A previously unreported function of beta1B integrin isoform in caspase-8-dependent integrin-mediated keratinocyte death

A novel function of β_1B integrin isoform in caspase-8-dependent integrin-mediated keratinocyte death

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Short title:

Pro-apoptotic role of β_1B integrin

Abbreviation list:

shFADD: short-hairpin-RNA-Fas-Associated-Death-Domain; IMD: integrin-mediated death; DISC: death-inducing signaling complex; SCC: Squamous-cell carcinoma.

Abstract

Integrins regulate adhesive cell-matrix interactions and mediate survival signals. On the other hand, unligated or free cytoplasmic fragments of integrins induce apoptosis in many cell types (integrin-mediated death). We have previously shown that β_1 integrins expression protects keratinocyte stem cells from anoikis, while the role of the β_1B integrin isoform has never been clarified. Here we report that suspended keratinocytes undergo apoptosis via the activation of caspase-8, independently of Fas/Fas Ligand system. Indeed, anti-β_1 integrin neutralizing antibodies induced apoptosis in short-hairpin-RNA-Fas-Associated-Death-Domain treated cells. Moreover, before and during suspension, caspase-8 directly associated with β_1 integrin, that in turn internalized and progressively degraded, shedding the cytoplasmic domain. β_1B was expressed only in the cytoplasm in a perinuclear fashion and remained unaltered during suspension. At 24 hrs, as β_1A located close to the nucleus, β_1B co-localized with β_1A and co-immunoprecipitated with caspase-8. Caspase-8 was activated earlier in β_1B integrin transfected keratinocytes, and these cells underwent a higher rate of apoptosis than mock cells. By contrast, caspase-8 was not activated in siRNA β_1B transfected cells. These results indicate that when β_1A is unligated, β_1B is responsible for “integrin-mediated death” in human keratinocytes.

Introduction

β_1 integrin is abundantly expressed in keratinocytes and is critical for keratinocyte adhesion to type IV collagen (Fleischmajer et al, 1998; Tiberio et al, 2002). A β_1B variant has also been isolated, in which a unique 12-amino-acid sequence replaces the last 21 COOH-terminal amino acids of wild-type β_1 integrin (Altruda et al, 1990). Although β_1B integrin is expressed not only in keratinocytes, but also in liver and in skeletal and cardiac muscles (Balzac et al, 1993), its functional properties remain largely unknown. β_1 integrins mediate binding to the extracellular matrix (ECM) and transduce an intracellular signal promoting cell survival in many tissues and cells, including keratinocytes (Howlett et al, 1995; Marconi et al, 2004). Cells denied adhesion because of integrin ligand deprivation undergo a form of cell death named anoikis (Frisch and

Francis, 1994). While anoikis occurs as the consequence of loss of integrin-mediated attachment, a form of integrin-mediated death (IMD) has been proposed, whereby integrins in the antagonized or unligated state, can promote apoptosis independently of cell detachment (Stupack et al, 2001; Brassard et al, 1999). In most instances, anoikis process involves the extrinsic apoptotic pathway that is typically triggered by death receptor ligands, which in turn recruit the adaptor protein FADD. The death receptor-bound FADD associates with caspase-8 in the death-inducing signaling complex (DISC) leading to caspase autoactivation (Muzio et al, 1998; Marconi et al, 2004) (Figure S1). In IMD, integrins seem to cluster with caspase-8 without the involvement of the DISC (Stupack et al, 2001), although the actual mechanisms underlying this process remain to be clarified. In this study, we demonstrate that β_1B integrin clusters with unligated wild-type β_1 integrin and (pro-)caspase-8 to induce IMD in human keratinocytes, providing evidence for a previously unidentified role of this integrin isoform.

Results

Death receptors are not involved in anoikis. To test the role of β_1 integrin and caspase-8 during anoikis, cells were suspended and treated with a neutralizing anti-β_1 integrin antibody. We show that suspension induces keratinocyte apoptosis that is significantly augmented by blocking β_1 integrin, indicating that this integrin plays a role in keratinocyte anoikis (Figure S1a) (Marconi et al, 2004). We also show that caspase-8 is activated early in keratinocyte anoikis (Figure S1b), suggesting the involvement of death receptors in this process. To evaluate whether death receptors are actually responsible for caspase-8 activation and anoikis in our system, we evaluated the modulation of Fas/FasL in suspended keratinocytes and after treatment with anti-β_1 integrin. During a 0 to 18 hr time course, no up-regulation of either Fas (Figure 1a) or FasL (Figure 1b) was detected in both suspended and anti-β_1 integrin-treated keratinocytes. In addition, no FasL was released by suspended keratinocytes up to 3 hrs (Figure 1c). To definitely rule out the involvement of death receptors in keratinocyte anoikis, we retrovirally infected HaCaT keratinocytes with short-hairpin-RNA-Fas-Associated-Death-Domain (shFADD). Western blot

analysis showed reduced levels of FADD in shRNA FADD-infected keratinocytes, as compared to mock cells (Figure 2a). We first tested the capability of shFADD to prevent apoptosis. As expected, TRAIL induced apoptosis in mock but not in shFADD keratinocytes (Figure 2b). By contrast, caspase-8 was activated in shFADD as well as in mock keratinocytes treated with anti-β_1 integrin, as shown by the appearance of the active fragment (23 kD) (Figure 2c). Furthermore, both shFADD and mock keratinocytes showed the same percent of sub G1 peak positive cells in presence or in absence of anti-β_1 integrin. At 18 hrs, while keratinocytes reached high degree of anoikis and caspase-8 was fully activated, blocking FADD did not prevent cell death in keratinocytes (Figure 2d).

Direct interaction of β_1 integrin and caspase-8 in adherent and suspended cells. If keratinocytes undergo anoikis without the involvement of FADD, we reasoned that there must be a direct interaction between β_1 integrin and caspase-8. To determine whether caspase-8 and β_1 integrin interact in keratinocytes, adherent cells were double stained with anti-β_1 integrin and anti-caspase-8 antibodies. Subconfluent keratinocytes expressed β_1 integrin both at the membrane and in the cytoplasm, while caspase-8 displayed a diffuse cytosolic localization. Double staining revealed that β_1 integrin and caspase-8 co-localize in the cytoplasm (Figure 3a). Consequently, we investigated the interaction between caspase-8 and β_1 integrin in keratinocyte anoikis. Western blotting on lysates from suspended keratinocytes showed the appearance of caspase-8 active fragment (18 kDa) starting at 1 hr (Figure 3b). The same lysates were immunoprecipitated with anti-β_1 integrin antibody. Procaspase-8 and β_1 integrin co-immunoprecipitated at 0 hrs, while procaspase-8 progressively decreased, indicating activation, up to 24 hrs. Furthermore, suspension affected β_1 integrin conformation starting at 1 hr, and precipitated protein decreased at 6 hrs, eventually disappearing at 24 hrs. FADD did not immunoprecipitate with β_1 integrin-procaspase-8 complex, further confirming that death receptors are not involved in keratinocyte anoikis (Figure 3c). Taken together, these results clearly indicate that β_1 integrin and caspase-8 directly interact, and that β_1 integrin is altered during anoikis.

β_1 integrin is internalized and degraded during suspension. As β_1 integrin is progressively altered during keratinocyte suspension, we wanted to investigate the fate and the function of β_1 integrin in this system. To this end, we incubated keratinocytes with neutralizing anti-β_1 integrin antibody and performed anoikis assay. Confocal images demonstrate that β_1 integrin was partially internalized after 30 min and almost completely at 2 hrs (Figure 4a). Indeed, as suspension proceeded, cell-to-cell contacts progressively decreased along with the internalization of integrin, in agreement with the observation that cells detached from each other are more prone to this kind of cell death (Frisch and Francis, 1994). Moreover, in suspended cells, the extracellular portion of β_1 integrin was progressively degraded up to 24 hrs, as shown by the use of an antibody directed against this integrin domain (Figure 4b). When suspended keratinocytes were blotted using an antibody raised against the full-length β_1A integrin, a band of approximately 75 kD was detected between 2 and 24 hrs (Figure 4b), representing the cytoplasmic fragment of β_1A integrin, likely derived from the degradation of the extracellular domain. β_1B differs from wild-type β_1 integrin (β_1A) in the cytoplasmic domain (Altruda et al, 1990) and is mainly detected within the endoplasmic reticulum (Balzac et al, 1993; Kee et al, 2000). In agreement with previous works, we show that β_1A variant was mostly expressed at the cell membrane. By contrast, we detected β_1B form at the cytoplasmic level in a perinuclear fashion (Figure S2a). Given the difference in the cytoplasmic tails, we used specific antibodies recognizing the different β_1 integrin domains. Although both β_1A and β_1B were expressed in human keratinocytes, the content of β_1A protein was markedly higher than that of β_1B integrin (Figure S2b). The role of this integrin form in keratinocytes is not fully understood. It has been proposed that β_1B acts as a dominant negative regulator of cell adhesion, by interfering with focal contact formation, thus decreasing adhesiveness (Retta et al, 1998). To better understand the role of this β_1 integrin variant, we suspended human keratinocytes and blotted lysates using an antibody raised against β_1B integrin isoform. Unlike β_1A, no protein modifications of β_1B were observed up to 24 hrs (Figure 4b), indicating that no degradation of this variant occurs during suspension.

β_1A and β_1B integrins cluster and immunoprecipitate with caspase-8 during suspension. Confocal images of cells at 0 hr suspension show that β_1A expression was strictly localized at the cell membrane, while active caspase-8 was still undetectable. At 1 hr, caspase-8 became activated, while β_1A began to internalize. After 24 hrs suspension, caspase-8 was clearly detected with a cytoplasmic punctate pattern. At this time, β_1A integrin was also observed in perinuclear location (Figure 5a). On the other hand, at 0 hr, β_1B was predominantly expressed at the perinuclear level. This expression pattern remained unchanged up to 24 hrs, when caspase-8 was fully activated (Figure 5b). These results suggest that during keratinocyte suspension, β_1A integrin internalizes and locates close to the nucleus, likely co-localizing with β_1B. As β_1A and β_1B integrins co-localize with caspase-8 at the cytoplasmic level, we analyzed whether caspase-8 co-immunoprecipitates with either integrin isoforms. At 0 hr, we observed a weak band indicating a small amount of procaspase-8 bound to both β_1 integrin variants. After 18 hrs suspension, binding of β_1A or β_1B to caspase-8 was markedly enhanced. As negative controls, no precipitates were observed when lysates were omitted nor when primary antibody was replaced by total rabbit serum (Figure 5c).

Pro-apoptotic role of β_1B integrin. To definitely assess the pro-apoptotic role of β_1B integrin, keratinocytes were transfected with a full-length β_1B expressing vector. β_1B was up-regulated at 24 hrs (Figure 6a), and it remained overexpressed until 48 hrs, both at the mRNA and the protein level (Figure 6a and b). β_1B integrin expression was strikingly increased at the intracellular level in transfected cells up to 48 hrs (Figure 6c). Moreover, keratinocytes that expressed high levels of β_1B integrin spontaneously detached from the culture dishes, as compared to mock cells (Figure 6e) and to keratinocytes transfected with a full-length β_1A expressing vector where adhesion and morphology were not affected (Figure 6d and e). Before inducing suspension, keratinocytes underwent cell death, as it appeared from the significant increase in the percent of TUNEL positive cells 24 and 48 hrs after transfection with β_1B, but not with β_1A (Figure 6f). When

keratinocytes were suspended, activation of caspase-8 at 0 hr occurred only in β_1B integrin transfected cells. During suspension, activation of caspase-8 was observed earlier in β_1B integrin overexpressing cells than in mock cells, as shown by the appearance of the active fragment at 3 and 6 hrs (Figure 6g). On the other hand, caspase-8 activation was not detected in keratinocytes transfected with siRNA β_1B at 12 hrs (Figure 6h). Finally, percentage of cells in subG1 peak at 12 hrs is higher in scramble than in siRNA β_1B transfected keratinocytes (Figure 6i).

Discussion

In this study, we report that the beta 1 B isoform cooperates with antagonized beta1 A integrin to induce a form of integrin-mediated cell death in human keratinocytes, independently of death receptors. Death-domain-containing proteins trigger anoikis in epithelial cells (Frisch, 1999; Rytömaa et al, 1999), and anoikis is mediated by Fas/FasL and by the activation of caspase-8 in endothelial cells (Aoudjit and Vuori, 2001). Furthermore, FADD plays a critical role in detachment-induced apoptosis (Frisch, 1999), and epithelial cell lines expressing a dominant-negative form of FADD are protected from anoikis (Rytömaa et al, 1999). By contrast, we demonstrate that, while blocking beta 1 integrin activates caspase-8, death receptors are not involved in this system. Indeed, either cell suspension or anti-beta1 integrin failed to up-regulate Fas and FasL, and induced apoptosis in shFADD infected keratinocytes, with an early activation of caspase-8. This is consistent with previous work demonstrating that caspase-8 activation can occur in a manner independent of FADD (Stupack et al, 2001). Since keratinocytes undergo anoikis following the early activation of caspase-8, without the involvement of FADD, one might postulate that caspase-8 is activated downstream of caspase-3, in agreement with previous reports of death receptor-independent apoptosis (Ryu et al, 2005). Alternatively, caspase-8 activation could occur downstream of caspase-9 as an amplification loop of the intrinsic apoptotic pathway (Grossmann et al, 2001). However, both explanations have been ruled out by our previous study where we

reported that caspase-9 as well as executioner caspases are activated downstream of caspase-8 during keratinocyte anoikis (Marconi et al, 2004).

Given the exclusion of death receptors from the apoptotic pathway, we pursued the hypothesis of a direct interaction between integrins and caspase-8. Indeed, this study provides evidence that β_1 integrin and caspase-8 co-localize in the cytoplasm and co-immunoprecipitate even before detachment. Immediately after suspension, caspase-8 is activated, while integrin is internalized and degraded. This suggests that keratinocyte suspension promotes β_1 integrin fragmentation of the extracellular domain. Interestingly, this fragment was previously shown to induce apoptosis via caspase-8 activation in myocytes (Menon et al, 2006) and β_5 cytoplasmic domain is pro-apoptotic in squamous cell carcinoma cell lines (Janes and Watt, 2004). Association of integrins with caspase-8 has been previously reported by Stupack and coworkers who, at variance with our study, observed a direct interaction of caspase-8 with β_3 integrin only in cell lines adherent to an inappropriate ligand (Stupack et al, 2001). Furthermore, $\alpha_v\beta_3$ integrin has been recently reported to associate with caspase-8 in osteoclasts plated on a non-specific matrix, and procaspase-8 was only detected in suspended cells (Zhao et al, 2005). To our knowledge, the present study describes a previously unreported form of IMD in normal human keratinocytes. However, at variance with the above publications, we show that a direct association between procaspase-8 and β_1 integrin occurs in keratinocytes still adherent to the proper ligand. Thus in keratinocytes, β_1 integrin-procaspase-8 complex exists in a “pre-apoptotic” state until a stimulus triggers IMD, further confirming that integrins themselves can play a proapoptotic role (Giancotti et al, 1990; Plath et al, 2000).

We also show that β_1B isoform plays a critical role in IMD. β_1B integrin is mostly expressed in the cytoplasm and, unlike β_1 integrin, does not undergo degradation during suspension, while caspase-8 is activated. Moreover, β_1A and β_1B integrins cluster at the perinuclear level and immunoprecipitate with caspase-8 during suspension. A direct association between caspase-3 and $\alpha_5\beta_1$integrin was detected in non-adherent rat fibroblasts (Rajeswari et al, 2006), but, at variance with our study, activation of the caspase was observed only when the complex was

translocated to the cell membrane, and not in the cytoplasm. While the study did not address the signal allowing translocation, we hypothesize that, in our system, the β_1B variant located in the cytoplasm cooperates with the cytoplasmic fragment of β_1A. This integrin fragment derives from the degradation of β_1A integrin, and proves the unligated state of this integrin. At the cytoplasmic level, the two integrin isoforms enhance the recruitment of caspase-8 and induce cell death, in good agreement with previous results on the proapoptotic role of integrins (Kozlova et al, 2001).

The pro-apoptotic role of β_1B integrin was definitely shown both in cells overexpressing the cDNA and in cells where expression of the isoform was silenced by β_1B siRNA. It appears that β_1B is responsible for keratinocyte apoptosis even before cell suspension, with the early activation of caspase-8. These results indicate that the expression of β_1B integrin is sufficient to induce IMD in human keratinocytes, even when cells are adherent to the appropriate ligand. We postulate that unligated β_1A integrin is internalized, recruits procaspase-8 and clusters with β_1B integrin that in turn is ligated to procaspase-8 at perinuclear level. Caspase-8 is then activated, leading to the initiation of the extrinsic apoptotic pathway.

Squamous-cell carcinomas (SCCs) are tumors that arise in multilayered epithelia such as the epidermis, esophagus, cervix and the oral cavity. While the tumors differ according to tissue of origin in their frequency, aetiology and prognosis, they are all characterized by the alteration of integrins. In the epidermis, SCC may originate from keratinocyte stem cells (Zhang et al, 2005), which are protected from anoikis by virtue of the high levels of β_1 integrin (Tiberio et al, 2002). SCCs are characterized by perturbation of integrin expression and extensive loss of the basement membrane (Ferreira et al, 2009; Savoia et al, 1994). Keratinocyte stem cells detach from the basement membrane, proliferate and may reattach to an inadequate location to eventually metastasize. We speculate that β_1B integrin works as a “sensor” of the detachment from the proper ligand and stimulates apoptosis to prevent spread of SCC, thus acting as a “fail-safe” device.

Materials and Methods

DNA constructs

Retroviral constructs pSUPER.Retro and pSUPER.Retro.shFADD were a kind gift from S.M. Frisch (West Virginia University, USA). A full-length β_1B or β_1A integrin cDNA were generated by RT-PCR and inserted in the pcDNA3.1/V5-His using the TOPO TA Expression kit (Invitrogen Corporation, Carlsbad, CA, USA).

Cell culture and infection

Normal human keratinocytes were obtained from foreskin and cultured as described previously (Pincelli et al, 1997). The spontaneously transformed keratinocyte line HaCaT was kindly provided by Dr. N. Fusenig (DKFZ Heidelberg) and cultivated as already described (Boukamp et al, 1988). Packaging lines for the described retroviral constructs were generated by transinfection in the ecotropic Phoenix and amphotropic GP+envAm12 cells, as previously described (Grande et al, 1999). HaCat cells were transduced by two cycles of infection (6 hrs each) with viral supernatant in presence of polybrene (8 μg/ml). Transduced cells were selected in presence of 1 μg/ml puromycin (Sigma, St Louis, MO). Anoikis assay was performed as previously described (Marconi et al, 2004).

Cell culture and transfection

HaCaT cells were transfected with described pcDNA3.1 plasmids with Lipofectamine 2000 and OptiMEM (Invitrogen) as suggested in the datasheet. Subconfluent normal human keratinocytes were double transfected with β_1B integrin siRNA (Sense: CAAAGUGGCUUAUAAAGUAUU; Antisense: 5'-PUACUUUAUAAGCCACUUUGUU; Thermo Scientific) with Lipofectamine 2000 and OptiMEM (Invitrogen) as suggested in the datasheet. Protein levels were detected by

Western Blotting as described below. Anoikis assay was performed as previously described (Marconi et al, 2004).

Antibodies

Rabbit polyclonal antibodies used were: anti-caspase 8 (AR-18; gift of J.C. Reed, The Burnham Institute, USA); anti-β_1 integrin (M-106; Santa Cruz Biotecnology, Inc.); anti-β_1A and anti-β_1B antisera (gift from G. Tarone, University of Turin, Italy). Mouse monoclonal antibodies used were: neutralizing anti-β_1 integrin (Lia 1/2; Immunotech, Marseile, France); anti-β_1 integrin (K20; Santa Cruz Biotecnology, Inc.); anti-caspase 8 (Ab-3; Calbiochem, Darmstadt, Germany); anti-active caspase 8 (C15; Alexis Biochemicals); anti-FADD (A66-2; BD PharMingen); anti-β actin (AC-15; Sigma). Secondary antibodies: Alexa Fluor 546 and 488-conjugated goat IgGs (Invitrogen); HRP-conjugated goat IgGs were also used (BioRad, Hercules, CA).

FACS, flow cytometry

For anoikis (sub G1 peak positive), cells were suspended in hypotonic fluorochrome solution: propidium iodide (PI) 50 mg/ml, 0.1% sodium citrate, 0.1% Triton X-100; labeled at 4°C for at least 15 minutes and then analyzed using Epics XL flow cytometer (Epics XL, Coulter, Hialeah, FL, USA). Plots were analyzed by WinMDI 2.8 software.

Both cells undergone anoikis or treated with PVIgG, as a positive control for Fas Ligand expression, were analyzed for extracellular antigen expression. Cells were labelled with monoclonal anti-human Fas antibody (clone UB2; Immunotech) and with biotin-coniugated rabbit anti-mouse immunoglobulin (Dako, Copenaghen, Denmark), or with biotin conjugated anti-human FasL monoclonal antibody (Clone NOK-1; BDPharMingen) diluted in PBS with 5% FBS. Cells were then labelled with streptavidin/RPE-Cy5 (Dako) and analyzed with Epics XL flow cytometer (Coulter). Plots were analyzed by WinMDI 2.8 software.

ELISA

To measure the release of soluble FasL during anoikis, we performed a two-site enzyme linked

immunoassay ELISA (MBL, Nagoya, Japan) on culture media according the manufacturer's instructions.

MTT assay

MTT assay was performed in selected infected keratinocyte. After treatment with TRAIL (kindly provided by Dr. Martin Leverkus, Magdeburg, Germany) and CHX 1μg/ml, cells were incubated with MTT (Sigma) as previously described (Truzzi et al, 2008). Results are expressed as the mean ± SD of three different experiments. Student's T-test was used for comparison of the means.

Co-immunoprecipitation and immunoblotting

Lysates were processed for IP and WB as described previously (Marconi et al, 2004) or with lysis buffer (50 mM Tris-HCl, pH 8.5, 150 mM NaCl, 1% deoxycolate, 1% Triton X-100, 0.1% SDS, 0.2% sodium azide and inhibitor cocktail [Roche]). Proteins were immunoprecipitated with anti-β_1 integrin (K20) or anti-β_1 antisera. Samples were resolved by 7 or 12% SDS-PAGE and immunoblotted as described previously (Marconi et al, 2004).

Immunofluorescence analysis

Sub-confluent cells were fixed in ice-cold methanol or PFA 4% and incubated with monoclonal anti-β_1 integrin antibody and the appropriate secondary antibody. Cells were then labelled with polyclonal anti-caspase 8 antibody and appropriate secondary antibody. Alternatively cells were first stained with rabbit anti-β_1 integrin variants antisera and with mouse anti-active caspase-8. Micrographs were taken on a Confocal Scanning Laser Microscopy (Leica TCS4D) (Leica, Exton,PA). In selected cases, a series of images of the same x/y plane was taken and the reconstruction made on the z-axis.

Internalization assay

Keratinocytes were pre-incubated with neutralizing anti-β_1 integrin antibody and anoikis assay was performed. At each time-point, cells were fixed in PFA 4% and immunofluorescence performed with the addition of the secondary antibody.

RT-PCR

Total RNA was extracted from transfected cells and RT-PCR was performed as previously described (Truzzi et al, 2008). Nucleotide sequences of the β_1B integrin oligomers used (MWG Biotech, Ebersberg, Germany) were: β_1B-DP: 5'-gggaacaacgaggtcatggttcat-3'; β_1B-RP: 5'-ttataagccactttgctttttggatg-3'.

TUNEL assay

Cells were fixed in PFA 4% both 24 and 48 hrs after transfection. Then cells were stained with the In Situ Cell Death Detection Kit (Roche Diagnostics, Basel, Switzerland) as recommended by manufacturer. Positive cells were analyzed as detailed previously (Truzzi et al, 2008).

Conflict of Interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1. Fas/FasL modulation in keratinocyte anoikis. (**a**) Fas expression. Keratinocytes were suspended in polypropylene tubes in presence or absence of anti-β_1 integrin neutralizing antibody. Cells were then incubated with anti-Fas antibody and analyzed by flow cytometry. (**b**) FasL expression. Keratinocytes were treated as before, incubated with anti-FasL antibody and analyzed by flow cytometry. Pemphigus vulgaris immunoglobulins G (PVIgG) were used as a positive control stimulus for FasL (Arredondo et al, 2005). (**c**) sFasL release. Keratinocytes were kept in suspension in polypropylene tubes for different time-points and culture media were collected. Soluble FasL in culture media was analyzed by ELISA. PVIgG were used as a positive control stimulus for FasL.

Figure 2. FADD is not required for keratinocyte anoikis. (**a**) FADD protein expression in infected HaCaT keratinocytes. Proliferating HaCaT cells were retrovirally infected with FADD shRNA (HaCaT shFADD) or with control shRNA (HaCaT mock). Cells were selected in presence of puromycin and lysed. Lysates were run on SDS-acrylamide gel and blotted onto nitrocellulose membrane. Blot was incubated against anti-FADD antibody. β-actin was used as loading control. (**b**) shFADD prevents TRAIL-induced apoptosis in keratinocytes. Infected HaCaT cells were treated with increasing doses of TRAIL and viability was analyzed by MTT assay after 24 hrs. (**c**) Caspase-8 activation in shFADD cells. Retrovirally infected HaCaT cells were suspended and treated with anti-β_1 integrin neutralizing antibody. Cells were lysed at different time points, and lysates were immunoblotted with anti-caspase-8 antibody. β-actin was used as loading control. (**d**) shFADD cells show the same percent of sub G1 peak positive cells. Mock and shFADD keratinocytes were kept in suspension in polypropylene tubes in presence or absence of anti-β_1 integrin antibody. After 18 hrs cells were stained in PI-solution. Sub-G1 peak positive cells (M1) were analyzed by flow cytometry. Data represent the mean from three independent experiments.

Figure 3. β_1 integrin associates with caspase-8. (**a**) β_1 integrin co-localizes with caspase-8. Subconfluent normal human keratinocytes were fixed in PFA 4%. Cells were immunostained with anti-β_1 integrin and anti-procaspase-8 antibodies. Bottom panel represents the merge. Cells were analyzed by confocal microscopy. Bar, 15 μm. (**b**) Early activation of caspase-8 during anoikis. Normal human keratinocytes were trypsinized (0 hr) and kept in suspension in polypropylene tubes for different time-points. At the end of single time-point cells were lysed and lysates were immunoblotted with anti-procaspase-8 antibody. β-actin was used as loading control. (**c**) β_1 integrin immunoprecipitates with procaspase-8. Keratinocytes were treated as in (b). Lysates were immunoprecipitated with anti-β_1 integrin antibody. Immunoprecipitates were blotted with anti-β_1 integrin, anti-procaspase-8 and anti-FADD antibodies. Control 1, immunoprecipitate with anti-β_1 integrin antibody without lysates; control 2, immunoprecipitate with a non-keratinocyte antigen antibody (anti-vimentin). Control samples were run simultaneously in a different gel.

Figure 4. β_1 integrin is internalized and degraded during keratinocyte suspension. (**a**) β_1 integrin internalization. Keratinocytes were incubated with the neutralizing anti-β_1 integrin antibody for 20 min at 4°C. Cells were then suspended for different time points, fixed in PFA 4% and analyzed by confocal microscopy. Images outside the x/y planes represent the z-line projection of the cross-identified area. Bar, 8,5 μm. (**b**) β_1 integrin degradation. Keratinocytes were suspended in polypropylene tubes and lysed at different time points. Lysates were probed against β_1 integrin, using an antibody directed to the extracellular portion. The same lysates were blotted with anti-β_1A and β_1B integrin antisera. β-actin was used as loading control.

Figure 5. β_1 integrin variants immunoprecipitate with caspase-8 during suspension. (**a-b**) β_1A and β_1B integrin localization during caspase-8 activation. Keratinocytes were suspended in polypropylene tubes and fixed in PFA 4% at different time points. Cells were spinned and immunostained at room temperature with anti-β_1A (a) and anti-β_1B integrin (b) antisera and anti-active caspase-8 antibody (a,b). Cells were analyzed by confocal microscopy. (a) Bar 21 μm. (b)

Bar 17 μm. (**c**) β_1 integrin variants immunoprecipitate with procaspase-8. Keratinocytes were suspended in polypropylene tubes and lysed after 18 hrs of suspension. Lysates were alternatively immunoprecipitated with anti-β_1B integrin, anti-β_1A integrin or anti-extracellular domain of β_1 integrin antibodies. Immunoprecipitates were blotted with anti-procaspase-8. Control 1: immunoprecipitate without lysates; Control 2: immunoprecipitate with total rabbit serum.

Figure 6. β_1B integrin is pro-apoptotic in keratinocytes. (**a**) HaCaT cells were transfected with a full-length β_1B integrin-containing vector (pcDNA3.1/β_1B) or with the empty vector (pcDNA3.1). m-RNA was extracted from cells after 24 and 48 hrs of transfection, β_1B integrin expression was analyzed by RT-PCR. β-actin was used as loading control. (**b**) HaCaT cells were transfected as in (a). Cells were lysed and protein extract were blotted with anti-β_1B integrin antibody. β-actin is the loading control. (**c**) HaCaT cells were transfected as before. Keratinocytes were fixed in PFA 4% after 24 and 48 hrs of transfection. Cells were spinned and stained with anti-β_1 integrin antibody and analyzed at confocal microscpy. Images outside the x/y planes represent the z-line projection of the cross-identified area. Bar 12 μm. (**d**) HaCaT cells were transfected with a full-length β_1A integrin-containing vector (pcDNA3.1/β_1A). Cells were lysed and protein extracts were blotted with anti-β_1A integrin antibody. β-actin is the loading control. (**e**) Micrograph pictures of transfected cells after 48 hrs of transfection. Bar 50 μm (**f**) HaCaT cells transfected with β_1B, β_1A or empty vector were stained with TUNEL assay 24 and 48 hrs after transfection. (**g**) β_1B transfected HaCaT cells were trypsinized 48 hrs after transfection and kept in suspension in polypropylene tubes for different time-points. Cells were lysed and lysates were blotted against anti-caspase-8 antibody. β-actin was used as loading control. (**h**) Primary human keratinocytes were transfected with siRNA β_1B or scramble and kept in suspension in polypropylene tubes for 12 hrs. Cell lysates were blotted against anti-β_1B integrin or anti-caspase-8 antibody. β-actin was used as loading control. (**i**) In the same experiment, cells were stained in PI-solution. Sub-G1 peak positive cells (M1) were analyzed by flow cytometry. Data represent the mean from three independent experiments.

Supplementary Materials