anoikis Assay

For blocking adhesion experiments, stem cells were trypsinized and suspended in polypropylene at 37°C with the addition of anti-β1 integrin-neutralizing antibody (1:250; Immunotech, Marseille, France, http://www.immunotech.cz) or in KBM alone for 0, 2, 4 and 6 hours.

Western Blot Analysis

Cells were washed with phosphate-buffered saline (PBS) and lysed on ice in RIPA buffer, pH 7.5, as described previously [21]. Twenty micrograms of total protein were analyzed under reducing conditions on 18% polyacrylamide gels 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 hours in blocking buffer (PBS buffer, pH 7.4, with 0.2% Tween 20 and 5% nonfat milk) and incubated overnight at 4°C with 1 μg/ml anti-survivin rabbit polyclonal antibody (Abcam, Cambridge, U.K., http://www.abcam.com), which recognizes both the cytoplasmic and nuclear pool of survivin [22], and with anti-β-actin mouse monoclonal antibody (1:5,000; Sigma-Aldrich) as a control. Then membranes were washed in PBS/Tween 20, incubated with peroxidase-conjugated goat anti-rabbit antibody and anti-mouse antibody (1:3,000; Bio-Rad, Hercules, CA, http://www.bio-rad.com) for 45 minutes at room temperature, washed, and developed using the ECL chemiluminescent detection system (Amersham Biosciences UK Limited, Little Chalfont Buckinghamshire, U.K., http://www.amersham.com).

Real Time Polymerase Chain Reaction

Total cellular RNA was extracted from the three populations using TRI Reagent method performed as described by Sigma-Aldrich. To verify the quality of the RNA, we ran an agarose minigel in RNase-free conditions. Quantitative real time polymerase chain reaction (PCR) was performed with an ABI 7500 (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com) for WT survivin, survivin-2B, survivin-3B, survivin-ΔEx3, and survivin-2α. As an internal control, housekeeping gene β-actin mRNA expression was measured in a separated tube. RNase-free water was used as a negative control. We performed three independent experiments, and each one was analyzed in a triplicate PCR. Then, 1 μl of RNA was subjected to retrotranscription and amplification in a 50-μl reaction mixture using the One-Step RT-PCR Master Mix Reagents kit (Applied Biosystems). To distinguish the five splice variants of survivin, the sequences of primers for WT survivin and survivin variants were designed to correspond to exon-exon borders of the complementary strand (Fig. 1). Real time probe sets were designed using the Primer Express program, version 2.0 (Applied

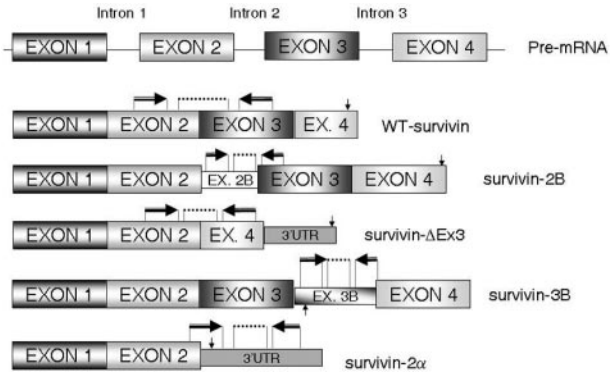

Figure 1. Real time polymerase chain reaction primer and probe position. Survivin pre-mRNA generates at least five mature mRNA transcripts. Exons are represented with boxes, and the black arrows indicate the stop codon. Primers and probes are designed to correspond to exon-exon borders to distinguish survivin variants. The double-line arrows represent primers; probes are represented by dotted lines. Abbreviations: ex., exon; UTR, untranslated region; WT, wild type.

Biosystems). The sequence used for primers and probes were designed as above for WT survivin (GenBank accession number BC065497; forward, 5′-CGAGGCTGGCTTCATCCA; probe, 5′-ACCCCATAGAGGAACATA; reverse, 5′-GCAACCGGACGAATGCTTT), survivin-2B (GenBank accession number NM_001012271; forward, 5′-CGGGCACGGTGGCTTA; probe, 5′-ACCAGCACTTTGGGAGG; reverse, 5′-CAACCGGACGAATGCTTTTT), survivin-3B (GenBank accession number AB154416; forward, 5′-GAAAAAATTGGAAGCCAGATTCAG; probe, 5′-CTGGAAGCAAAAGAA; reverse, 5′-ACAGACCCTGGCAAACATCAG), survivin-ΔEx3 (GenBank accession number NM_001012270; forward, 5′-GCTGGGAGCCAGATGACG; probe, 5′ -CCCCATGCAAAGGAAACCAACAATAAGAA; reverse, 5′-TCCGCAGTTTCCTCAAATTCTTT), and survivin-2α (forward, 5′-TGACGACCCCATGTAAGTCTTCT; probe, 5′-TGGCCAGCCTCGAT; reverse, 5′-TTGACAACTCAGTTCAAAACAAAGC). MGB probes were FAM dye-labeled. For β-actin, we used predeveloped TaqMan Assay Reagents (MGB probe was VIC dye-labeled; Applied Biosystems). Thermal cycling conditions for one-step reverse transcription-PCR were: initial reverse transcription at 48°C for 30 minutes, then DNA polymerase activation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute. Data from each sample were compared with stem cells or untreated control or WT survivin expression, as calibrators, using the Sequence Detection Software, version 1.2.3, according to the Relative Quantification Study method (Applied Biosystems). Results were obtained as the mean from three independent experiments. A double-sided Student's t test was performed between samples and calibrator.

Confocal Immunofluorescence

Stem and TA cells growing on collagen-coated coverslips for 96 hours were washed in PBS and fixed in situ in formaldehyde (3.6%) for 20 minutes, whereas PM cells were collected, fixed, and cytospun onto slides precoated with 0.01% poly(L-lysine) and air-dried at 48 hours. After a rehydration in PBS, cells were permeabilized by incubation for 10 minutes with 0.5% Triton X-100, for 15 minutes with 0.5% bovine serum albumin and 5% goat serum, and then for 60 minutes at 37°C with the rabbit polyclonal anti-survivin antibody (1 μg/ml) (Abcam), which recognizes both the cytoplasmic and nuclear pool of survivin [22], and with FBS buffer alone as a control. After a brief washing in PBS, the cells were incubated for 60 minutes with the secondary antibody anti-rabbit tetramethylrhodamine B isothiocyanate (DakoCytomation, Glostrup, Denmark, http://www.dako.com) at 1:50 dilution. Fluorescent specimens were analyzed by a confocal scanning laser microscope (Leica TCS SP2; Leica, Heerbrugg, Switzerland, http://www.leica.com).

Flow Cytometry

After 96 hours, cells were trypsinized and resuspended (1,000,000 cells) in 1 μl of hypotonic fluorochrome solution: 50 μg/ml pro-

pidium iodide, containing 0.1% sodium citrate, and 0.5% Triton X-100 (Sigma). After a 15-minute incubation, cells were analyzed using an Epics XL flow cytometer (Coulter Electronics Inc., Hialeah, FL, Beckman Coulter, Fullerton, CA, http://www.beckmancoulter.com). This univariate analysis of proliferation index (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 (G_1 vs. S vs. G_2/M) and makes it possible to detect apoptotic cells with fractional DNA content. The PI of cells was calculated by the following formula: PI (%) = ([S + G_2/M]/[G_0/G_1 + S + G_2/M]) × 100%.

Immunohistochemistry

Five-micron sections cut from formalin-fixed, paraffin-embedded normal skin were deparaffinized in xylene and rehydrated in graded alcohol. The slides were first boiled in citrate buffer, pH 6, for 20 minutes in a standard pressure cooker. Quenching of the endogenous peroxidase activity was obtained by treatment with 6% H_2O_2 in PBS. Samples were then incubated either with 10 μg/ml anti-survivin rabbit polyclonal antibody (antinuclear and anticytoplasmic survivin; Abcam) or with 200 μg/ml anti-survivin mouse monoclonal antibody (anti-nuclear survivin; Abcam) at 4°C overnight. After three 5-minute washes in PBS, slides were incubated with secondary antibody anti-rabbit biotinylated (DakoCytomation) at 1:300 dilution and anti-mouse biotinylated (DakoCytomation) at 1:200 dilution for 45 minutes at room temperature, followed by incubation with conjugated streptavidin-biotinylated-alkaline phosphatase complex for 30 minutes (BioSPA Division; Società Prodotti Antibiotici S.P.A., Milan, Italy, http://www.spaspa.it/biospa/home.htm) at 1:100 dilution. After three washes of 5 minutes in PBS, the slides were stained with 0.1% New Fuchsin (Sigma-Aldrich). Negative controls were obtained by omitting primary antibody.

RESULTS

Survivin Is Expressed in a Subpopulation of Normal Basal Keratinocytes

Contradictory results have been reported on the expression of survivin in normal human skin. A number of works indicate that normal human keratinocytes do not express survivin [23, 24], but some results are inconsistent with this observation [13, 25]. To clarify survivin expression in normal epidermis, we stained skin section with an antibody recognizing the nuclear as well as the cytoplasmic pool of survivin. Survivin expression was detected only in the basal layer of normal human epidermis. In particular, survivin appeared to be localized only in a subpopulation of basal keratinocytes. Within this population, keratinocytes expressed a cytoplasmic staining (Fig. 2A). No reactivity was observed when the antibody against the nuclear pool of survivin was used (Fig. 2B) or when the primary antibody was omitted (Fig. 2C).

Expression of Survivin in TA and PM Cells and KSCs

To determine the precise expression of survivin in human epidermis, we isolated three populations of basal keratinocytes, based on their adhesive capacity to type IV collagen, as previously described [16, 26]. We obtained KSCs characterized by highest levels of β1 integrin and by a high CFE. We also selected a population of TA cells with a significant decrease in β1 integrin levels and in CFE. Finally, we obtained a population of PM cells with undetectable β1 integrin and with no CFE [16].

We first investigated whether the keratinocyte subpopulations were characterized by different expression patterns of survivin. Survivin protein was overexpressed in KSCs, whereas it was almost absent in TA cells and not detectable in PM cells (Fig. 3).

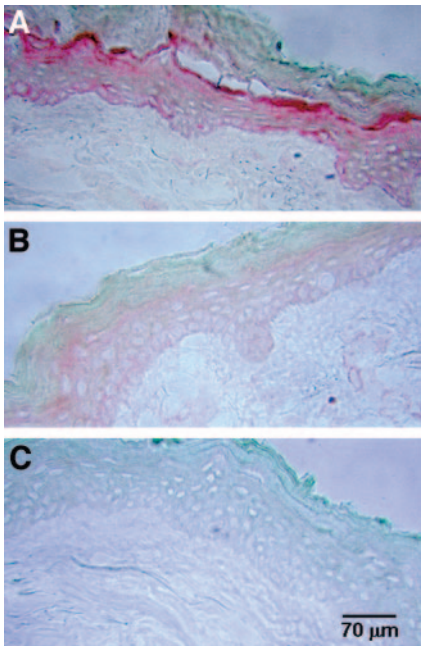

Figure 2. Survivin in normal human skin. Normal human skin was formalin-fixed, paraffin-embedded, and stained with anti-survivin polyclonal antibody able to recognize both nuclear and cytoplasmic survivin **(A)** and with a monoclonal antibody against the nuclear pool of survivin **(B)**. Control was obtained by omitting the primary antibody **(C)**.

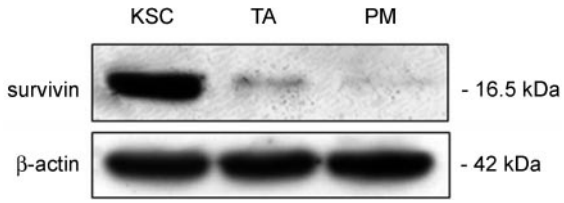

Figure 3. Expression of survivin in keratinocyte subpopulations. Western blotting was performed on protein extracts from KSCs and TA and PM cells. Nitrocellulose membrane was blotted against polyclonal anti-survivin antibody. A protein of approximate molecular mass, 16.5 kDa, corresponding to the predicted size of survivin, was detected in keratinocyte subpopulations. β-Actin was used as internal control. Abbreviations: KSC, keratinocyte stem cell; PM, postmitotic; TA, transit amplifying.

Expression of Survivin-Spliced Variants in TA and PM Cells and KSCs

We analyzed the expression of the different survivin isoforms and measured the difference in the three keratinocyte subpopulations by quantitative real time PCR. First, keratinocytes expressed all of the survivin isoforms. In particular, in KSCs, survivin-3B and survivin-2α levels are scarce and significantly lower than WT survivin, survivin-ΔEx3, and -2B (Fig. 4A). On the other hand, in TA cells, survivin-3B and survivin-2α are significantly more expressed than the other variants (Fig. 4B). In PM cells, survivin-2α and survivin-3B are highest and significantly more expressed than WT survivin, survivin-ΔEx3, and survivin-2B (Fig. 4C).

We next compared the expression pattern of each survivin variant in relation with the three keratinocyte subpopulations. WT survivin mRNA was significantly more expressed in KSCs than in TA cells (Fig. 5A). Unlike the protein level, the difference in WT survivin mRNA expression between KSCs and TA cells is less marked, suggesting possible post-transcriptional regulations. Similarly to WT survivin, survivin-2B was more expressed in KSCs than in TA cells (Fig. 5B). Survivin-ΔEx3

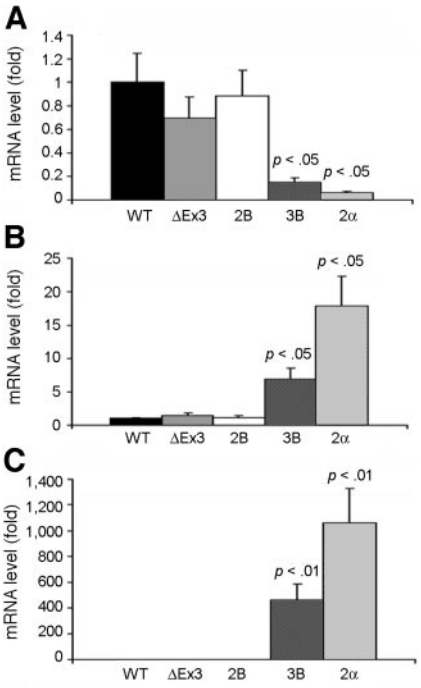

Figure 4. Relative quantification of survivin spliced variants in individual keratinocyte subpopulations. RNA extracts from keratinocyte stem cells (KSCs) and transit amplifying (TA) and postmitotic (PM) cells were amplified by real time polymerase chain reaction to quantify the spliced variants of survivin. Isoforms (ΔEx3, 2B, 3B, 2α) were quantified in the individual keratinocyte subpopulations using WT survivin expression as a calibrator. KSC **(A)**, TA **(B)**, and PM **(C)**. A double-sided Student's *t* test was performed between samples and calibrator. Abbreviations: ΔEx3, survivin-ΔEx3; 2α, survivin-2α; 2β, survivin-2β; 3β, survivin-3β; WT, wild type.

expression appeared to be similar in KSCs and TA cells, whereas its expression almost disappeared in PM cells (Fig. 5C). Finally, survivin-2α and survivin-3B expression was higher in TA and PM cells compared with KSCs (Fig. 5D, 5E).

Subcellular Localization of Survivin in TA and PM Cells and KSCs

Survivin exists in distinct subcellular pools, and a ratio of 1:6 for nuclear to cytosolic survivin has been obtained by quantitative fractionation [22]. Moreover, survivin translocates to the nucleus and initiates the cell cycle entry by interacting with cyclin-dependent kinases [27]. Using an antibody that recognizes both nuclear and cytosolic pool, by confocal microscopy, survivin was predominantly localized in the nucleus of KSCs (Fig. 6A). On the other hand, TA cells expressed mostly cytosolic survivin (Fig. 6B), whereas no survivin expression was detected in PM cells (Fig. 6C). No staining was observed when the primary antibody was omitted (Fig. 6D). To evaluate whether the nuclear localization correlates with the rate of proliferation, we measured the proliferation index by flow cytometry at the time when subcellular distribution was studied. The rate of proliferation in KSCs was significantly higher than in TA cells (Fig. 6E).

Survivin Is Downregulated During Anoikis

KSCs are protected from anoikis because of the high levels of β1 integrin [16]. As we present evidence that KSCs express the highest levels of survivin, we wanted to investigate the expression of survivin in keratinocytes undergoing anoikis following anti-β1 integrin antibody treatment. By real time PCR, WT survivin expression levels significantly decreased in suspended cells and more strikingly in cells treated with anti-β1 integrin antibody (Fig. 7A). Within 4 hours of suspension, survivin protein levels were slightly decreased, whereas anti-β1 integrin antibody markedly enhanced this effect. At 6 hours, survivin was hardly detectable in suspended keratinocytes and completely disappeared in anti-β1 integrin-treated cells (Fig. 7B). To investigate survivin isoform expression in KSCs during anoikis, we evaluated mRNA levels of each isoform in KSCs treated with anti-β1 integrin. Compared with untreated KSCs, KSCs undergoing anoikis were characterized by a marked upregulation of survivin-2α and survivin-3B expression and by a downregulation of WT survivin, survivin-2B, and survivin-ΔEx3 expression (Fig. 7C).

DISCUSSION

In this study, we have definitely shown that survivin is expressed in normal adult human epidermis. This is consistent with the localization of survivin in other normal human epithelia, such as the colonic mucosa and the endometrium [28, 29], two renewal-type tissues characterized by a constant turnover of cells. In colonic mucosa, survivin is expressed in a subpopulation of cells that include stem cells [28], in agreement with the previous description of survivin distribution in areas containing stem cells in fetal human epidermis and gastrointestinal tract [12]. Indeed, we report that isolated KSCs strongly express survivin, whereas differentiated cells do not. This might suggest that survivin contributes to preventing apoptosis and to conferring prolonged survival in the stem population, which drives the renewal process of the epidermis.

To our knowledge, survivin is the first antiapoptotic protein definitely distinguishing KSCs from TA cells. Other classic apoptotic proteins are differently regulated in the two populations as a function of susceptibility to cell death but are not mutually exclusive [16, 17]. The presence of a small amount of survivin in TA is not surprising if we consider that the method of isolation does not allow us to obtain pure populations. In the absence of a specific KSC marker, survivin can be considered a good candidate not different from previously defined proteins, such as β1 integrin [30] or p63 [31]. Both proteins have been detected in high quantities of KSCs, whereas some protein is also expressed in TA cells, even when clonal separation was used [31]. We propose that survivin, in addition to these markers, can be used to identify KSCs.

Studying the function of this newly identified marker will shed light on the property of KSCs. In this respect, we have shown that the antiapoptotic WT survivin strongly identifies KSCs. This is in keeping with the proposed function of survivin and with the small degree of susceptibility to apoptosis of this population [16].

To further evaluate the survivin expression and function in keratinocytes, we have studied survivin isoforms in keratinocyte subpopulations. Survivin variants are differently expressed in keratinocyte subpopulations. The high survivin-2B expression in KSCs is at variance with previous literature that claims this isoform is a potential proapoptotic protein [5] with dominant-negative effects on WT survivin [3, 32]. Since the precise function of the cryptic exon 2B [3] is not clearly defined, survivin-2B activity remains to be fully elucidated [33]. Moreover, in the survivin-2B, the break point in the BIR domain is the same as in survivin-ΔEx3, which maintains antiapoptotic function. Based on our data, it is therefore conceivable to consider survivin-2B an antiapoptotic protein. Survivin-ΔEx3 is equally expressed in KSCs and in TA cells, although it strikingly decreases in PM cells, in agreement with the proposed antiapoptotic function of this isoform [3]. The high expression levels of survivin-ΔEx3 in TA cells are consistent with the

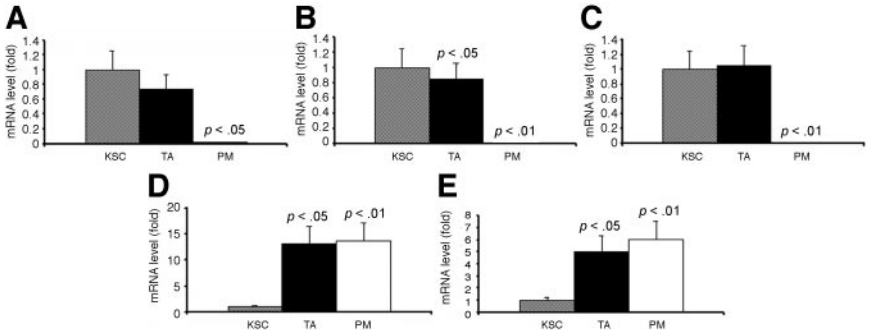

Figure 5. Comparison of individual survivin-spliced variants in keratinocyte subpopulations. RNA extracts of the three keratinocyte subpopulations were amplified by real time PCR to quantify the spliced variants of survivin, using KSCs as a calibrator. WT survivin **(A)**, survivin-2B **(B)**, survivin-ΔEx3 **(C)**, survivin-2α **(D)**, and survivin-3B **(E)**. A double-sided Student's *t* test was performed between samples and calibrator. Abbreviations: KSC, keratinocyte stem cell; PM, postmitotic; TA, transit amplifying.

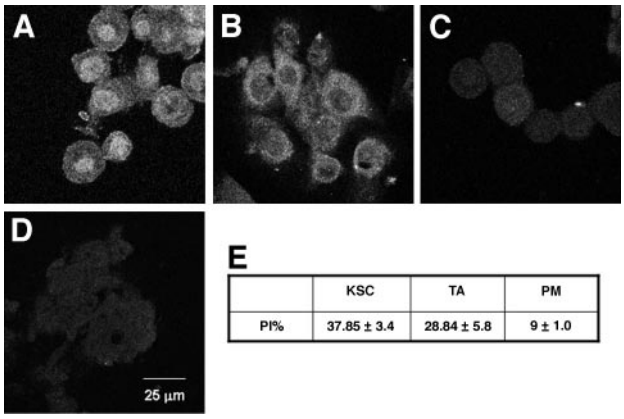

Figure 6. Subcellular localization of survivin in keratinocyte subpopulations. The three subpopulations were fixed and stained in situ with anti-survivin polyclonal antibody 96 hours after plating (**A**, KSC; **B**, TA; **C**, PM). As a negative control, primary antibody was omitted **(D)**. Cells were observed by confocal microscopy. The three subpopulations were trypsinized, stained at 96 hours with propidium iodide, and analyzed by flow cytometry. PI was then calculated, and a double-sided Student's *t* test was used for comparison of the means **(E)**. KSC versus TA, $p < .05$; KSC versus PM, $p < .01$. Abbreviations: KSC, keratinocyte stem cell; PI, proliferation index; PM, postmitotic; TA, transit amplifying.

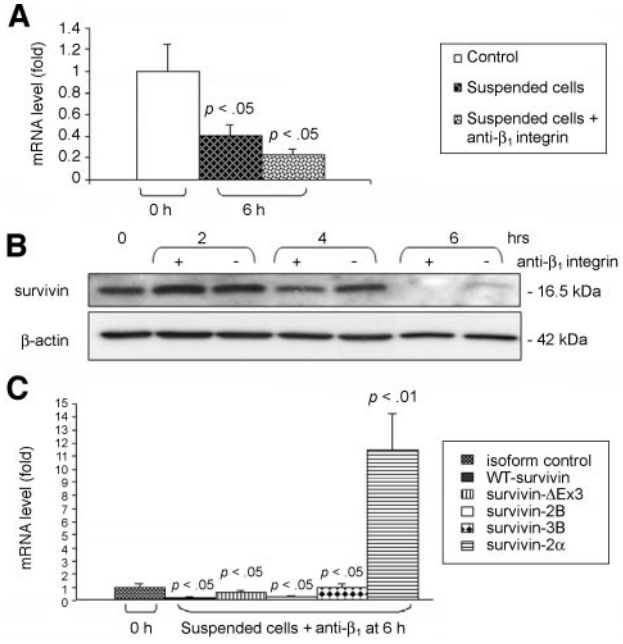

Figure 7. Expression of survivin in KSCs undergoing anoikis. KSCs were suspended in polypropylene with or without anti-β1 integrin antibody and then lysed at different time points. A quantitative relative real time PCR was performed with RNA extracts after 0- and 6-hour treatment, using KSCs at 0 hours as a calibrator for WT survivin **(A)** and for each isoform **(C)**. A double-sided Student's *t* test was performed between samples and calibrator. Protein extracts were analyzed by Western blotting with anti-survivin antibody at 0, 2, 4, and 6 hours **(B)**. β-Actin was used as internal control. Abbreviations: cntrl, control; h/hrs, hour(s).

partial resistance of these cells to apoptosis [17]. The availability of a specific antibody will allow us to evaluate the possible differences in the protein expression between KSCs and TA cells. The high levels of survivin-2α in TA and PM cells, which are more susceptible to apoptosis, are consistent with the hypothesis of a possible proapoptotic activity of this isoform. Survivin-2α could indeed interfere with WT survivin action by physically interacting with it and by changing its intracellular localization. Moreover, survivin-2α lacks the entire form of the BIR domain, which is responsible for the antiapoptotic activity [4]. The low expression levels of survivin-3B in KSCs coupled with its increase in TA and PM cells seems to indicate that this isoform is characterized by a low antiapoptotic activity. Although survivin-3B structure is characterized by the entire form of the BIR domain and the absence of coiled-coil domain at C-terminal position, there are not yet available data relative to its function [4].

KSCs are more protected from apoptosis compared with TA cells [16]. Although survivin has already been shown to protect keratinocytes from ultraviolet-B (UVB)-induced apoptosis [7], and UVB downregulates survivin expression in these cells (unpublished observation), this study first suggests a role for survivin in homeostatic programmed cell death in human epidermis [34].

Survivin not only exerts antiapoptotic activities but is also involved in cell-cycle regulation [27, 35]. Whether survivin controls cell division in keratinocytes has not been addressed in the present study, even though anoikis is a cell cycle-independent apoptotic stimulus [34]. However, it is interesting to note that survivin is overexpressed in KSCs that share with other normal cells, such as basal colonic mucosa [28] and $CD34^+$ bone marrow-derived stem cells [36], as well as with cancer cells the high proliferative potential. Indeed, we present evidence that nuclear survivin is mostly restricted to KSCs, which present a high proliferation rate. This is consistent with the concept that nuclear survivin is involved in cell-cycle regulation.

The overexpression of survivin in most tumors renders them resistant to cell death, thus maintaining cancer cell viability [37]. KSCs are the target of skin carcinogenesis, and mutated cells are normally located to the stem cell niche [38]. It is plausible that high levels of survivin not only protect KSCs in their niche under physiological conditions but also during skin tumor formation. We present evidence that survivin expression is downregulated during anoikis. Anoikis appears to be a critical mechanism to eliminate mutated cells [39], and anoikis resistance favors skin carcinogenesis [20]. Moreover, integrin protects squamous cell carcinoma from anoikis [19] and α3 inte-

grin, which is overexpressed in this skin tumor, and anchors KSCs to the niche [40]. Blocking β1 integrin completely abolishes survivin in KSCs, suggesting that survivin partially contributes to the integrin survival signal.

CONCLUSION

Stem cells are critical in tissue homeostasis as well as in cancer development. Moreover, the use of stem cells as a novel therapy for the treatment of several diseases is promising. However, the use of stem cells is hampered by the difficulties in identifying this population in most adult tissues, including epidermis, because of the lack of a specific marker. In addition, the mechanisms underlying stem cell regulation at the epidermal level are not fully understood; in particular, it is still unclear how KSCs lose their stemness and escape the niche protection. There is evidence that KSCs leave this microenviroment not only through differentiation but also via an apoptotic mechanism [16]. Survivin as a molecule that identifies KSCs and their involvement in the protection of these cells from cell death is an important contribution to the understanding of the correlation between KSC phenotype and function. To further clarify the role of survivin in KSC isolation and activity, experiments are now in progress to correlate survivin isoforms expression and function. The discovery of new markers and pathways for the characterization of KSCs is of paramount importance for the clinical use of epidermal cell cultures and possibly for gene therapy.

ACKNOWLEDGMENTS

We thank Marco Pignatti for the valuable assistance in the preparation and submission of the manuscript. This work was supported by a grant from Ministry of Instruction, University and Research 2003061751_004 (2003–2005). A.M. and K.D. contributed equally to this article.

DISCLOSURES

The authors indicate no potential conflicts of interest.

REFERENCES

1 Li F. Survivin study: What is the next wave? J Cell Physiol 2003;197:8–29.
2 Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med 1997;3:917–921.
3 Mahotka C, Wenzel M, Springer E et al. Survivin-deltaEx3 and survivin-2B: Two novel splice variants of the apoptosis inhibitor survivin with different antiapoptotic properties. Cancer Res 1999;59:6097–6102.
4 Caldas H, Honsey LE, Altura RA. Survivin 2alpha: A novel Survivin splice variant expressed in human malignancies. Mol Cancer 2005;4:11.
5 Badran A, Yoshida A, Ishikawa K et al. Identification of a novel splice variant of the human anti-apoptopsis gene survivin. Biochem Biophys Res Commun 2004;314:902–907.
6 Xing Z, Conway EM, Kang C et al. Essential role of survivin, an inhibitor of apoptosis protein, in T cell development, maturation, and homeostasis. J Exp Med 2004;199:69–80.
7 Grossman D, Kim PJ, Blanc-Brude OP 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.
8 Dohi T, Okada K, Xia F et al. An IAP-IAP complex inhibits apoptosis. J Biol Chem 2004;279:34087–34090.
9 Dohi T, Beltrami E, Wall NR et al. Mitochondrial survivin inhibits apoptosis and promotes tumorigenesis. J Clin Invest 2004;114:1117–1127.
10 Li F, Ambrosini G, Chu EY et al. Control of apoptosis and mitotic spindle checkpoint by survivin. Nature 1998;396:580–584.
11 Altieri DC. Survivin in apoptosis control and cell cycle regulation in cancer. Prog Cell Cycle Res 2003;5:447–452.
12 Adida C, Crotty PL, McGrath J et al. Developmentally regulated expression of the novel cancer anti-apoptosis gene survivin in human and mouse differentiation. Am J Pathol 1998;152:43–49.
13 Chiodino C, Cesinaro AM, 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.
14 Lajtha LG. Haemopoietic stem cells: Concept and definitions. Blood Cells 1979;5:447–455.
15 Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol 1994;124:619–626.
16 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.
17 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;117:5815–5823.
18 Escaffit F, Perreault N, Jean D et al. Repressed E-cadherin expression in the lower crypt of human small intestine: A cell marker of functional relevance. Exp Cell Res 2005;302:206–220.
19 Janes SM, Watt FM. Switch from alphavbeta5 to alphavbeta6 integrin expression protects squamous cell carcinomas from anoikis. J Cell Biol 2004;166:419–431.
20 Zhang Y, Lu H, Dazin P et al. Squamous cell carcinoma cell aggregates escape suspension-induced, p53-mediated anoikis: Fibronectin and integrin alphav mediate survival signals through focal adhesion kinase. J Biol Chem 2004;279:48342–48349.
21 Pincelli C, Haake AR, Benassi L et al. Autocrine nerve growth factor protects human keratinocytes from apoptosis through its high affinity receptor (TRK): A role for BCL-2. J Invest Dermatol 1997;109:757–764.
22 Fortugno P, Wall NR, Giodini A et al. Survivin exists in immunochemically distinct subcellular pools and is involved in spindle microtubule function. J Cell Sci 2002;115:575–585.
23 Grossman D, McNiff JM, Li F et al. Expression of the apoptosis inhibitor, survivin, in nonmelanoma skin cancer and gene targeting in a keratinocyte cell line. Lab Invest 1999;79:1121–1126.
24 Bowen AR, Hanks AN, Murphy KJ et al. Proliferation, apoptosis, and survivin expression in keratinocytic neoplasms and hyperplasias. Am J Dermatopathol 2004;26:177–181.
25 Aziz MH, Ghotra AS, Shukla Y et al. Ultraviolet-B radiation causes an upregulation of survivin in human keratinocytes and mouse skin. Photochem Photobiol 2004;80:602–608.
26 Jones PH, Watt FM. Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. Cell 1993;73:713–724.
27 Suzuki A, Hayashida M, Ito T et al. Survivin initiates cell cycle entry by the competitive interaction with Cdk4/p16(INK4a) and Cdk2/cyclin E complex activation. Oncogene 2000;19:3225–3234.
28 Zhang T, Otevrel T, Gao Z et al. Evidence that APC regulates survivin expression: A possible mechanism contributing to the stem cell origin of colon cancer. Cancer Res 2001;61:8664–8667.
29 Konno R, Yamakawa H, Utsunomiya H et al. Expression of survivin and Bcl-2 in the normal human endometrium. Mol Hum Reprod 2000;6:529–534.
30 Jones PH, Harper S, Watt FM. Stem cell patterning and fate in human epidermis. Cell 1995;80:83–93.
31 Pellegrini G, Dellambra E, Golisano O et al. p63 identifies keratinocyte stem cells. Proc Natl Acad Sci U S A 2001;98:3156–3161.
32 Islam A, Kageyama H, Hashizume K et al. Role of survivin, whose gene is mapped to 17q25, in human neuroblastoma and identification of a novel dominant-negative isoform, survivin-beta/2B. Med Pediatr Oncol 2000;35:550–553.
33 Li F. Role of survivin and its splice variants in tumorigenesis. Br J Cancer 2005;92:212–216.
34 Reed JC. The Survivin saga goes in vivo. J Clin Invest 2001;108:965–969.
35 O'Connor DS, Grossman D, Plescia J et al. Regulation of apoptosis at cell division by p34cdc2 phosphorylation of survivin. Proc Natl Acad Sci U S A 2000;97:13103–13107.
36 Fukuda S, Foster RG, Porter SB et al. The antiapoptosis protein survivin is associated with cell cycle entry of normal cord blood CD34(+) cells and modulates cell cycle and proliferation of mouse hematopoietic progenitor cells. Blood 2002;100:2463–2471.

37 Hassounah M, Lach B, Allam A et al. Benign tumors from the human nervous system express high levels of survivin and are resistant to spontaneous and radiation-induced apoptosis. J Neurooncol 2005;72: 203–208.
38 Morris RJ. A perspective on keratinocyte stem cells as targets for skin carcinogenesis. Differentiation 2004;72:381–386.
39 Wang LH. Molecular signaling regulating anchorage-independent growth of cancer cells. Mt Sinai J Med 2004;71:361–367.
40 Kurata S, Okuyama T, Osada M et al. p51/p63 controls subunit alpha3 of the major epidermis integrin anchoring the stem cells to the niche. J Biol Chem 2004;279:50069–50077.

Survivin Identifies Keratinocyte Stem Cells and Is Downregulated by Anti-ß1 Integrin During Anoikis

Alessandra Marconi, Katiuscia Dallaglio, Roberta Lotti, Cristina Vaschieri, Francesca Truzzi, Fabrizio Fantini and Carlo Pincelli

Stem Cells 2007;25;149-155; originally published online Sep 28, 2006;
DOI: 10.1634/stemcells.2006-0165