Keratinocytes enriched for stem cells are protected from anoikis via an integrin signaling pathway in a Bcl-2 dependent manner

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Abstract Because inhibition of integrin signaling induces apoptosis, we investigated whether keratinocytes expressing β1 and α6β4 integrins (enriched for stem cells) are protected from cell death. Keratinocytes rapidly adhering to type IV collagen expressed highest levels of β1 and α6β4 and of the anti-apoptotic stem cell marker p63. Apoptotic cells were significantly higher in slowly adhering than in rapidly adhering keratinocytes. Anti-β1 integrin caused a significant increase in apoptotic cells, while it decreased Bcl-2 levels in stem keratinocytes. Bax and Bad proteins were higher in slowly adhering than in rapidly adhering cells. By contrast, Bcl-2, Bcl-x and Mcl-1 proteins were highest in rapidly adhering keratinocytes and nearly absent in slowly adhering cells. After addition of anti-β1 integrin, the apoptotic rate was significantly higher in HaCaT cells not expressing Bcl-2 than in controls. These results indicate that keratinocytes enriched for stem cells are protected from apoptosis via β1 integrin, in a Bcl-2 dependent manner. **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Adhesion; Extracellular matrix; Anoikis; Epidermis; p63; α6β4

1. Introduction

The ability of human epidermis to maintain a constant turnover of cells is based on the presence in the basal layer of a population of keratinocyte stem cells that retains a high capacity of self-renewal throughout life [1,2]. Stem cells generate transit amplifying (TA) cells that terminally differentiate after a discrete number of cell divisions [3,4]. A third population ('young' TA) of basal keratinocytes behaves in an intermediate way between stem and TA cells [5]. Epidermal keratinocytes with the characteristics of stem and TA cells can be identified and isolated on the basis of their adhesive capacity [6]. Basal keratinocytes which rapidly adhere to type IV collagen and express highest levels of β1 integrin, have the greatest colony forming efficiency (CFE), thus showing characteristics of stem cells. On the other hand, basal cells expressing lower amounts of β1 integrin, slowly adhering to the extracellular matrix (ECM), tend to be committed to terminal differentiation after few divisions [7,8]. Recently, a stem cell population has been isolated based on the expression of high levels of α6β4 integrin and low levels of a proliferation-related marker [9]. Finally, the transcription factor p63, a p53 homolog, has recently been shown to identify keratinocyte stem cells [10].

Apoptosis plays a fundamental role in epidermal homeostasis by counterbalancing cell proliferation, thus maintaining the proper cell number [11]. In the skin, although apoptosis can be induced by different stimuli [12], it can also be physiologically activated [13], while apoptotic cells are consistently observed in normal human epidermis [14].

It has been shown that cell survival requires interaction with the ECM and that integrin signaling controls cell death in many cell settings [15]. Apoptosis occurs in certain cell types, such as epithelial and endothelial cells following detachment from the ECM [16,17].

In the present study, we show that keratinocytes most rapidly adhering to type IV collagen, expressing highest levels of β1 and α6β4 integrins, and identified by p63, are protected from cell death. We also present correlative evidence that the balance of pro- and anti-apoptotic members of the Bcl-2 family in the three keratinocyte populations plays a critical role in preventing 'anoikis' in keratinocyte stem cells.

2. Materials and methods

2.1. Cell cultures

Keratinocytes were obtained from neonatal foreskin, as described in [14]. Recovered keratinocytes were washed in serum-free medium and plated onto plastic dishes, coated for 2 h at 37°C with 100 μg/ml type IV collagen (Sigma, St. Louis, MO, USA). Keratinocytes were divided into three populations and cultured in serum-free medium (KGM, Clonetics, San Diego, CA, USA). They were first allowed to adhere to type IV collagen for 5 min (population 1), and the non-adherent cells were then transferred to fresh collagen-coated dishes and allowed to attach overnight (population 2). Finally, keratinocytes not yet attached after one night were plated onto type IV collagen to obtain a third population (population 3). The three populations were then used for further experiments. HaCaT keratinocytes, HaCaT cells transfected with *bcl-2*(pcDNA1/Neo plus Bcl-2), HaCaT cells transfected with pcDNA1/Neo minus Bcl-2 and control transfectants (a kind gift of Anne Haake, University of Rochester, NY, USA [18]) were cultured in Dulbecco's modified Eagle's medium plus 10% fetal calf serum and were used for certain adhesion experiments. For blocking adhesion experiments, keratinocytes were pre-incubated for 1 h at

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Abbreviations: ECM, extracellular matrix; CFE, colony forming efficiency; TA, transit amplifying

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37°C with anti-β1 integrin neutralizing antibody (1:250, Immunotech), anti-vimentin antibody (1:250, Dako, Copenhagen, Denmark) or medium alone.

2.2. Immunofluorescent staining and FACS

Cells from the three populations were resuspended in phosphate-buffered saline (PBS) at 4°C, incubated on ice with anti-β1 integrin monoclonal antibody (1:20, Immunotech, Marseille, France) for 30 min. Cells were then washed in PBS at 4°C and incubated with the anti-mouse secondary antibody FITC-conjugated for 30 min on ice. After washing, cells were analyzed using a FACScan flow cytometer (Becton-Dickinson, San José, CA, USA) with Lysys II software (Becton-Dickinson).

2.3. Determination of CFE

Keratinocytes from the three different populations were harvested by treatment with a trypsin/EDTA solution (0.05%/0.02%). Ten dishes (6 mm ∅) were plated for each cell population at a density of 100 cells per dish. Keratinocytes were cultured on mitomycin C-treated 3T3 cells (2.4×10^4/cm^2; ATCC, Rockville, MD, USA) and cultivated in Dulbecco's modified Eagle's medium and Ham's F12 media for 2 weeks. Culture medium was changed after 6 days. Fourteen days later, dishes were fixed and stained with crystal violet. Colonies that contained more than 10 cells were counted and CFE was calculated. The colony number was expressed as a percentage of the number of basal cells plated in each dish.

2.4. Cell death analysis

Cells from populations 1 and 2 were fixed in situ in para-formaldehyde (4% in PBS), while cells from population 3 were collected and cytospun onto slides precoated with 0.01% poly-L-lysine and air-dried at 48 h. All cells were then stained with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method, as described in [12]. Fluorescent specimens were analyzed by confocal scanning laser microscopy (Leica TCS4D) in conjunction with a conventional optical microscope (Leica DM IRBE). For flow cytometric analysis, cells were trypsinized after 48 h and resuspended (1×10^6 cells) in 400 μl hypotonic fluorochrome solution: propidium iodide (PI) 50 mg/ml in 0.1% sodium citrate containing 0.1% Triton X-100, Sigma. After a 60 min incubation in this solution, cells were analyzed using a FACScan.

2.5. Western blot analysis

Cells were washed with PBS and lysed on ice in RIPA buffer pH 7.5. Total protein (40 μg) was analyzed under reducing conditions on 12% polyacrylamide gels and blotted onto nitrocellulose membranes. The blots were blocked for 2 h in blocking buffer (PBS buffer, pH 7.4 with 0.2% Tween 20 and 5% non-fat milk) and incubated with anti-human Bcl-2 (1:1000), Bax (1:1000), Bcl-x (1:1000), Mcl-1 (1:1000), Bad (1:3000), p63 (ΔNp63α, 1:400, Santa Cruz Biotechnology, Santa Cruz, CA, USA), α6β4 integrin (1:100, Santa Cruz Biotechnology) and β-actin (1:1000, Sigma) overnight at 4°C. Then membranes were washed in PBS/Tween 20, incubated with peroxidase-conjugated goat anti-mouse antibodies (1:800, Bio-Rad, Hercules, CA, USA) for p63, α6β4 and β-actin for 45 min at room temperature, and with peroxidase-conjugated goat anti-rabbit (1:3000) for Bcl-2, Bax, Bcl-x, Mcl-1 and Bad. Finally, membranes were washed and developed using the ECL chemiluminescent detection system (Amersham).

3. Results

3.1. Characterization of keratinocytes enriched for stem cells

Because epidermal stem cells have previously been isolated based on their β1 integrin expression [19], we selected three populations according to their different adhesive capacities to type IV collagen. Cells that rapidly (5 min) attached to type IV collagen (population 1) expressed highest levels of β1 integrin, whereas β1 integrin protein was significantly lower in population 2 and almost undetectable in population 3 (Fig. 1A). Moreover, α6β4 integrin was strongly expressed in population 1, to a lesser degree in population 2 and absent in population 3 (Fig. 1B). We also demonstrated that population

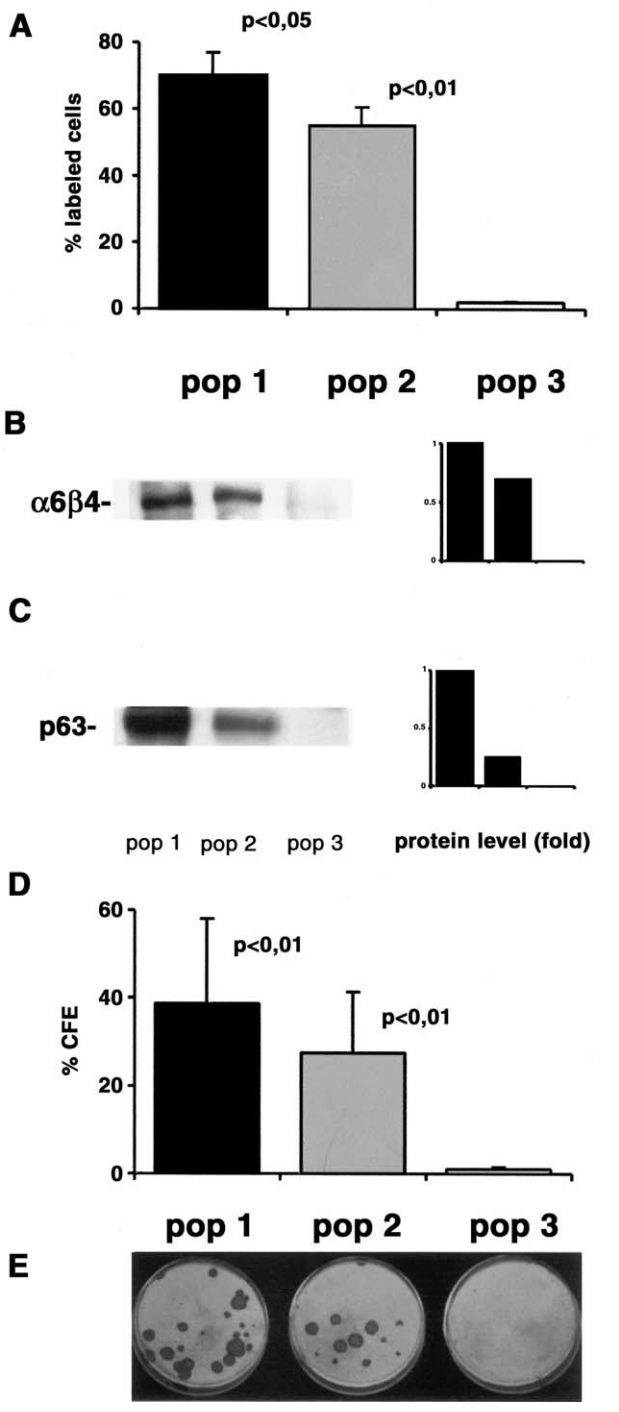

Fig. 1. Isolation of keratinocytes enriched for stem cells. Cell suspensions from the three keratinocyte populations were immunostained using antibody specific for β1 integrin, as described in Section 2. Cells were analyzed by flow cytometry. The results are expressed as the mean ± S.D. of three experiments. Student's *t*-test was used for comparison of the means (A). Cells from the three populations were lysed at 48 h and protein analyzed by Western blotting using anti-α6β4 (B) and anti-p63 (C) monoclonal antibodies, as described in Section 2. Relative intensity of bands on autoradiograms was quantified by scanning laser densitometry. CFE was determined on the three keratinocyte populations, as described in Section 2. Results are expressed as the mean ± S.D. of three different experiments. Student's *t*-test was used for comparison of the means (D, E).

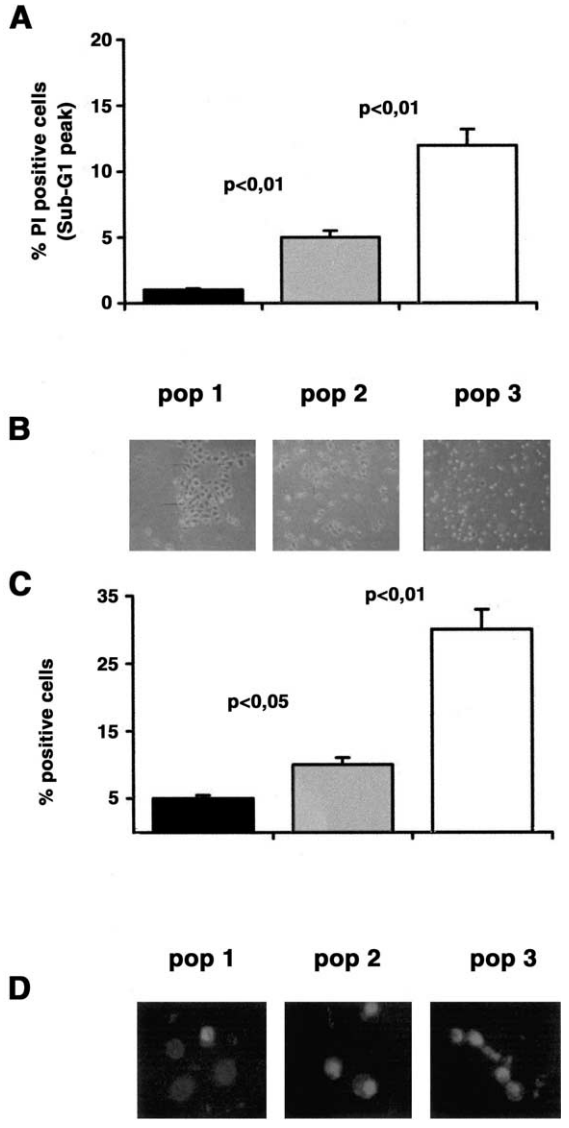

Fig. 2. Keratinocytes enriched for stem cells are protected from apoptosis. Cells were stained with PI and analyzed by flow cytometry. Results are expressed as the mean ± S.D. of PI-positive cells in the sub-G1 peak, indicating apoptosis, from three different experiments. Student's *t*-test was used for comparison of the means (A). Keratinocyte colonies from the three populations were observed and photographed at 48 h under phase contrast (B). Cells from the three populations were stained by the TUNEL technique. Approximately 100 cells were evaluated, in randomly selected high power fields and the percentage of TUNEL-positive cells was counted. Each experiment was repeated three times. Results are expressed as the mean percentage ± S.D. from three experiments. Student's *t*-test was used for comparison of the means (C, D).

1 expressed highest levels of p63, whereas this protein was barely detectable in population 2 and absent in population 3 (Fig. 1C), in agreement with previous reports [10]. Finally, cells from population 1 had the highest CFE, while CFE was significantly decreased in population 2 and virtually undetectable in population 3 (Fig. 1D). Taken together, these results indicate that we isolated a population of keratinocytes enriched for stem cells, a population of 'young' TA cells and a population of terminally differentiated cells.

3.2. Apoptosis ('anoikis') and adhesiveness

Because lack of matrix attachment can cause apoptosis in other cell systems [20], we reasoned that keratinocytes from population 1, that most rapidly adhered to type IV collagen, could be protected from programmed cell death by survival signals generated via integrin activation [21]. Indeed, apoptotic keratinocytes were almost undetectable in population 1 and significantly more numerous in populations 2 and 3 (Fig. 2A). Fig. 2B illustrates large colonies formed by cells from population 1 at 48 h, while keratinocytes from population 2 formed only small colonies. As expected, population 3 showed only detached cells (Fig. 2B). Moreover, the percentage of TUNEL-positive cells was significantly higher in populations 2 and 3 than in population 1 (Fig. 2C,D). The above findings show that keratinocytes enriched for stem cells are effectively protected from apoptosis.

3.3. Protection from apoptosis ('anoikis') is mediated by integrins

Because integrin signaling is a general requirement for cell survival [22], we evaluated the role of β1 integrin in mediating protection from apoptosis in keratinocytes rapidly adhering to

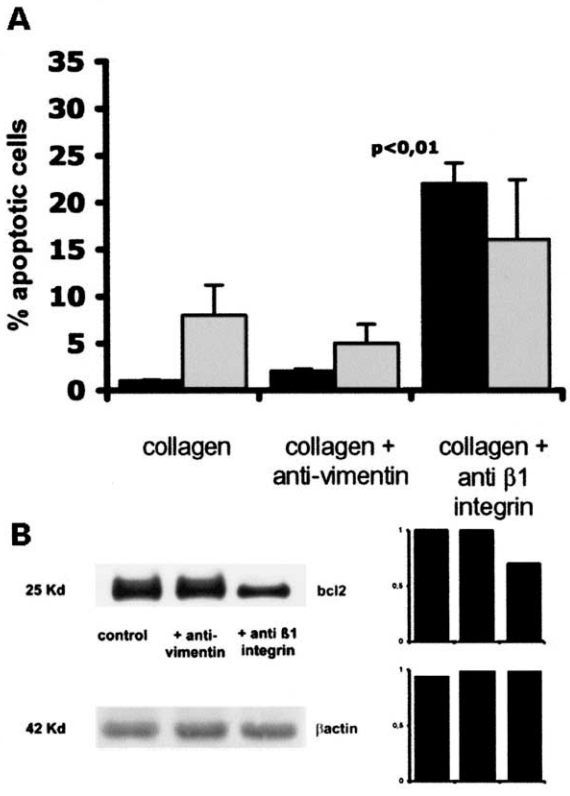

Fig. 3. Integrins mediate protection of keratinocyte stem cells from apoptosis. Keratinocytes from populations 1 (black bar) and 2 (gray bar) were pre-incubated for 1 h at 37°C with anti-β1 integrin neutralizing antibody, anti-vimentin antibody or medium alone, as described in Section 2. Then keratinocytes were allowed to adhere to type IV collagen and stained by the TUNEL technique at 24 h. Cell counting and statistics were performed as before (A). Keratinocytes from population 1 were treated as above and Bcl-2 protein level analyzed by Western blot, as described in Section 2. β-Actin levels were evaluated to assess equal loading of the protein. The relative intensity of bands on autoradiograms was quantified by scanning laser densitometry. Values are expressed as fold variations compared to control cells (B).

type IV collagen. To this purpose, we treated keratinocytes from populations 1 and 2 with anti-β1 integrin neutralizing antibody. This antibody induced a significantly increased percentage of apoptotic cells both in population 1 and in population 2, as compared to untreated keratinocytes. By contrast, the addition of anti-vimentin antibody failed to augment the number of apoptotic cells (Fig. 3A). As integrin mediates cell survival by increasing the expression of Bcl-2 protein in other cell systems [23], we evaluated the levels of this anti-apoptotic protein after treating keratinocytes enriched for stem cells with anti-β1 integrin neutralizing antibody. Addition of anti-β1 integrin resulted in a clear decrease in Bcl-2 levels (Fig. 3B).

3.4. The Bcl-2 family regulates apoptosis

Various members of the Bcl-2 family are expressed in basal keratinocytes [23,24] and have been shown to regulate apoptosis in these cells [25,26,27]. Therefore, we measured the levels of pro- and anti-apoptotic proteins of the Bcl-2 family in the three populations. Levels of Bcl-2, Bcl-X and Mcl-1 proteins were markedly higher in population 1 than in population 2, while they were nearly absent in population 3. By contrast, Bax and Bad were inversely correlated to the anti-apoptotic proteins in the same populations, in that their levels were markedly lower in population 1 than in population 2, while they were highest in population 3 (Fig. 4A,B).

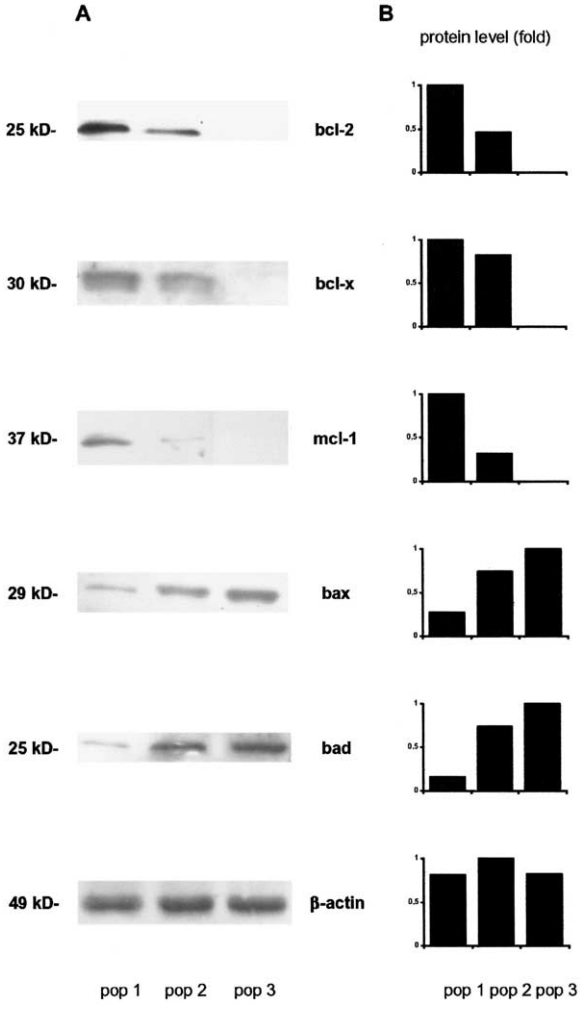

Fig. 4. Bcl-2 family expression. Cells from the three populations were lysed at 48 h and protein analyzed by Western blotting using anti-Bcl-2, Bcl-x, Mcl-1, Bax, Bad and β-actin polyclonal antibodies, as described in Section 2 (A). The relative intensity of bands on autoradiograms was quantified by scanning laser densitometry (B).

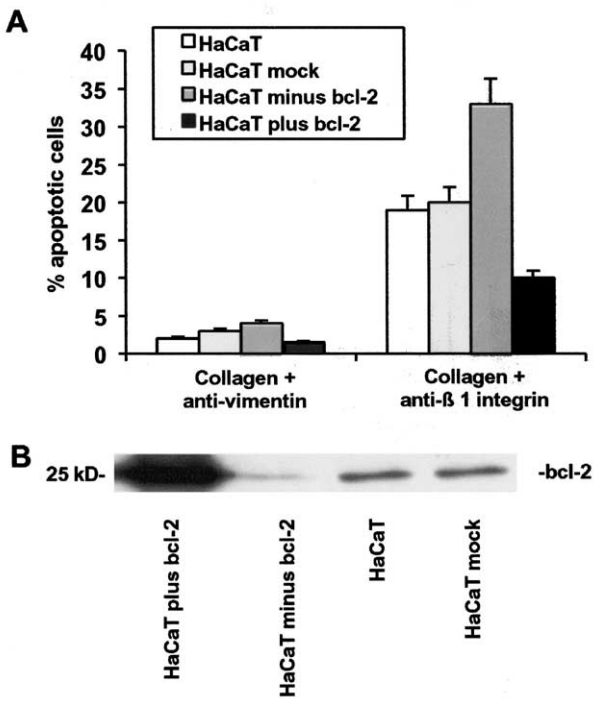

Fig. 5. Bcl-2 protects keratinocyte stem cells from apoptosis. HaCaT cells not expressing Bcl-2, HaCaT cells overexpressing Bcl-2, parental HaCaT cells and control transfectants were pre-incubated with anti-β1 integrin antibody or with anti-vimentin, as a control. HaCaT cells were then allowed to adhere to type IV collagen and stained with the TUNEL technique at 24 h. Cell counting and statistics were performed as before. HaCaT minus Bcl-2 vs. HaCaT cells and control transfectants (mock): $P < 0.01$; HaCaT plus Bcl-2 vs. HaCaT cells and control transfectants (mock): $P < 0.01$ (A). HaCaT cells not expressing Bcl-2, HaCaT cells overexpressing Bcl-2, parental HaCaT cells and control transfectants were lysed at subconfluency and protein analyzed by Western blotting using anti-Bcl-2 polyclonal antibody (B).

To further define the role of Bcl-2 in protection from 'anoikis', the spontaneously immortalized HaCaT cell line overexpressing Bcl-2 (HaCaT plus), HaCaT cells not expressing Bcl-2 (HaCaT minus), parental HaCaT cells and control transfectants (mock) (Fig. 5B) were pretreated with anti-β1 integrin antibody and plated onto type IV collagen. While few apoptotic cells were detected in untreated HaCaT cells or in HaCaT cells pretreated with anti-vimentin antibody, following treatment with anti-β1 integrin antibody, the number of apoptotic HaCaT keratinocytes was significantly reduced in cells HaCaT plus Bcl-2 as compared to both parental HaCaT cells and to control transfectants. On the other hand, upon treatment with anti-β1 integrin antibody, the number of apoptotic cells in HaCaT minus Bcl-2 was significantly higher than in controls (Fig. 5A).

4. Discussion

Keratinocyte stem cells have been defined by various characteristics, such as location within the tissue [28], mitotic be-

havior [29,30], morphology [31] and expression of protein markers [32,33]. However, little is known of their resistance to apoptosis. We report here that a subpopulation of keratinocytes enriched for stem cells is protected from cell death, while keratinocytes with the characteristics of 'young TA' and TA cells are significantly more susceptible to apoptosis. Previous studies have indicated that the apoptotic process may indeed regulate the fate of stem cells in other epithelial systems such as the intestinal crypts [34,35]. In the murine epidermal system in vitro, upon TPA treatment, while maturing basal cells undergo apoptosis, slowly cycling, label-retaining cells that correlate to stem keratinocytes remain attached and seem to be resistant to TPA [36].

Keratinocytes enriched for stem cells in our system express highest levels of the transcription factor p63, which has been reported as a keratinocyte stem cell marker [10]. Moreover, the ΔN isoform of p63 [37], used in our study, decreases in basal keratinocytes after UV-B irradiation and such a down-regulation is required for UV-B-induced apoptosis in epidermis [38]. These findings point to an anti-apoptotic role for p63 in keratinocytes. Most of all, this well correlates with the observation, reported in this study, of a subpopulation of keratinocytes enriched for stem cells, strongly expressing p63, that are protected from apoptosis.

We also provide evidence that keratinocyte stem cell survival is supported by cell–matrix interaction. Indeed, the method of sorting stem cell population used in our study is based on the adhesiveness mediated by integrins [19]: keratinocytes most rapidly adhering to type IV collagen are more resistant to apoptosis. Secondly, inhibition of β1 integrin function increases apoptosis in keratinocyte stem cell populations. Control of cell survival by integrins occurs in other cell systems. Inhibition of integrin-mediated cell–ECM adhesion induces apoptosis in bronchial epithelial cells [39] and integrin αvβ3 rescues melanoma cells from apoptosis [40]. While blocking keratinocyte adhesion to ECM was shown to increase the sensitivity of these cells to UV-induced apoptosis [41], our study first demonstrates that spontaneous apoptosis is prevented in keratinocyte stem cells, by virtue of their rapid attachment to type IV collagen via β1 integrin. Although an anti-apoptotic role of other integrins in keratinocyte stem cells cannot be ruled out, the addition of anti-β1 integrin antibody clearly increases the rate of cell death in our cell populations. This indicates that β1 integrin is a key adhesion molecule for protecting keratinocyte stem cells from apoptosis.

It is interesting to note that β1 integrin protects cells from apoptosis through the induction of Bcl-2 expression [20]. Bcl-2 expression is often topographically restricted to long-lived or proliferating cell compartments [42]. Furthermore, a small subset of slowly cycling basal epithelial cells expressing high levels of bcl-2 has recently been characterized [43]. Consistent with this observation, we show that a subpopulation of basal keratinocytes enriched for stem cells expresses high levels of anti-apoptotic members of the Bcl-2 family, while levels of pro-apoptotic proteins are nearly absent in these cells. Furthermore, Bcl-2 overexpression prevents apoptosis caused by the inhibition of cell–ECM interaction, while blocking Bcl-2 expression in anti-β1 integrin-treated cells induces a significantly higher rate of apoptosis as compared to controls. Finally, anti-β1 integrin reduced the levels of Bcl-2 protein in stem keratinocytes. This indicates that keratinocyte stem cells are protected from 'anoikis' through a Bcl-2 dependent pathway, as previously reported for epithelial cell lines [16].

Rescue from apoptosis in epidermal stem cells could contribute to skin carcinogenesis [44], while integrin signaling functions may offer targets for tumor therapy [45].

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