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# Integrin α8β1–fibronectin interactions promote cell survival via PI3 kinase pathway

Eduardo Farias [a], Min Lu [a], Xianwu Li [b], Lynn M. Schnapp [a,b,*]

[a] Department of Medicine, Mount Sinai School of Medicine, New York, NY, USA
[b] Division of Pulmonary and Critical Care Medicine, Harborview Medical Center, University of Washington, Seattle, WA, USA

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**Abstract**

Integrin signaling plays a critical role in many aspects of normal growth, differentiation, and injury response. In the adult, α8β1 is expressed in alveolar myofibroblasts and is upregulated in pulmonary fibrosis and other models of organ injury. Following injury, survival of fibronectin-producing myofibroblasts cells is an important determinant of development of fibrosis. Using stable α8-transfected cell lines, we show that interactions of α8β1 with its ligand, fibronectin, promote cell survival during serum deprivation. Multiple cell signaling pathways were activated following fibronectin adhesion, including PI3 kinase and MAP kinase. However, the α8-mediated cell survival was blocked by LY294002, a PI3 kinase inhibitor, but not by staurosporine, a PKC inhibitor, or PD98059, a MAPK kinase inhibitor. A dominant negative construct of PI3 kinase also inhibited α8-mediated cell survival. Therefore, α8-mediated survival appears to be mediated by the PI3 kinase pathway. Survival of α8-expressing myofibroblasts may contribute to persistent fibrosis following injury.


*Keywords:* Integrin; Apoptosis; Signaling pathway

Integrins, a class of cell adhesion molecules, mediate cell–extracellular matrix (ECM) interactions and regulate many cell behaviors such as cell survival, adhesion, migration, proliferation, and differentiation, which are critical to the wound-healing response [1]. The integrin α8 subunit forms a heterodimer exclusively with β1 subunit [2–4]. In the adult, α8β1 is expressed in mesenchymal cells, including alveolar myofibroblasts, kidney mesangial cells and hepatic stellate cells, and vascular and visceral smooth muscle [3]. α8β1 expression is upregulated after injury in models of lung, liver, kidney, and myocardial injury [5–7].

In the lung, α8β1 is expressed in the contractile interstitial cells and is upregulated following injury. During kidney development, α8β1 is selectively expressed in the condensed mesenchyme [8] and is critically important for the epithelial conversion of mesenchymal cells. Disruption of the α8 gene results in severely impaired kidney development and early postnatal death due to kidney failure [8]. α8β1 recognizes the tripeptide sequence arginine-glycine-aspartic acid (RGD) in several extracellular matrix proteins including fibronectin, tenascin, vitronectin, nephronectin, and osteopontin [4,9–13]. However, the signaling pathways activated following ligand interaction remain unknown.

Interactions between interstitial cells and ECM proteins play an essential role in the fibrotic phase of acute injury. During fibrosis, there is proliferation of interstitial fibroblasts and an increase in ECM production. Fibronectin, as well as other components of the provisional wound-healing matrix, provides a scaffold on which fibroblasts migrate. Fibronectin also provides a

* Corresponding author. Fax: +1 206 341 5392.
*E-mail address:* lschnapp@u.washington.edu (L.M. Schnapp).

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chemotactic and proliferative signal for fibroblasts. Fibroblast proliferation and migration towards injured tissues is part of the normal repair response following injury. However, at times the normal repair process goes awry, resulting in persistent fibrosis. There is increasing evidence that failure of interstitial fibroblasts to undergo apoptosis contributes to persistent fibrosis.

Since α8β1 is expressed on many interstitial cells and is upregulated following injury, we asked whether binding of α8β1 to its ligand, fibronectin, affected cell survival. In addition, we asked what signaling pathways were activated following fibronectin adhesion. We found that α8–fibronectin interaction protected cells from serum deprivation induced apoptosis. While multiple signaling pathways were activated by fibronectin adhesion, the cell survival signal appears to be primarily mediated through the PI3 kinase pathway.

## Materials and methods

*Cells and reagents*. AtT20 cells were a generous gift from Dr. Jean Schwarzbauer (Princeton University, NJ) and maintained in DMEM/Ham's F12, 10% fetal calf serum (FCS), 10% Nu-serum (Sigma), 200 μm Hepes, and penicillin/streptomycin. Cells were transfected with pCDNAIneoα8 (α8-transfected cells) or pCDNAIneo alone (mock-transfected cells) using the Lipofectin reagent (Gibco-BRL) according to the manufacturer's instructions. Stably transfected cell lines were selected in medium containing the neomycin analog G418 (0.4 mg/ml). The inhibitors LY294002, staurosporine, bisindolylmaleimide, and PD98059 were purchased from Sigma.

Generation and characterization of rabbit polyclonal antibody to α8 subunit was previously described [9,14]. Antibodies to Bcl-2, Bax, p-Akt (which recognizes phosphorylated S472 and S473), Akt, and PARP (which recognizes the intact 116 kDa protein and the 85 kDa proteolytically cleaved fragment) were obtained from PharMingen. Antibodies to ERK and actin were obtained from Santa Cruz. Fibronectin (FN) was purchased from Boerhinger–Mannheim, collagen type I (Col I) and poly-L-lysine (PLL) were purchased from Sigma.

*Immunoprecipitation*. In some experiments, cell surface proteins were biotinylated with Sulfo-NHS-biotin (Pierce) according to manufacturer's recommendation. Cells were lysed in IP buffer (100 mM Tris-base, 150 mM NaCl, 1 mM $CaCl_2$, 1% Triton-X, 0.1% SDS, and 0.1% NP-40) and samples were incubated with antibodies overnight at 4 °C. Immune complexes were captured with protein A–Sepharose and beads were washed five times, boiled in Laemmli's sample buffer, and separated by SDS–PAGE. Gels were transferred to Immobilon, blocked overnight, and developed with enhanced chemiluminescence (ECL) technique (Amersham) or processed for Western blot analysis.

*Western blot analysis*. Cells were plated in serum-free media onto Petri dishes coated with fibronectin (10 μg/ml) or 0.01% poly-L-lysine. At indicated timepoints, cells were lysed in buffer containing 50 mM Tri–HCl (pH 7.4), 150 mM NaCl, 0.25% sodium deoxycholate, 1% IGEPAL CA-630, 1 mM EGTA, 1 mM PMSF, 1 mM sodium vanadate, 1 mM sodium fluoride, and 1 mg/ml each of aprotonin, leupeptin, and pepstatin. Equal amounts of protein were separated by SDS–PAGE, transferred to Immobilon, and blocked for 1 h. Blots were incubated with primary antibody for 1 h, followed by peroxidase-conjugated secondary antibody for 1 h and then developed with ECL.

*Adhesion assays*. The assays were performed as previously described [9]. Briefly, untreated polystyrene 96-well flat-bottomed microtiter plates (Evergreen) were coated with fibronectin (10 μg/ml) for 1 h at 37 °C. As a negative control, wells were coated with 1% BSA. After washing with phosphate-buffered saline (PBS), non-specific protein binding sites were saturated with 1% BSA for 30 min at 37 °C. Cells were detached with 2 mM EDTA, washed with PBS, and resuspended in serum-free DMEM (pH 7.4) with or without 5 mM $Mn^{2+}$. Fifty thousand cells were added to each well, centrifuged at 10*g* for 3 min to ensure uniform settling of cells, and incubated for 1 h at 37 °C. Non-adherent cells were then removed by centrifugation (top-side down) at 10*g* for 5 min. The attached cells were fixed and stained with 1% formaldehyde/0.5% crystal violet/20% methanol for 30 min at RT. After washing with PBS, adherence was determined by measuring absorption at 595 nm in a Microplate Reader (Bio-Rad, Richmond, CA). The data were reported as the mean absorbance of triplicate wells ± SE, minus the mean absorbance of BSA-coated wells.

*Flow cytometry analysis*. AtT20-α8, AtT20 mock transfected or wild type AtT20 cells were plated on fibronectin (10 μg/ml) for 24 h in serum-free medium. Adherent and suspended cells were collected, pelleted and resuspended in cold PBS, and added dropwise to 800 μl ice-cold ethanol and fixed at −20 °C for 30 min. Cells were spun at 800 rpm for 5 min, resuspended in 800 μl cold PBS, passed through a 25 gauge needle to remove clumps, and incubated with RNAse (1 mg/ml) and 100 μl propidium iodide (PI) (400 mg/ml). Cells (10,000 per condition) were analyzed by FACscan (Becton–Dickinson) and data were analyzed with CellQuest software (Becton–Dickinson).

*Confocal laser scanning microscopy*. Cells were examined using a Leica TCS-SP (UV) confocal laser scanning microscope (Heidelberg, Germany) equipped with a 100× 1.4 n.a. objective lens. The pinhole size was adjusted such that resultant "optical sections" were less than 0.5 μm in thickness. The sensitivity of the photomultiplier detectors was set such that the intensity levels of the output signal in the plane of maximum fluorescence intensity were distributed in a linear fashion (via a glow over/under lookup table) over 256 gray levels (with the dimmest pixel, black = 0 and the brightest pixel, white = 255).

*Cell death ELISA*. $1 \times 10^4$ cells were plated in serum-free medium in 96-well plates coated with fibronectin (10 μg/ml) or PLL for 24 h. In some experiments, the inhibitors LY294002, staurosporine, bisindolylmaleimide, or PD98059 (20 μM) (Sigma) were added to cells. Apoptosis was measured with Cell Death Detection ELISA-plus System (Roche), per manufacturer's instructions. All experiments were performed in triplicate and presented as means ± SE.

*Transfections*. The dominant negative PI3 kinase construct (DN p85) was a generous gift of Dr. Julian Downward, Imperial Cancer Research fund, London, UK [15,16]. DN p85 and the surface selection marker CD20 (gift from Dr. James Manfredi, (Mount Sinai School of Medicine) were co-transfected at a 10:1 ratio using FuGene (Roche Diagnostics, IN). Control consisted of cells co-transfected with empty vector and CD20. After 48 h, CD20 positive cells were selected with anti-CD20 antibody (BD Pharmingen, San Diego, CA) and immunomagnetic beads (Dynabeads M-450 conjugated to goat anti-mouse IgG, Dynal Biotech, Brown Deer WI) following the manufacturer's protocol. Transfected (DN p85 or vector) α8-AtT20 cells were plated on Fn (10 μg/ml) in serum-free media for 24 h. Cells were then stained with acridine orange (5 μM/ml) to identify apoptotic (red) cells and counterstained with ethidium bromide (5 μM/ml). Fluorescence microscopy was performed; apoptosis was quantitated by counting 200 cells in four independent fields. Two independent experiments were performed.

## Results

### *Characterization of α8-transfectants*

Most cell lines, including fibroblasts, express additional fibronectin receptors, such as α5β1 or αv-containing integrins. AtT20 cells, a neuroendocrine cell line, do

not express any fibronectin receptors, do not adhere to fibronectin, and do not secrete fibronectin [17]. Therefore, we transfected AtT20 with full-length human α8 construct driven by a CMV promoter to enable us to directly assess the contribution of α8β1 to behaviors observed on fibronectin. To verify cell-surface expression of α8β1 in stably transfected cells, we biotinylated surface proteins of several clones of α8-transfected cells and immunoprecipitated with a polyclonal α8 antibody. Several clones of AtT20 cells were obtained with high levels of surface expression of α8β1 (Fig. 1A). To verify that surface expression of α8β1 resulted in a functional receptor, α8-transfected clones were examined for adhesion to fibronectin, a known ligand [4,9]. We found a significant increase in the ability of α8-transfected cells to adhere to and spread on fibronectin (Fig. 1B). α8β1-mediated binding to fibronectin was increased in the presence of 5 mM $Mn^{2+}$ (Fig. 1B), similar to previous reports that α8β1 binding to osteopontin was increased in the presence of $Mn^{2+}$ [12]. The level of surface expression of α8β1 correlated roughly with increased fibronectin adhesion.

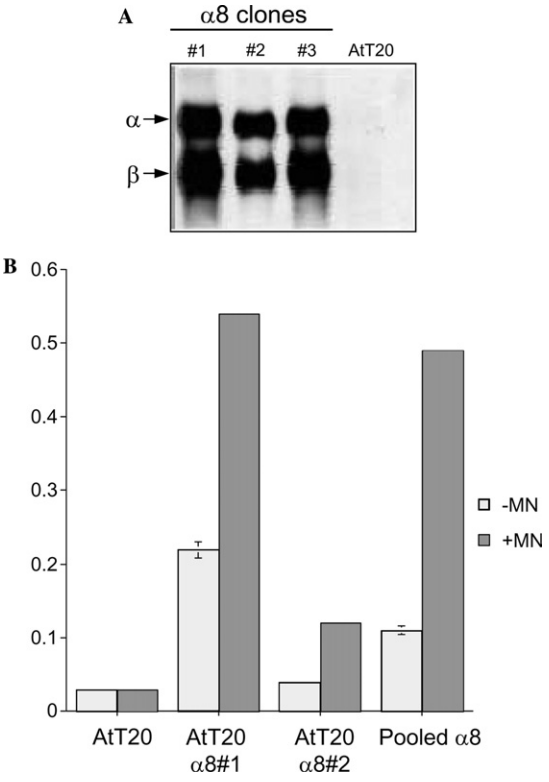


Fig. 1. (A) Immunoprecipitation of α8β1 from transfected AtT20 cell clones. Aliquots of biotinylated surface labeled lysates from three independent transfected clones and parental AtT20 cells were immunoprecipitated with rabbit polyclonal anti-α8 antibody. Proteins were analyzed by SDS–PAGE under non-reducing conditions. Positions of α and β subunits are indicated by arrows. (B) Increased adhesion of α8-transfected cells to fibronectin. Mock-transfected AtT20 cells, α8-transfected clones #1, #2 or pooled α8-transfectants were allowed to attach to wells coated with 10 μg/ml fibronectin in the absence or presence of 5 mM manganese. After 1 h, non-adherent cells were removed by washing and adherent cells were fixed and stained with crystal violet. Adherent cells were quantitated by measuring the absorbance of wells at OD 595 nm.

*α8β1 decreases apoptosis on a fibronectin matrix*

Apoptosis of fibrogenic cells is an important determinant of fibrosis resolution in an injured tissue [18]. Therefore, we examined whether α8-expression conferred protection from apoptosis in cells grown on fibronectin. Apoptosis in α8-expressing cells grown on fibronectin in serum-free medium was significantly decreased compared to wild type or mock-transfected AtT20 cells, as indicated by cell cycle analysis by flow cytometry (Fig. 2A). We correlated these findings with PARP cleavage as a biochemical marker of apoptosis. During apoptosis, active caspase 3 cleaves intact PARP (116 kDa) into 85 and 25 kDa fragments [19]. Thus, an increase in cleaved PARP is an indicator of apoptosis. We confirmed that α8-AtT20 cells grown in serum-free media for 24 h resulted in less apoptosis (less 85 kDa PARP fragment) than wild type or mock-transfected cells grown on fibronectin (Fig. 2B). When α8-AtT20 cells were grown on collagen I or polylysine, there was no protection from apoptosis (Fig. 2).

We then examined the phosphorylation of several components of different signaling pathways to determine which may be involved in α8-mediated signaling. FAK is a tyrosine kinase that is a component of focal adhesions phosphorylated following integrin-mediated adhesion and can suppress apoptosis in several cell types [20–22]. We previously showed that α8β1-mediated adhesion of cells to fibronectin resulted in phosphorylation of FAK [17]. FAK can activate PI3 kinase, either directly or indirectly through Src kinase [23]. To determine whether the PI3 kinase pathway was involved in the α8 cell survival signal, we examined the phosphorylation of Akt, a downstream effector of PI3 kinase. We found an increase in phosphorylated Akt in α8-AtT20 cells (but not wild type) after 30 min on fibronectin, compared to polylysine or collagen I (Fig. 3A). We previously showed that α8β1-mediated signaling resulted in phosphorylation of extracellular signal regulated kinase (ERK). We now show that ligation of α8β1 by fibronectin results in the translocation of ERK from the cytoplasm to the nucleus, another hallmark of ERK activation (Fig. 3B).

We also examined the expression of Bax and Bcl-2, pro- and anti-apoptotic proteins, respectively. Previous reports indicated that the prosurvival signal mediated through α5β1-fibronectin interactions was due to increased Bcl-2 levels [24]. However, we found no change in Bcl-2 and Bax expression in α8-transfected and wild type AtT20 cells (Fig. 3C). Thus, in contrast to α5β1, the improved survival of α8-transfected AtT20 cells

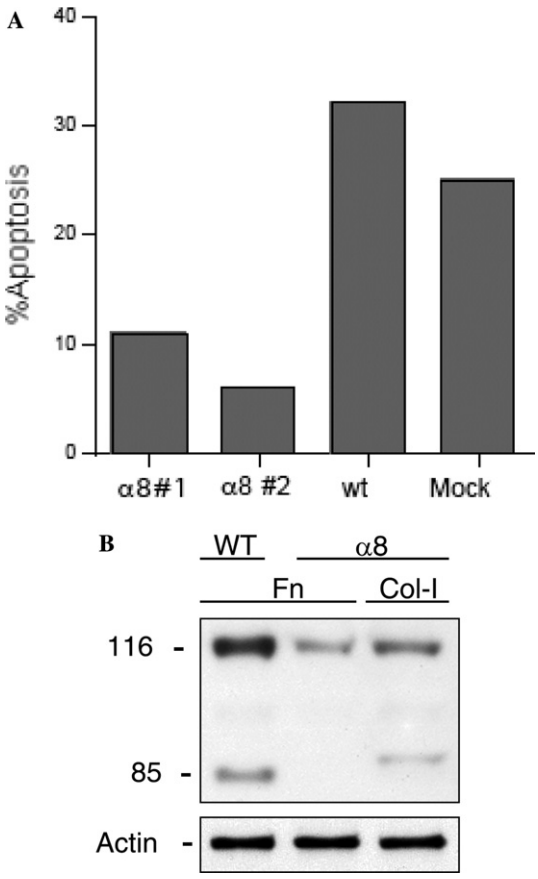


Fig. 2. (A) α8-transfected cells show decreased apoptosis when grown on fibronectin. WT, mock-transfected or α8-transfected AtT20 cells were plated on fibronectin-coated dishes in serum-free media for 24 h. Cells were treated with RNAse and stained with PI for 30 min at 37 °C. Cells (10,000 per condition) were analyzed with argon-ion laser at 488 nm, and forward and orthogonal light scatter and red fluorescence were measured. Cell cycle analysis was performed with Becton–Dickinson FACScan and CellQuest Software. (B) PARP cleavage. $10^6$ AtT20 or α8-AtT20 cells were plated on fibronectin (Fn) or collagen (Col I) in serum-free media for 24 h. Cells were lysed and equal amounts of protein were loaded and blotted with an antibody to PARP. The full length PARP (116 kDa) and cleaved PARP (85 kDa) are indicated. Blots were probed with actin antibody to control for loading.

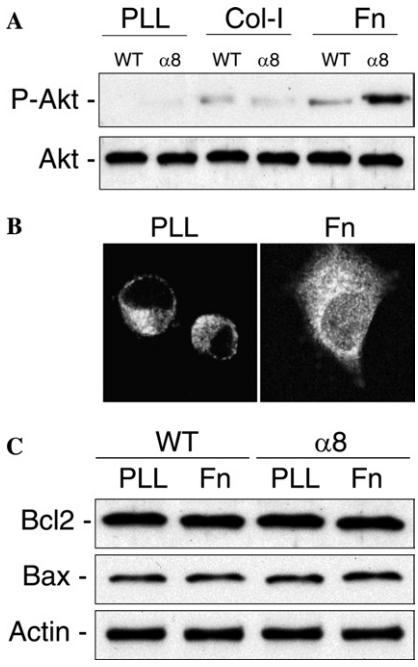


Fig. 3. (A) Adhesion of α8-transfected cells to fibronectin results in phosphorylation of Akt. Wild type or α8-transfected AtT20 cells were plated on polylysine (PLL), collagen type I (Col I) or fibronectin (Fn) (10 μg/ml) for 30 min, 37 °C. Cells were lysed in buffer containing phosphatase inhibitors. Equal amounts of protein were loaded and blotted with the antibody to phospho-Akt (top) or Akt (bottom). (B) Translocation of ERK to nucleus after adhesion to fibronectin. AtT20-α8 cells were plated on Fn (10 μg/ml) or PLL for 30 min, then fixed, permeabilized, and incubated with anti-ERK antibody, followed by FITC-conjugated anti-mouse antibody. Confocal microscopy was performed to visualize nuclear staining. (C) No change in Bcl-2 and Bax expression. AtT20-α8 or wild type AtT20 cells were grown for 24 h in serum-free media on fibronectin (FN) or polylysine (PLL). Cells were lysed and equal amounts of protein were loaded and probed with antibody to Bcl-2 or Bax. To control for loading, blots were re-probed with actin antibody.

grown on fibronectin is not due to a change in Bcl-2/Bax ratio.

To determine the signaling pathways involved in α8-mediated survival, we grew cells in the presence of several pharmacological inhibitors. We found that α8-transfected cells grown on fibronectin in the presence of LY294002, a specific inhibitor of PI3 kinase, underwent apoptosis to a similar degree as mock-transfected cells (Fig. 4A). Other inhibitors, including PKC inhibitor, staurosporine, and bisindolylmaleimide, and the MAPK kinase inhibitor, PD98059, had no significant effect. This suggests that the α8-mediated cell survival signal involves the PI3 kinase pathway. To confirm the involvement of the PI3 kinase pathway, we co-transfected cells with a selection marker (CD20) and a construct of PI3 kinase (DN p85) that contains a mutation in the peptide required to activate the catalytic subunit p110 (Fig. 4B) [16]. Therefore, the regulatory subunit p85 binds to the catalytic subunit p110 but is unable to activate it. As expected, AtT20-α8 cells transfected with the dominant negative PI3 kinase did not phosphorylate Akt when grown on fibronectin (Fig. 4C). Furthermore, they were no longer protected from apoptosis when grown on fibronectin (Figs. 4D and E). Taken in concert, the data show a role for PI3 kinase pathway in the cell survival signal mediated by α8.

## Discussion

Our data suggest that engagement of α8β1 by fibronectin protects cells from pro-apoptotic stimuli, such as serum deprivation. Ligation of α8β1 by fibronectin

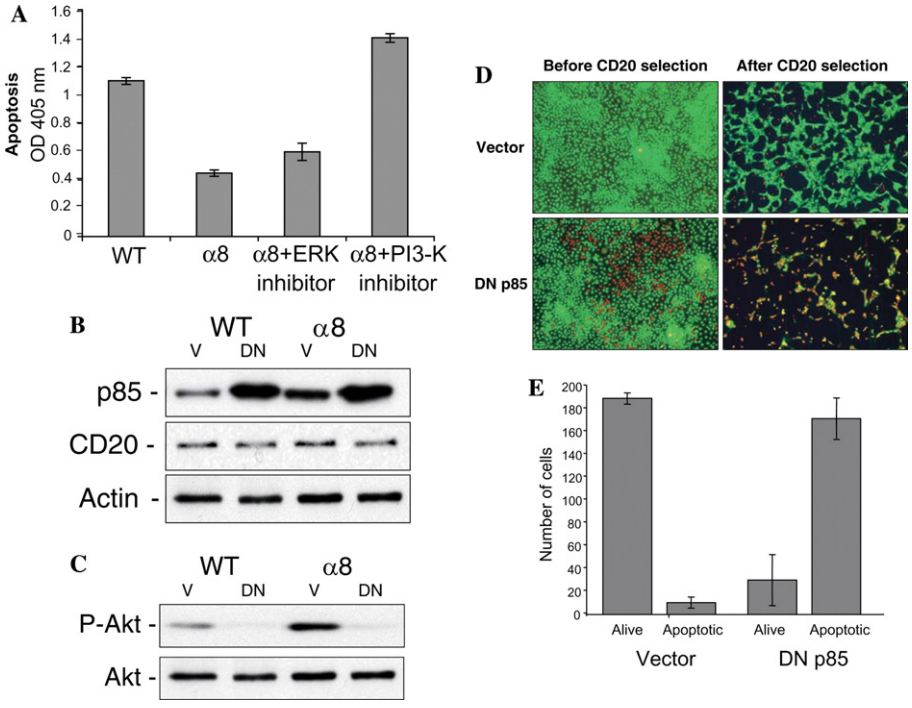


Fig. 4. PI3 kinase signaling pathway is required for α8-mediated survival. (A) Wild type or α8-transfected AtT20 cells were grown for 24 h in serum-free media on fibronectin alone or with the MAP kinase inhibitor PD98059 or PI3 kinase inhibitor LY294002. Apoptosis was measured using the Cell Death Detection ELISA-plus System (Roche). All experiments were done in triplicate and are reported as means ± SD. (B) α8 and wild type AtT20 cells were co-transfected with CD20 (for selection) and vector alone (V) or dominant negative PI3 kinase construct (DN). To verify transfection, cell lysates were separated by SDS–PAGE and probed with antibodies to p85, CD20, and actin (loading control). (C) Dominant negative PI3 kinase (DN) eliminates Akt phosphorylation. Transfected cells were serum starved, plated on fibronectin for 30 min, and then lysed, and equal amounts of protein were separated by SDS–PAGE and blotted with antibody to phospho-Akt (top) or Akt (bottom). (D) Dominant negative PI3 kinase inhibits α8-mediated survival. α8-transfected AtT20 cells, transfected with DN or control vector, were grown on fibronectin in serum-free media for 24 h. Cells were stained with acridine orange to identify apoptotic cells and counterstained with ethidium bromide. Representative fields are shown before and after CD20 selection. (E) Apoptosis was quantitated by counting apoptotic and total number of cells/field in four independent fields. Two independent experiments were performed.

resulted in enhanced phosphorylation of Akt, suggesting that that PI3 kinase/Akt pathway regulates the cell survival pathway. As confirmation, the protective effect of α8 ligation was eliminated by a pharmacological inhibitor of PI3 kinase. In addition, expression of a dominant negative form of PI3 kinase reversed the protective effects of α8 ligation. Several studies have implicated the PI3 kinase pathway in integrin-mediated cell survival. PI3 kinase is a heterodimer composed of a catalytic p110 subunit and a regulatory p85 subunit. FAK binds to PI3 kinase [25] which can activate the protein kinase B/Akt. Akt has multiple targets for is protective effect, including inactivation of caspases, activation of NFκB, inactivation of GSK, and direct phosphorylation of Bad [26]. All of these actions can result in an anti-apoptotic signal.

Integrins can influence cell survival in several ways. The signaling mechanisms involved in cell survival depend on both the integrin and cell type. FAK phosphorylation can cause integrin-mediated cell survival by activation of MAPK pathway, in addition to the PI3 kinase pathway [21]. Ligation of integrins α5β1, αvβ3, and α1β1 leads to phosphorylation of the adapter protein Shc, which may activate the ERK pathway [27] and lead to cell survival. Signaling through the integrin α3β1 in keratinocytes activates ERK pathway which promotes survival [28]. Although ERK was activated in our system, it was not the major pathway responsible for cell survival. Several integrins including the fibronectin receptor α5β1 exert their prosurvival effects through alterations in Bcl-2/Bax levels [24]. In contrast to theses studies, α8β1–fibronectin cell survival was not associated with alterations of Bcl-2/Bax ratio. However, it is possible that protein kinase B/Akt phosphorylates Bax, which interferes with its ability to heterodimerize with Bcl-2 and exerts its pro-death effect [29–31].

Bieritz et al. [32] showed that mesangial cells derived from α8-deficient mice had decreased proliferation on fibronectin compared to wild type mesangial cells as measured by BrDU and thymidine incorporation. Since the mechanisms and signaling pathways of cell survival

and cell proliferation are separable, our results are compatible. Indeed, there is a suggestion that presence of α8-expressing mesangial cells is necessary for glomerular tuft stability [33]. In addition, integrin-activated pathways may also depend on the cell type studied.

In summary, ligation of the integrin α8β1 to its ligand, fibronectin, results in enhanced cell survival, which is mediated through the PI3 kinase pathway in AtT20 cells. Mesenchymal cells expressing α8β1 are in a unique position to relay information and transduce signals critical to the development of organ fibrosis. Since α8β1-expressing cells are in position to interact with increased fibronectin deposition during the development of fibrosis, this may result in persistence of fibrogenic cells. In a model of liver injury, clearance of activated myofibroblasts by apoptosis is essential to remove cells expressing TIMP, thereby unmasking latent collagenase activity [18]. Thus, the balance between cell survival and cell death is an important determinant of the end result of organ injury. α8β1 promotes cell survival through adhesion to fibronectin and therefore may play a role in the continued survival of matrix producing cells during fibrosis.

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