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ORIGINAL PAPER

An altered fibronectin matrix induces anoikis of human squamous cell carcinoma cells by suppressing integrin alpha v levels and phosphorylation of FAK and ERK

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Abstract Fibronectin regulates many cellular processes, including migration, proliferation, differentiation, and survival. Previously, we showed that squamous cell carcinoma (SCC) cell aggregates escape suspension-induced, p53-mediated anoikis by engaging in fibronectin-mediated survival signals through focal adhesion kinase (FAK). Here we report that an altered matrix, consisting of a mutated, nonfunctional high-affinity heparin-binding domain and the V region of fibronectin (V^+H^-), induced anoikis in human SCC cells; this response was blocked by inhibitors of caspase-8 and caspase-3. Anoikis was mediated by down-regulation of integrin alpha v in a panel of SCC cells and was shown to be proteasome-dependent. Overexpression of integrin alpha v or FAK inhibited the increase in caspase-3 activation and apoptosis, whereas suppression of alpha v or FAK triggered a further significant increase in apoptosis, indicating that the apoptosis was mediated by suppression of integrin alpha v levels and dephosphorylation of FAK. Treatment with V^+H^- decreased the phosphorylation of extracellular signal-regulated kinase (ERK) 1 and 2, and direct activation of ERK by constitutively active MEK1, an ERK kinase, increased ERK1 and ERK2 phosphorylation and inhibited the increase in apoptosis induced by V^+H^-. ERK acted downstream from alpha v and FAK signals, since alpha v and FAK overexpression inhibited both the decrease in ERK phosphorylation and the increase in anoikis triggered by V^+H^-. These findings provide evidence that mutations in the high-affinity heparin-binding domain in association with the V region of fibronectin, or altered fibronectin matrices, induce anoikis in human SCC cells by modulating integrin alpha v-mediated phosphorylation of FAK and ERK.

Keywords Anoikis · Fibronectin fragments · Integrins · Focal adhesion kinase

Abbreviations

ECM	Extracellular matrix
FAK	Focal adhesion kinase
MAPK	Mitogen-activated protein kinase
V^+	V region of fibronectin
H^+	Heparin-binding region of fibronectin
H^-	Mutated, nonfunctional heparin-binding region of fibronectin

Introduction

Fibronectin, a large extracellular matrix (ECM) glycoprotein found in plasma and other body fluids, is composed of homologous type I, type II, and type III repeating subunits. These subunits form binding sites for other ECM proteins and for cells that interact with fibronectin via integrin and proteoglycan receptors. Multiple isoforms arise through alternative splicing at numerous sites. Fibronectin participates in cell adhesion, migration, invasion, and survival by activating integrin and proteoglycan receptors that engage specific signaling networks.

Integrins are cell-surface receptors that mediate cell–cell and cell–ECM interactions [1]. Several bind to fibronectin. The classic alpha 5 beta 1 integrin [2] binds to fibronectin's central cell-binding domain, which has an arginine-

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glycine-aspartic acid (RGD) sequence, a site recognized by other beta integrins as well [3–5]. Integrins also recognize the heparin-binding domain and alternatively spliced II-ICS/V segment [6, 7].

Integrins regulate cell adhesion, migration, and survival by activating complex signaling networks that involve molecules such as focal adhesion kinase (FAK), a nonreceptor protein tyrosine kinase and MAPK members, such as ERK [8]. FAK localizes to sites of integrin clustering through carboxyl-terminal domain-mediated interactions with integrin-associated proteins such as paxillin and talin [9]. In vitro, its amino-terminal domain binds to sequences in the cytoplasmic domain of beta integrin subunits [10]. Elevation of the phosphotyrosine content of FAK increases cell adhesion [11].

When ECM molecules are altered by inflammation [12], during development or wound healing [13], or by metastatic processes [14], cell adhesion to the ECM can be disrupted, triggering apoptosis [15]. Under such conditions, ECM proteins such as fibronectin undergo proteolytic cleavage or alternative splicing. Cell-adhesive interactions with the ECM can also be disrupted with blocking antibodies or peptides or by culturing cells in suspension [16]. Recently, fibronectin fragments that are produced during inflammatory processes by the activity of proteinases have been implicated in apoptosis [17]. Our group has further found that specific fibronectin proteins induce a specific form of detachment-induced apoptosis, called anoikis [18].

Resistance to anoikis has been described in mammary tumors, colon cancers, osteosarcomas, and lung cancer [19–22]. However, little is known about its role in the progression of human oral squamous cell carcinoma (SCC), the sixth most common solid tumor. SCC accounts for 5.5% of all malignancies [23] and 96% of oral cancers, and many patients with these tumors die from metastatic disease [24].

Although some information has been reported regarding integrin alpha v's regulation of cell migration, proliferation, and invasion in squamous cell carcinoma [25–28] and in other cancer cell systems [29, 30], there are no published reports on the role of integrin alpha v in the regulation of anoikis signaling in SCC. Furthermore, there is no literature, other than our own work on the molecular mechanisms involving the ECM and integrin alpha v-mediated apoptosis in SCC. Published reports indicate that integrin alpha 5 but not alpha v regulate apoptosis induced by serum deprivation through upregulation of Bcl2 in Chinese hamster ovary cells [31] and colon cancer cells [32]. However, again integrin alpha v was not implicated in this mechanism.

Previously, we showed that survival signals mediated by fibronectin and integrin alpha v through FAK enable SCC cell aggregates to escape suspension-induced, p53-mediated anoikis [33]. Although many domains of fibronectin have been implicated in mediating tumor cell functions, the contributions of each domain and the relative importance of each family of receptors to these processes are difficult to assess. However, these evaluations are important since they may suggest potential therapeutic interventions by targeting the portions of the fibronectin molecule that play the greatest role in tumor cell processes. These inquiries are often best made using recombinant proteins that exhibit altered function because of specific point mutations rather than deletions of large protein segments, which may alter protein function nonspecifically. In this study, we used an altered fibronectin protein, that contained an alternatively spliced V region and function-perturbing point mutations in the high affinity heparin-binding region, ($V^{+}H^{-}$) [34] to disrupt cell adhesion to the ECM and examine the potential of the heparin-binding domain and the alternatively spliced V region of fibronectin to regulate anoikis in SCC cells. Through their roles in regulating cellular adhesion, spreading, and migration, these regions are likely important in cancer cell invasion, migration, metastasis, and survival [35–39]. For the first time, we found that mutations in the high-affinity heparin-binding domain in association with the V region of fibronectin, or altered fibronectin matrices, induce anoikis in human SCC cells by modulating integrin alpha v-mediated phosphorylation of FAK and ERK.

Materials and methods

Cell lines and culture

The highly invasive human oral SCC cell line HSC-3 [40] was kindly provided by Dr. Randy Kramer (University of California, San Francisco). The human oral SCC cell lines, UM-SCC-11A and UM-SCC-17B were gifts from Dr. Tom Carey (University of Michigan, Michigan) [41]. Cells were plated and maintained on uncoated cell culture dishes or plates and incubated with Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum, 1% penicillin, and 1% streptomycin in a 5% CO_2 atmosphere at 37°C. In experiments, however, cells were plated in serum free conditions.

Plasmids and DNA constructs

The FAK construct (pRc/CMV-FAKHA) was provided by Dr. Steven K. Hanks (Vanderbilt University). An HA-tagged FAK mutant construct in which the tyrosine-397 phosphorylation site was substituted with phenylalanine (Y^{397}F-FAK) was provided by Dr. Ken Yamada (NIH). A pCMV plasmid expressing constitutively active MEK1 was provided by Dr. Kunliang Guan (University of Michigan). A pcDNA1/NEO construct encoding integrin alpha v was

provided by Dr. David Cheresh (Moores University of California San Diego Cancer Center, La Jolla).

Recombinant fibronectin proteins

We tested two recombinant fibronectin proteins [34, 42] that contained an alternatively spliced V region and either an unmutated [V^+H^+] or a mutated, nonfunctional [V^+H^-] high-affinity heparin-binding domain [43]. Both proteins also contained the RGD cell-binding site and the alternatively spliced EIIIA domain.

DAPI nuclear staining

Nuclear staining of DNA was used to assess the quality of the nucleus in cells incubated with the recombinant fibronectin proteins in serum-free conditions. Briefly, cells were grown on 22-mm glass coverslips in six-well plates. After treatment with fibronectin proteins, the cells were fixed with ice-cold 100% methanol for 15 min, stained with a fluorescent, groove-binding DNA probe, 4′,6-diamidino-2-phenylindole (DAPI, Sigma), for 10 min in darkness. Cells were photographed at 400× magnification with a Nikon Eclipse 50i photomicroscope equipped with a DAPI filter.

Flow cytometry

The percentage of apoptotic cells was determined by flow cytometry [44]. Briefly, cells were grown in 12-well plates and treated as indicated in serum-free conditions. In some cases, cells were pre-treated with AC-IETD-CHO or AC-DEVD-CHO (Bachem) for 1 h. Adherent cells were detached by incubation with enzyme-free dissociation buffer (Invitrogen), pelleted by centrifugation, and stained with annexin V-FITC (BD Pharmingen) for analysis by flow cytometry (FACSDiVA Cell Sorter, Becton Dickinson).

Western blot analysis

For Western blot analysis, cells were grown in 12-well plates, treated as indicated in serum-free conditions, washed once with phosphate-buffered saline, and lysed in RIPA lysis buffer containing protease inhibitors [50 mM Tris/HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1% protease inhibitor cocktail (P8340, Sigma), 1 mM Na_3VO_4, and 1 mM NaF] on ice for 30 min. Insoluble material was removed by centrifugation at 12,000 × *g* for 10 min at 4°C. Lysates were adjusted for protein concentration with the BCA protein assay kit (Bio Rad), resolved by SDS-PAGE, and transferred to Immobilon-P membranes (Millipore). Membranes were probed with antibodies against integrins alpha 4 (H-210), alpha 5 (H-104, Santa Cruz Biotechnology), alpha v (AB1930, Chemicon), and active beta 1 (HUTS-4, chemicon), total beta 1 (4B7R, Santa Cruz), phospho p42/44 ERK (Thr 202/Tyr 204, No. 9101, Cell Signaling) or total p42/44 ERK (No. 9102, Cell Signaling), FAK phosphorylated at Tyr-397 (No. 07-012, Upstate Biotechnology) and Try-925 (No. 11766, Santa Cruz Biotechnology), total FAK (No. 05-182, Upstate Biotechnology), or caspase-3 (N-19, Santa Cruz Biotechnology) and developed with the ECL-Plus detection system (Pierce). To demonstrate equal protein loading, membranes were stripped and reprobed with an anti-beta-actin antibody (Santa Cruz Biotechnology). Lactacystin was purchased from Roche. Intact plasma fibronectin and all other reagents were from Sigma.

Transient transfection of cells

HSC-3 cells at 60–70% confluency in 12-well plates were transiently transfected with cDNA for alpha v (1 and 1.5 μg), FAK (0.5–1.5 μg), or CA-MEK1 (0.5 μg) or with control vector in 500 μl of serum-free medium containing Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. For gene suppression experiments, 500 or 1000 nM of phosphothioated FAK antisense oligonucleotides (AS-FAK, 5′-TTTCAACCAGATGGTCATTC-3′) or scrambled FAK oligonucleotides (Scr-FAK, 5′-TTTTAATCATATTGTTATTC-3′) (Oligos Etc.,) [33, 45–47] and 100 pM of alpha v small interfering RNA (siRNA) (sequence, 5′–3′: sense, GCAUUGAUUUUACUAAAGCtt; antisense, GCUUUAGUAAAAUCAAUGCtg) or negative controls (Ambion) were transfected into cells using Oligofectamine or Lipofectamine according to the manufacturer's instructions (Invitrogen). After 6 h of incubation at 37°C, the cells were maintained in normal medium for 30 h. Transfection efficiency was assessed by measuring the levels of alpha v, FAK, or phospho p42/44 (ERK) in transfected and control cells by Western blotting.

Statistical analysis

Values are expressed as means ± S.D. Intergroup differences were determined by two-way analysis of variance (ANOVA) and Scheffe's multiple-comparison test; $P < 0.001$ was considered statistically significant. All experiments were repeated at least three times.

Results

An altered fibronectin matrix induces anoikis in a caspase-dependent manner

To assess the effects of an altered fibronectin matrix on resistance to apoptosis (anoikis), we treated SCC cells with recombinant fibronectin proteins. V^+H^- fibronectin significantly increased apoptosis (Fig. 1A), as shown by DAPI staining. As shown by annexin V staining and flow cytometry, the apoptotic effect was dose dependent (Fig. 1B) and was suppressed by inhibitors of caspase-8 (death-receptor initiator caspase) and caspase-3 (executioner caspase) (Fig. 1C). To assess whether this mechanism was generalizable to SCC, we tested an additional two different human SCC cells. V^+H^- significantly increased apoptosis in 11A and 17B cells (Fig. 1D).

Anoikis is mediated by down regulation of integrin alpha v and is proteasome-dependent

Next, we explored the role of integrins in V^+H^--induced anoikis of SCC cells, since integrins mediate fibronectin signals and we have shown that SCC cells express these receptors on their surface [48]. In the present study, treatment with V^+H^- decreased the level of integrin alpha v, but not of integrin alpha 4 or alpha 5, as assessed by Western blotting; integrin levels were unchanged in controls (Fig. 2A). Overexpression of integrin alpha v inhibited the increase in apoptosis triggered by V^+H^- (Fig. 2B). In contrast, suppression of integrin alpha v with siRNA significantly increased apoptosis (Fig. 2C). To examine whether downregulation of integrin alpha v is generalizable in SCC, we tested an additional two human SCC cells. V^+H^- significantly downregulated integrin alpha v in 11A and 17B cells, suggesting that alpha v downregulation is an important and general mechanism in V^+H^--induced anoikis in SCC (Fig. 2D). To further examine whether the V^+H^--induced alpha v downregulation was due to degradation through a proteasome pathway, we pretreated HSC and 11A cells with lactacystin, a specific proteasome inhibitor, followed by treatment with V^+H^-. Lactacystin significantly increased the accumulation of alpha v and also prevented the downregulation of alpha v in response to V^+H^- treatment in these cells (Fig. 2E). To further explore the molecular mechanism involved in alpha v degradation, we determined the levels of ubiquitinated alpha v. We found that degradation of alpha v is through ubiquitination (Fig. 2E, bottom panel). V^+H^--treated cells also had lower levels of active beta 1, but no changes in total beta 1 integrin levels (Fig. 2F). We examined the

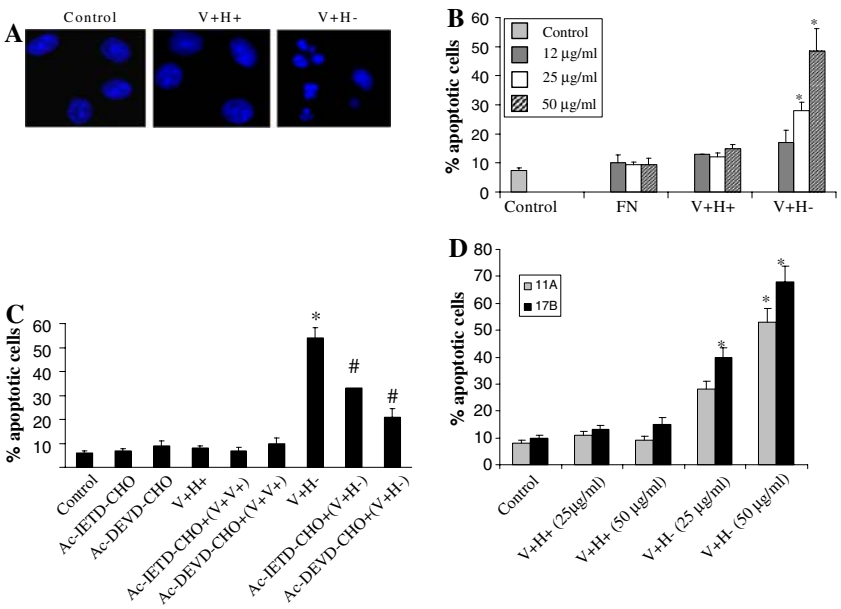

Fig. 1 Apoptosis induced in HSC-3 cells by an altered fibronectin matrix is caspase mediated. (**A**) Nuclear morphology of DAPI-stained cells treated with V^+H^+ or V^+H^- (50 µg/ml) for 16 h. (**B**) Percentage of apoptotic cells determined by flow cytometry after treatment with V^+H^-, V^+H^+, intact plasma fibronectin, or medium for 16 h. Values are mean ± S.D. of three independent experiments. $^*P < 0.001$ vs. control. (**C**) Effects of caspase-3 and caspase-8 inhibition on apoptosis. Cells were pretreated or not with Ac-IETD–CHO (caspase-8 inhibitor, 25 µM) or AC-DEVD–CHO (caspase-3 inhibitor, 25 µM) for 1 h and exposed to V^+H^+ or V^+H^- (50 µg/ml) for 16 h. Apoptosis was assessed by flow cytometry. Values are mean ± S.D. of three independent experiments. $^*P < 0.001$ vs. control; $^{\#}P < 0.001$ vs. V^+H^- alone. (**D**) Percentage of apoptotic cells determined by flow cytometry after treatment with V^+H^-, V^+H^+ or medium for 16 h in UM-SCC-11A and UM-SCC-17B cells. Values are mean ± S.D. of three independent experiments. $^*P < 0.001$ vs. control

changes in beta 6 integrin levels by V^+H^- and found no difference; therefore these data were not included.

Integrin-mediated downstream signaling by FAK regulates V^+H^--induced anoikis

To assess downstream signaling in V^+H^--induced anoikis, we investigated the role of FAK in SCC cells. In V^+H^--treated cells, FAK phosphorylation at Tyr-397 and Tyr-925 decreased (Fig. 3A). In control cells, however, it increased steadily, likely because matrix deposition increased in response to serum depletion. Under all conditions, total FAK protein levels were unchanged. Overexpression of FAK inhibited caspase-3 activation (Fig. 3B) and suppressed apoptosis (Fig. 3C) triggered by V^+H^-. Caspase 3 activation was demonstrated by the presence of caspase-3 cleaved products in cell lysates analyzed by Western blotting. In Fig. 3B and C, the presence of two bands in the Western blots for total FAK protein detected after FAK overexpression of a triple HA-tagged cDNA in SCC cells represents the ability of the anti-FAK antibody to detect both HA-tagged and endogenous FAK in these lysates. The decrease in FAK phosphorylation was integrin mediated,

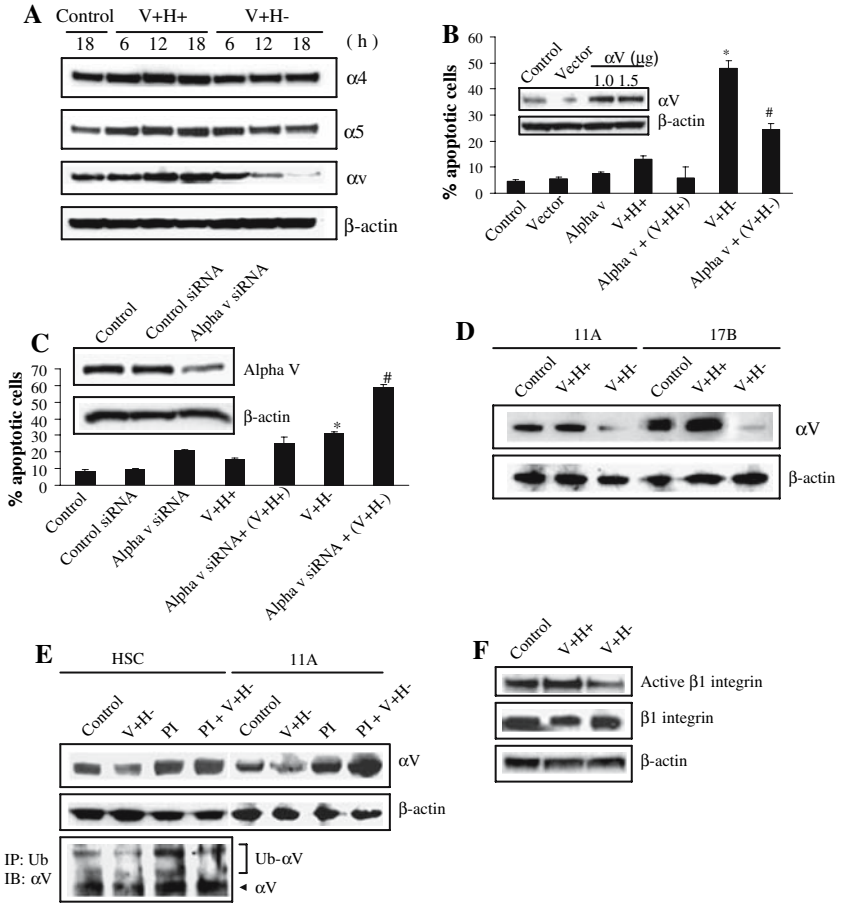

Fig. 2 Induction of apoptosis by V^+H^- in HSC-3 cells is mediated by integrin alpha v. (**A**) Immunoblots showing expression of alpha 4, alpha 5, and alpha v in cells treated with V^+H^+ or V^+H^- (50 μg/ml), or medium for 6, 12, or 18 h. (**B**) Percentage of apoptotic cells determined by flow cytometry after treatment of control and alpha v-transfected cells with V^+H^- or V^+H^+ (50 μg/ml) for 16 h. Values are mean ± S.D. of three independent experiments. $P < 0.001$ vs. control; $^{\#}P < 0.001$ vs. V^+H^- alone. *Inset*, Immunoblot showing integrin alpha v expression in untransfected cells (control) and cells transfected with empty vector (vector) or vector encoding alpha v (αv; 1.0 and 1.5 μg/ml of DNA). (**C**) Percentage of apoptotic cells after transfection with alpha v siRNA or negative control and treatment with V^+H^- or V^+H^+ (25 μg/ml) for 16 h. Apoptosis was assessed by flow cytometry. Values are mean ± S.D. of three independent experiments. $^*P < 0.001$ vs. control; $^{\#}P < 0.001$ vs. V^+H^- alone. *Inset*, Immunoblots showing integrin alpha v expression in cells transfected with control or alpha v siRNA. (**D**) Immunoblots showing integrin alpha v expression in 11A and 17B cells treated with V^+H^- or V^+H^+ (50 μg/ml) for 18 h. (**E**) Immunoblots showing integrin alpha v expression in cells pretreated or not with lactacystin (5 μM), a proteaosomal inhibitor (PI), for 1 h and exposed to V^+H^- (50 μg/ml) for 12 h. (*bottom panel*), lysates were immunoprecipitated with ubiquitin and immunoblotted with alpha v. (**F**) Immunoblots showing active and total integrin beta 1 in cells treated with V^+H^- or V^+H^+ (50 μg/ml) for 5 h

since overexpression of integrin alpha v inhibited the V^+H^--induced decrease in FAK phosphorylation (Fig. 3D). None of the treatments affected total FAK levels.

Transient FAK suppression further augments apoptosis induced by an altered fibronectin matrix. Suppressing FAK with an antisense oligonucleotide increased V^+H^--induced apoptosis in dose-dependent fashion, as shown by flow cytometry (Fig. 4A). Western blots confirmed the dose-dependent suppression of total FAK protein levels (Fig. 4 A, B). FAK antisense and V^+H^- treatment appeared to have more than an additive effect, since the total increase in apoptosis was more than the sum of the effects of either treatment alone, suggesting that V^+H^- potentiates other FAK-related downstream signaling pathways that lead to apoptosis. Treatment with control scrambled FAK or with an antisense FAK oligonucleotide alone or with V^+H^+ did not affect apoptosis. Similarly, transient suppression of FAK induced a dose-dependent increase in caspase-3 activation by V^+H^- (Fig. 4B). The experiments represented in Fig. 4B were performed after an additional 16 h of treatment with the recombinant fibronectin proteins. Therefore, it is expected that the robust inhibition of FAK seen in Fig. 4A will be diminished to some degree in Fig. 4B. It is obvious that cells treated with FAK AS together with V^+H^- significantly suppress FAK levels beyond that in cells treated with V^+H^- alone, underscoring the effectiveness of the anti-sense treatment.

ERK1 and ERK2 dephosphorylation are downstream signaling events in this cascade

To further examine the signaling networks in V^+H^--induced anoikis, we assessed ERK phosphorylation signaling events. After treatment of cells with V^+H^-, ERK1 and ERK2 phosphorylation levels increased slightly at 3 and 5 h, however, the increase was significantly lower than that of control V^+H^+ treated cells (Fig. 5A). To further confirm the role of ERK in this pathway, we overexpressed a constitutively active form of the ERK kinase MEK1. ERK1 and ERK2 phosphorylation increased, inhibiting the increase in apoptosis induced by V^+H^- (Fig. 5B). In both experiments, total ERK1 and ERK2 protein levels were unaffected.

Next, we sought to determine if ERK signaling was downstream of FAK and alpha v signaling in V^+H^--induced anoikis. As expected, overexpression of FAK increased ERK phosphorylation significantly (Fig. 5C). Furthermore, overexpression of integrin alpha v, and FAK, but not a FAK phosphorylation mutant (Y^{397}F-FAK),

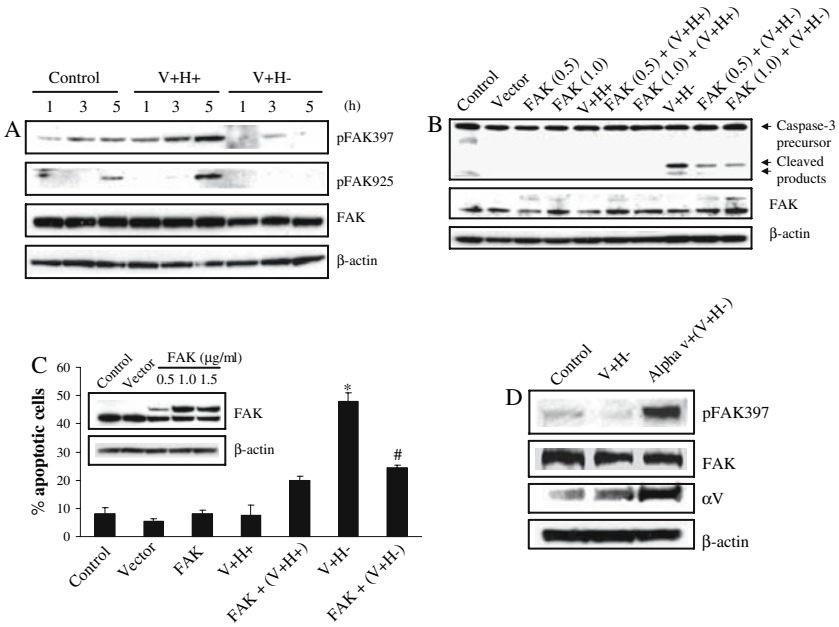

Fig. 3 V^+H^--induced apoptosis in HSC-3 cells is mediated by FAK phosphorylation. (**A**) Immunoblots showing the levels of pFAK (phosphorylated at Tyr 397 and Tyr 925) and FAK after treatment with V^+H^- or V^+H^+ (50 μg/ml) for 1, 3, or 5 h. (**B**) Immunoblots showing the activation of caspase-3 and FAK levels in cells after transfection with FAK (0.5 or 1.0 μg/ml of DNA) or vector control and treatment with V^+H^- or V^+H^+ (50 μg/ml) for 16 h. (**C**) Apoptosis levels after transfection with FAK (1 μg/ml) or vector control and treatment with V^+H^- or V^+H^+ (50 μg/ml) for 16 h. Apoptosis was assessed by flow cytometry. Values are mean ± S.D. of three independent experiments. $^*P < 0.001$ vs. control; $^{\#}P < 0.001$ vs. V^+H^- alone. *Inset*, Immunoblots showing the levels of FAK in cells transfected with FAK or vector control. (**D**) Immunoblots showing the levels of pFAK (phosphorylated at Tyr 397), FAK, and alpha v in cells transfected with alpha v and treated with V^+H^- (50 μg/ml) for 5 h

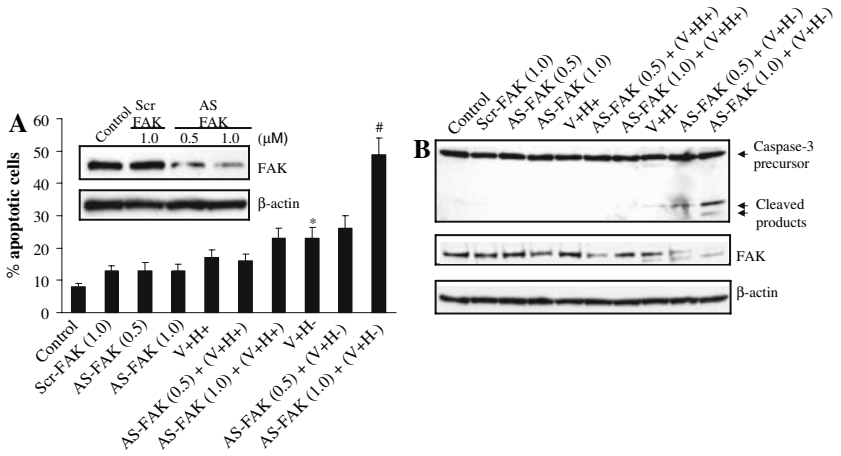

Fig. 4 Downregulation of FAK enhances V^+H^--induced apoptosis and caspase-3 activation. (**A**) Percentage of apoptotic cells determined by flow cytometry after transfection with an antisense (AS) FAK or a scrambled (Scr) FAK oligonucleotide and treatment with V^+H^- or V^+H^+ (25 μg/ml) for 16 h. Values are means ± S.D. of three independent experiments. $^*P < 0.001$ vs. control; $^{\#}P < 0.001$ vs. V^+H^-. *Inset*, Immunoblots showing FAK levels in HSC-3 cells transfected with AS-FAK or Scr-FAK oligonucleotide. (**B**) Immunoblots showing caspase-3 activation and FAK levels after transfection with AS-FAK or Scr-FAK oligonucleotide and treatment with V^+H^- or V^+H^+ (25 μg/ml) for 16 h

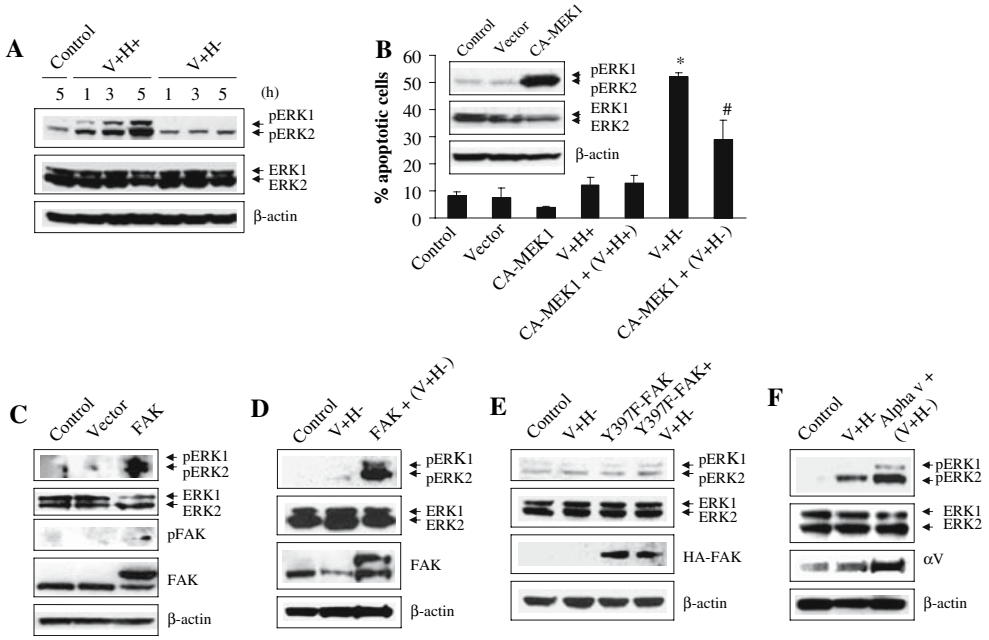

Fig. 5 Apoptosis in HSC-3 cells is regulated by ERK phosphorylation. (**A**) Immunoblots showing the levels of phospho p42/44 (ERK) (Thr 202/Tyr 204) and total p42/44 (ERK) in cells treated with V^+H^- or V^+H^+ (50 μg/ml) for 1, 3, or 5 h. (**B**) Percentage of apoptotic cells determined by flow cytometry after transfection with empty vector or constitutively active MEK1 cDNA and treatment with V^+H^+ or V^+H^- (50 μg/ml) for 16 h. Values are means ± S.D. of three independent experiments. $^*P < 0.001$ vs. control; $^{\#}P < 0.001$ vs. V^+H^-. *Inset*, Immunoblots showing the levels of phospho p42/44 (pERK) (Thr 202/Tyr 204) and total p42/44 (ERK) in cells transfected with empty vector or constitutively active MEK1 cDNA. (*Top panel*) The lower band overlaps with the upper band due to a shorter running time for this specific gel. (**C–F**) Immunoblots showing the levels of phospho p42/44 (pERK) (Thr 202/Tyr 204), total p42/44 (ERK), phospho FAK (phosphorylated at Tyr 397), total FAK, and alpha v after transfection with FAK (**C** and **D**), mutant FAK (**E**) or alpha v (**F**) and treatment with $V^+(^-$ (50 μg/ml) for 5 h

inhibited the decrease in ERK phosphorylation triggered by V^+H^-, supporting their roles as upstream modulators of ERK signaling in V^+H^--mediated anoikis. The overexpression of FAK, the FAK mutant, and alpha v, did not affect the total levels of ERK1 and ERK2 as confirmed by Western blots (Fig. 5C–F). In addition, these blots confirmed the overexpression of FAK, mutant FAK, and alpha v achieved with these constructs (Fig. 5C–F).

Discussion

Several studies have shown that fibronectin/integrin signaling promotes cell survival [49, 50]. In this study, we showed that V^+H^-, a recombinant fibronectin protein consisting of a mutated, nonfunctional high-affinity heparin-binding domain and the V region, induced anoikis in different types of human SCC cells that was inhibited by inhibitors of caspase-8 and caspase-3. These results indicate that V^+H^- induces caspase-mediated anoikis by disrupting cell adhesion. V^+H^- decreased the levels of integrin alpha v and active beta 1 protein, but not integrin alpha 4 or alpha 5. Overexpression of alpha v inhibited the V^+H^--induced increase in apoptosis, whereas suppression of alpha v with siRNA led to a significant increase in apoptosis. Further, V^+H^- induced the downregulation of alpha v by a proteasome-dependent mechanism via ubiquitination. This proteasome-mediated degradation of integrins constitutes a relatively novel pathway for regulating integrin expression; since our search of the literature identified only one other such finding. This other study showed regulation of integrin beta 4 levels by a proteasome-dependent pathway [51]. These findings underscore the importance of integrin alpha v in the survival of SCC cells and in the anoikis induced by V^+H^-. Consistent with these results, a neutralizing monoclonal antibody to alpha v beta 6 strongly interferes with squamous tumor growth in vivo and with migration and proliferation in vitro [25]. Moreover, in oral SCC cell lines, an anti-alpha v beta 6 antibody partially inhibits migration on fibronectin [26]. Further, the alpha v beta 3 integrin has been implicated in the growth of lymphoid tumor cells [29] and melanoma, and a neutralizing antibody to the alpha v subunit effectively blocks the growth of melanoma cell lines in vivo [30].

Although alpha 5 beta 1 is the key receptor for the central cell binding domain of fibronectin its protein levels were unchanged in our system as indicated in our data. Furthermore, blocking antibody experiments indicated that there are no additional effects mediated by alpha 5 beta 1 in this mechanism. However, ectopic expression of alpha 5 beta 1 integrin, but not alpha v beta 1 integrin, suppresses anoikis of fibronectin-bound Chinese hamster ovary cells [31] and HT29 carcinoma cells under serum-free conditions [32], indicating that specific ECM-bound integrins suppress anoikis in specific cell types. Thus, cell–ECM interactions mediated by integrin alpha v are important in regulating SCC cell survival and in mediating anoikis triggered by an altered fibronectin matrix, and may be important in regulating cell-matrix-cell [33] interactions in oral SCCs.

Integrin alpha v-associated downstream signaling via FAK helps mediate V^+H^--induced anoikis. The role of FAK in apoptotic signaling mechanisms and cell adhesion and spreading, has been demonstrated [52, 53]. Specifically, cell spreading onto a ligand substrate involves integrin-induced FAK activation. In our V^+H^+-treated cells, FAK was rapidly phosphorylated; however, after treatment with V^+H^-, FAK phosphorylation at Tyr-397 and Tyr-925 decreased as early as 1 h after treatment. Previously, we showed that decreases in FAK phosphorylation precede the cell rounding noted in apoptotic fibroblasts at very early time points [54]. In this study, overexpression of integrin alpha v inhibited the decreases in FAK phosphorylation triggered by V^+H^-, suggesting that the decreases in FAK phosphorylation in this apoptotic mechanism are mediated by integrin alpha v. Similarly, FAK has been reported to transduce integrin signals, including those regulating survival and migration [1, 52, 55, 56]. In addition, we and others have shown that detachment of epithelial cells from their substratum or matrix led to lower integrin levels and a decrease in integrin-mediated survival signaling, resulting in anoikis [15, 33].

A key step in the anoikis induced by V^+H^- in SCC cells was the reduction in FAK phosphorylation triggered by the altered fibronectin matrix. Overexpression of FAK rescued the apoptotic cells, and suppression of FAK led to a dose-dependent increase in apoptosis after treatment with V^+H^-. Consistent with these findings, inhibition of FAK induces apoptosis in breast cancer as well as other cells [57, 58], while constitutively active FAK confers resistance to anoikis in epithelial cells [59]. In a study of carcinoma cells, apoptosis was accompanied by dephosphorylation of FAK at Tyr-397, and overexpression of FAK rescued cellular rounding mediated by the FAK-N-terminal domain [60]. Additionally, suppression of FAK promoted anoikis and suppressed metastasis in pancreatic carcinoma [61], whereas overexpression correlated with tumor invasiveness and metastasis [62] in esophageal SCC cells. In contrast, in our study, a FAK antisense oligonucleotide alone had no appreciable effect on anoikis. However, addition of V^+H^- after antisense oligonucleotide treatment increased anoikis significantly, suggesting that suppression of FAK increases the sensitivity of SCC cells to V^+H^--induced anoikis. Furthermore, FAK overexpression in human tumors provides a survival signal function by binding to receptor-interacting protein and inhibiting its interaction with the death receptor complex [56]. Since a Fas antagonistic antibody rescues V^+H^--induced apoptosis, we cannot rule out the possibility that a FAK–death receptor interaction or mechanism may be involved in V^+H^--induced apoptosis (Kapila lab unpublished data).

Our findings suggest that V^+H^--induced anoikis in SCC cells is regulated by a caspase cascade. Treatment with V^+H^- activated caspase-3, and the activation was

significantly inhibited by overexpression of FAK. In contrast, suppression of FAK with siRNA further increased the apoptotic effects of V^+H^-, suggesting that caspase-3 is an executioner caspase that mediates V^+H^--induced apoptosis. FAK and caspase-3 are also involved in anoikis in breast cancer [63] and COS cells [64]. Previously, we showed that caspase-1 and caspase-3 are involved in V^+H^--induced anoikis in fibroblasts [42].

Integrin-mediated signaling through the ERK pathway promotes cell survival in adherent epithelial cells [65]. We found that V^+H^- treatment decreased phosphorylation of ERK1 and ERK2, suggesting that ERK signaling contributed to V^+H^--induced anoikis. Moreover, in control cells treated with V^+H^+, ERK phosphorylation increased in a time-dependent manner similar to FAK phosphorylation, suggesting that ERK could be downstream of FAK phosphorylation. The decrease in ERK signaling resulted from changes in upstream integrin alpha v levels and FAK phosphorylation. It should be noted that although the total protein for alpha v showed apparent degradation after 12 h and pFAK and pERK changes occurred earlier, overexpression of alpha v clearly rescued the decreases in pFAK and pERK. In addition, other facets of alpha v regulation that occur earlier, such as its activation state, are likely also critical to this mechanism. Furthermore, disruption of FAK phosphorylation by expression of a mutant FAK did not influence ERK activation even in the presence of V^+H^-. These findings suggest that alpha v and FAK are upstream modulators of ERK signaling in the anoikis induced by V^+H^-. In agreement with these findings, activating ERK by transfecting the cells with a constitutively active form of MEK1, the kinase upstream of ERK, led to a reduction in V^+H^--induced anoikis, further supporting the central role of ERK in this mechanism.

Upregulation of fibronectin and activation of ERK are associated with tumorigenesis and tumor progression in esophageal SCC [66]. Mounting evidence support the importance of ERK and its signaling pathway in the proliferation and invasion of oral SCC cells [66, 67]. In many cell lines, integrin-dependent activation of MAPK requires FAK signaling [68–70]. Furthermore, intrinsic FAK catalytic activity and Y925 FAK phosphorylation have a novel role in promoting a MAPK-associated angiogenic switch during tumor progression [71]. Finally, in hepatic stellate cell cultures, a neutralizing antibody to alpha v beta 3 or alpha v siRNA inhibited both proliferation and activation of ERK1/2 [72]. Taken together, these findings provide evidence that mutations in the high-affinity heparin-binding domain in association with the V region of fibronectin (V^+H^-), or altered fibronectin matrices, modulate the integrin alpha v-mediated phosphorylation of FAK and ERK to induce anoikis in human SCC cells.

Conclusion

From our studies and those of others, fibronectin fragments that are produced during inflammatory processes have been implicated in apoptosis, functions not normally observed with intact FN [17, 18]. Comparable fragments to the V^+H^- protein have been found in periodontal disease and arthritis and correlate with disease severity [12, 73]. Our lab is currently focused on identifying the role of these regions of fibronectin in human oral SCC in tissue specimens and this work is in progress. There is already evidence suggesting the importance of the heparin-binding domain and RGD site in tumor pathogenesis [74]. In addition, an antibody to the anti-anti-EDB domain of fibronectin has been shown to inhibit cell growth and induce apoptosis in tumors [75]. These in vitro and in vivo studies help lay the ground work for investigations leading toward new therapeutic approaches for SCC. In summary, these data support the idea that fibronectin fragments have distinct functions from those of intact fibronectin, which may have profound implications for wound-healing dynamics, inflammatory diseases and cancer therapy.

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