Mutations in the Heparin Binding Domain of Fibronectin in Cooperation with the V Region Induce Decreases in pp125<sub>FAK</sub> Levels Plus Proteoglycan-mediated Apoptosis via Caspases*

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Intact fibronectin (FN) protects cells from apoptosis. When FN is fragmented, specific domains induce protease expression in fibroblasts. However, it is not known whether specific domains of FN can also regulate apoptosis. We exposed fibroblasts to four recombinant FN fragments and then assayed for apoptosis using criteria of cellular shape change, condensed nuclear morphology, and DNA fragmentation. The fragments extended from the RGD-containing repeat III10 to III15; they included (V<sup>H</sup>) or excluded (V<sup>−</sup>) the alternatively spliced V region and contained either a mutated (H<sup>−</sup>) or an unmutated (H<sup>+</sup>) heparin binding domain. Only the V<sup>H</sup> fragment triggered decreases in pp125<sub>FAK</sub> levels and apoptosis, which was rescued by intact FN and inhibitors of caspase-1 and caspase-3. This apoptotic mechanism was mediated by a chondroitin sulfate proteoglycan, since treating cells with chondroitin sulfate reversed the apoptotic cell shape changes. The α4 integrin receptor may also be involved, since using a blocking antibody to α4 alone induced apoptotic cell shape changes, whereas co-treatment with this antibody plus V<sup>H</sup> reversed these effects. These results demonstrate that the V and heparin binding domains of FN modulate pp125<sub>FAK</sub> levels and regulate apoptosis through a chondroitin sulfate proteoglycan and possibly α4 integrin-mediated pathway, which triggers a caspase cascade.

The extracellular matrix molecule fibronectin (FN) is composed of several domains that mediate multiple cell functions through cell surface integrin and proteoglycan receptors. When isolated, specific domains of FN display activities not exhibited by the intact molecule. For example, the central cell binding domain of FN (FN 120) induces rabbit synovial fibroblasts (1) and human fibroblasts (2) to express elevated levels of matrix metalloproteinases, whereas fragments from the amino-terminal and gelatin binding domains induce chondrolysis in vitro, the latter effect presumably through matrix metalloproteinase and serine proteinase induction (3, 4). These FN fragments are also associated with chronic inflammatory states in vivo, since high levels of such fragments have been found in synovial fluids from arthritic patients (5–7) and in gingival crevicular fluid from patients with periodontitis (8, 9).

Another function recently attributed to FN is protection against programmed cell death, or apoptosis. Apoptosis is generally characterized by cell rounding, nuclear condensation, and DNA fragmentation and by signaling pathways that activate a cascade of cell death proteases. Intact FN as a substrate for cell adhesion can rescue cells from apoptosis, although it is not known whether specific domains of FN regulate this function. The mechanism underlying the protective effect of FN seems to involve integrin-mediated signaling and activation of the focal adhesion kinase (pp125<sub>FAK</sub> (10–13)) and/or activation of the Bcl-2 cell survival pathway, which is independent of pp125<sub>FAK</sub> (14). This difference in utilization of pp125<sub>FAK</sub> as the signaling molecule may depend on cell type.

We tested the hypothesis that specific domains of FN are important in protecting fibroblasts from undergoing apoptosis. Specifically, we tested the roles of the high affinity heparin-binding domain and the alternatively spliced V region of FN in this process.

**Experimental Procedures**

**Fibroblast Cell Culture**—Primary cultures of human periodontal ligament fibroblasts were routinely obtained from human teeth extracted from patients undergoing therapeutic removal of third molars or orthodontic treatment. To obtain cells, the periodontal ligament (PD1) was scraped from the midroot section of extracted teeth with a scalpel blade using standard protocols (2). The tissue was placed under a coverslip and kept in culture medium (α-minimal essential medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin/Fungizone) until cells grew out of the explant and covered the bottom of the tissue culture plate. Once the cells became confluent, they were trypsinized and passaged. Cells from passages 2 to 6 were used for all experiments. Five different PD1 cell isolates derived from five different patients were used for these experiments.

**Recombinant FN Proteins**—Four recombinant FN proteins were tested in these experiments (see Fig. 1). These proteins, described elsewhere (15), either included (V<sup>H</sup>) or excluded (V<sup>−</sup>) the alternatively spliced V region and contained either an unmutated (H<sup>−</sup>) or mutated, nonfunctional high affinity heparin binding domain (H<sup>−</sup>). In brief, the heparin binding domain was mutated and rendered nonfunctional by changing two arginines (Arg-6 and Arg-7) to threonines (Thr-6 and Thr-7) in the heparin binding consensus sequence (16) found in type III-13 of FN. All four proteins also contained the arginine, glycine, aspartic acid (RGD) cell binding site and the alternatively spliced EIIIA domain of FN. These proteins were designated as V<sup>H</sup>, V<sup>+</sup>H<sup>−</sup>, V<sup>−</sup>H<sup>+</sup>, and V<sup>−</sup>H<sup>−</sup>.

**Plating of Cells**—Cells were trypsinized, pelleted under centrifugation, washed twice with phosphate-buffered saline (PBS), and suspended in culture medium (serum-free α-minimal essential medium supplemented with 0.2% lactalbumin hydrolysate (Life Technologies, Inc.) and 1% penicillin/streptomycin). For experiments, the four recombinant FN proteins were either added to the wells immediately before...

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† The abbreviations used are: FN, fibronectin; FAK, focal adhesion kinase; ICE, interleukin 1β-converting enzyme; RGD, arginine-glycine-aspartic acid; DAPI, 4′, 6-diamidino-2-phenylindole; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter.
cells were plated (see Fig. 2A only) or added approximately 2 h after cell spreading, yielding a final protein concentration of 0.1 mm. Unless otherwise specified, cells were plated in 100 μl of medium at a density of 3.0 × 10^4 cells/well in a 96-well tissue culture plate and subsequently incubated at 37 °C in a humidified 5% CO2 incubator. Cell shape changes were assessed by microscopy at 400× magnification. To test the effect of the V'-H' protein, cells were plated with the recombinant proteins. To test the effect of the V'-H' protein, cells were plated with the recombinant proteins.

In experiments testing whether the V'-H' protein could induce cells that had already spread to round up, the V'-H' protein was added in culture medium 1.5 and 2.5 h after cell spreading on plastic, intact FN (30 μg/ml plasma FN, Roche Molecular Biochemicals), collagen type I (5, 10, and 50 μg/ml, Upstate Biotechnology, Lake Placid, NY), or vitronectin (22 and 44 μg/ml, Life Technologies, Inc.).

In experiments testing whether plasma FN could reverse the phenotype triggered by the V'-H' protein, the medium containing the protein was replaced with medium containing 0.1 mM FN. The FN-containing medium was added 2 h after adding V'-H'.

For ICE (casepase-1) and caspase-3 inhibitor experiments, the V'-H' protein and inhibitors (ICE Inhibitor III: acetyl-Tyr-Val-Ala-Asp-acycloxy-methylketone; caspase-3 inhibitor III: acetyl-Asp-Glu-Val-Asp-carboxy-benzyloxycarbonyl-Val-Ala-Asp-acycloxy-methylketone) were added simultaneously immediately after cell plating. The inhibitors were used at concentrations of 2.5, 1.25, 0.125, and 0.0125 μg/ml. Cell shape changes for the caspase inhibitor experiments were photographed 5 or 6 h after plating. Cells were fixed for nuclear staining after 6 and 14 h of incubation time. DNA fragmentation was assayed after 14 h of incubation.

To identify the receptor(s) triggering V'-H' -mediated apoptosis, several blocking experiments were performed. To evaluate the possible role of the integrin α4, α5, and αv subunits in this mechanism, blocking antibodies (0.1 mg/ml for clones P4G9 (anti-α4), P1D6 (anti-α5), and P3G8 (anti-αv), Chemicon, Temecula, CA) to these subunits were incubated with prespread cells for 1 h, and then cells were treated with V'-H' or V'-H' to determine whether apoptotic cell shape changes could be reversed. Before testing on cells, the blocking antibodies anti-α4 and α5 were first dialyzed against 1 mg/ml bovine serum albumin in PBS using the Tube-O-Dialyzer (1-kDa cut-off, Chemicon) to remove sodium azide from the antibody packaging solution. To determine the possible role of proteoglycans, cells in suspension were first pretreated with 0.1 mg/ml plasma FN, Roche Molecular Biochemicals), collagen type I (5, 10, and 50 μg/ml, Upstate Biotechnology, Lake Placid, NY), or vitronectin (22 and 44 μg/ml, Life Technologies, Inc.).

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**Nuclear Staining**—Nuclear staining of DNA was used to assess the quality of the nucleus in cells incubated with the recombinant FN proteins or with control medium in 16-well chamber slides for 6 or 14 h. After incubation, cells were fixed with ice cold 100% methanol for 15 min, stained with a fluorescent groove binding probe for DNA, 4',6-diamidino-2-phenylindole (DAPI; Sigma) for 10 min, rinsed three times with 95% ethanol, and stained with 10 μg/ml of 4',6-diamidino-2-phenylindole (DAPI; Sigma) for 10 min. Finally, cells were treated with calcium- and magnesium-free PBS, dried, and sealed with a coverslip using mounting medium. Cells were photographed at 400× magnification with an Axioskop photomicroscope equipped with a filter for DAPI stain detection (Zeiss, West Germany).

**DNA Fragmentation**—DNA fragmentation in cell lysates and supernatants was assayed by 2% agarose gel electrophoresis with a 300-bp ladder (Promega). The integrity of the DNA was visualized with ethidium bromide.

**Immunoprecipitation**—Levels of pp125FAK protein were assessed by standard immunoprecipitation. Cells were plated in 100 μl of culture medium at a density of 3.0 × 10^4 cells/well in a 96-well tissue culture plate. The test wells contained 0.1 μg/ml anti-human FAK monoclonal antibody (clone AIIB2, Santa Cruz Biotechnology, Santa Cruz, CA). The primary antibody was removed by washing with PBS three times, and 100 μl of dilution buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol) were added to the wells. After overnight incubation at 4 °C, the plates were washed four times with TNE buffer, pelleted again, and eluted from the beads by the addition of sample buffer (0.5 μg/ml protein, 62.5 mM Tris, pH 6.8, 10% glycerol, 5% SDS, 0.1% 2-mercaptoethanol, and 0.05% bromphenol blue) and heated for 2 min. Samples were loaded and electrophoresed on a 7.5% SDS-PAGE gel. The gel was transferred to nitrocellulose by standard methods. The immunoblot was probed with a secondary antibody (anti-rabbit IgG, Zymed Laboratories Inc.) for 1 h. Bound antibody was detected using the ECL-plus detection system (ECL-plus detection system, Amersham Pharmacia Biotech). Bound antibody was detected using the ECL-plus detection system.

**Flow Cytometry**—The integrin subunits expressed on the cell surface were determined by flow cytometry. Trypsinized cells were harvested from tissue culture plates, rinsed twice with PBS, then resuspended (3 × 10^6 cells/ml) with the following primary antibodies: 1:40 antibody dilution in PBS containing 10% serum, a carrier protein for the antibody, and 0.1% sodium azide. Monoclonal antibody (clone P4C2, Life Technologies, Inc.) specific for mouse αv integrin (clone VNR 147, Life Technologies, Inc.), anti-rat α5 (BII-GII, gift from Dr. Carolinne Damsky), and anti-rat α1 (AIIB2, gift from Dr. Carolinne Damsky). Nonimmune mouse and rat IgGs (Life Technologies, Inc.) were used as negative controls. The antibody-containing cell suspension was rocked for 1 h at room temperature. Cells were then rinsed three times with PBS plus 0.05% Tween and incubated with the secondary fluorescein-labeled antibodies (1:1000 antibody dilution in PBS with 1% serum; fluorescein isothiocyanate-rabbit anti-mouse IgG1 (Zymed Laboratories Inc., South San Francisco, CA) and fluorescein isothiocyanate-Russian rabbit anti-mouse IgG2a (Zymed Laboratories Inc.) for 30 min at room temperature. Finally, cells were washed three times with 1× fix buffer made up of 1:10 dilution of 4% formaldehyde, 1.8 dilution of 2% sodium azide in PBS), and analyzed on a flow cytometer (Lysis II Version 1.0, FACSCAN, Becton Dickinson, San Jose, CA). Fluorescence intensity was expressed on a log scale for a replicate of three experiments.

**RESULTS**

To determine the cellular response to the high affinity heparin binding domain and the alternatively spliced V region of FN, we first examined their effects on cell spreading. Cells were rounded, and cell membranes exhibited blebbing when incubated with the V'-H' protein (Fig. 2A, e) but not when incubated with control serum-free medium or medium containing the other three FN proteins tested (Fig. 2A, a–d). Although photographs were typically taken after 2 h of incubation to
show early events, cells were observed up to 48 h. Up to this time, cells incubated with the V\(^+\)H\(^-\) protein failed to spread. In contrast, the V\(^+\)H\(^-\) protein accelerated cell spreading during the initial 2 h after plating (Fig. 2A, d) compared with the other proteins tested or the control (Fig. 2A, a–c). The effects of the V\(^+\)H\(^-\) protein on cell shape were triggered only when the protein was provided in soluble form (Fig. 2B, d) and not as a coated substrate (Fig. 2B, c). In addition, as we and others have shown (15, 17, 18), the heparin binding domain of FN is critical for cell spreading; however, the characteristic cell rounding and membrane blebbing triggered by V\(^+\)H\(^-\) were not due to the inability of cells to spread, since cells that had been allowed to spread first on plastic, intact FN, collagen type I, or vitronectin (Fig. 3, a, c, e, and g, respectively) still exhibited the V\(^+\)H\(^-\)-mediated cell rounding and membrane blebbing (Fig. 3, b, d, f, and h, respectively). These data demonstrate that the V\(^+\)H\(^-\) protein triggers apoptotic-like cell rounding and membrane blebbing irrespective of cell shape.

Adhesion to FN promotes cell survival in a number of cell types (11, 14, 19–22). Given the cell shape changes in these cells, we hypothesized that the mutation in the high affinity heparin binding domain in conjunction with the alternatively spliced V region in the V\(^+\)H\(^-\) protein triggered an apoptotic response in these cells and that the wild-type heparin binding domain was therefore critical to cell survival. We thus investigated the effects of the V\(^+\)H\(^-\) protein on nuclear morphology and integrity. The V\(^+\)H\(^-\) protein induced nuclear condensation and some disintegration by 6 h, seen as intensely staining, small nuclei (Fig. 4, c). By 14 h, more distinct nuclear fragmentation was evident. Nuclei appeared as condensed, bright, and lobulated fragments (Fig. 4, f). Cells exposed to the other fragments as well as control cells retained the larger oval-shaped, pale nuclei characteristic of living cells (Fig. 4, a–d). More DNA fragmentation, a hallmark of apoptosis, was induced in cell lysates and supernatants by the V\(^+\)H\(^-\) protein than by the other proteins tested or the control medium (Fig. 5). These nuclear changes support our hypothesis that the V\(^+\)H\(^-\) protein triggers apoptosis in fibroblasts.

Because FN has been shown to rescue cells from apoptosis, we asked whether intact plasma FN might also rescue cells from the rounded cell phenotype and nuclear breakdown triggered by the V\(^+\)H\(^-\) protein. We found that FN is indeed capable of reversing the rounded phenotype up to 2 h after incubation of cells with the V\(^+\)H\(^-\) protein (Fig. 6, c and d). However, if FN and the V\(^+\)H\(^-\) protein were co-cultured in the wells simultaneously (Fig. 6, a and b), cells spread more than if cells were incubated first with the V\(^+\)H\(^-\) protein alone (Fig. 6, compare a or b with d). Intact FN also reversed the nuclear morphology exhibited by cells incubated in the presence of the V\(^+\)H\(^-\) protein (Fig. 8, B, b, and D, b). These data indicate that intact plasma FN opposes the apoptotic effects of the V\(^+\)H\(^-\) protein in fibroblasts.

Others have shown (10, 13) that inactivation of pp125\(^{FAK}\) function can trigger apoptosis in anchorage-dependent fibroblasts. We therefore tested whether pp125\(^{FAK}\) was differentially altered by V\(^+\)H\(^-\). Indeed, we found that in cells incubated with the V\(^+\)H\(^-\) fragment, pp125\(^{FAK}\) levels were decreased within minutes, and before any changes in cell shape (Fig. 7).

Since the interleukin 1\(\beta\)-converting enzyme (ICE)/caspase family of cell death proteases is a key component in the signaling pathways of apoptosis (23), we tested whether effector caspases such as caspase-1/ICE and caspase-3 were critical to the mechanism by which V\(^+\)H\(^-\) triggers apoptosis in fibroblasts. A caspase-1/ICE- and caspase-3 inhibitor were tested for their ability to reverse the apoptotic phenotype induced by the V\(^+\)H\(^-\) protein. The ICE inhibitor partially rescued the cells from the rounding (Fig. 8A, c) and nuclear disintegration (Fig. 8B, d) induced by the V\(^+\)H\(^-\) protein. The caspase-3 inhibitor almost completely reversed the cell rounding (Fig. 8C, c) and nuclear disintegration (Fig. 8D, d) induced by the V\(^+\)H\(^-\) protein. The partial rescue with the ICE inhibitor may be explained by the fact that at the highest concentration of ICE inhibitor (2.5 \(\mu\)g/ml), the vehicle required to dissolve the inhibitor was itself inhibitory.

To determine the receptor involved in triggering V\(^+\)H\(^-\)-mediated apoptosis, we used several approaches. To evaluate whether integrins were involved in this mechanism, we used blocking antibodies to integrins \(\alpha_4\), \(\alpha_5\), and \(\alpha\nu\) to determine whether these antibodies could rescue the apoptotic cell shape changes induced by V\(^+\)H\(^-\). The data demonstrated that anti-\(\alpha_4\) on its own induced the rounded cell shape phenotype, similar to
that induced by the V\(^+\)H\(^-\) protein, and co-treatment of cells with this antibody and V\(^+\)H\(^+\) partially rescued the cell shape changes. The other antibodies tested, anti-\(\alpha_5\) and -\(\alpha_v\), had no or a minimal effect on cell shape, either on their own or after treatment with V\(^+\)H\(^-\) (Fig. 9).

To verify the expression of the \(\alpha_4\) integrin, we performed FACS analysis on these cells. The FACS data demonstrated that indeed these cells express not only \(\alpha_4\) but also \(\alpha_5\), \(\alpha_v\), and \(\beta_1\) on their cell surface. The data further revealed that there is relatively more \(\alpha_v\) present on these cells than any of the other three integrin subunits. The \(\alpha_5\) and \(\beta_1\) subunits are the next most abundant, and the \(\alpha_4\) subunit is the least abundant (Fig. 10).

To explore the possible role of glycosaminoglycan receptors in this mechanism of apoptosis, we treated cells with glycosaminoglycan digestive enzymes (chondroitinase, heparinase, and heparitinase) and with glycosaminoglycans themselves (chondroitin sulfate, heparan sulfate, and heparin) to determine whether any of these treatments could rescue the apoptotic cell shape changes triggered by V\(^+\)H\(^-\). Both chondroitinase treatment and chondroitin sulfate substantially rescued the apoptotic cell shape changes induced by V\(^+\)H\(^-\) (Fig. 11, A and B). The other treatments had a more minimal ability to rescue the apoptotic cell shape changes (Fig. 11, A and B).

DISCUSSION

Several studies have shown that intact FN promotes cell survival (11, 14, 19–22). We have found that a fragment of FN, V\(^+\)H\(^-\), induces apoptosis. The decrease in pp125\(^{FAK}\) levels in
the presence of V'H' suggests that this fragment interferes with transduction of extracellular matrix survival signals mediated by pp125FAK, which have been shown to be essential in anchorage-dependent fibroblasts cultured in the absence of serum (10, 13). Our data further suggest that a caspase cascade is activated in V'H'-mediated apoptosis via a chondroitin sulfate proteoglycan receptor and the α4 integrin. Taken together, these data support our original hypothesis that specific domains of FN regulate cell survival. Specifically, our data demonstrate that the high affinity heparin binding domain plus the V region of FN are critical to the mechanism by which FN promotes survival, since mutating the heparin binding domain in a V-containing fragment triggers apoptosis in fibroblasts.

The combined functions of the heparin binding domain and the V region of FN are an important regulatory component in cell survival. This is suggested by the fact that the V'H' protein, which is exactly the same as V'H'' except for the mutations in the heparin binding domain, promotes survival. In addition, since the V''H'' protein does not induce apoptosis, the V region is critical to this apoptotic mechanism. Therefore, these findings suggest that the signal (or lack of signal) triggered by the V'H' protein that leads to apoptosis is likely the result of at least two features specific to this protein: 1) the two point mutations in its heparin binding domain, which abrogate heparin-binding function, and 2) the potential loss of cooperative interactions between its heparin binding domain and V region. Therefore it is the combined presence of the heparin binding mutation and the V region that is critical to this ability of the protein to trigger the apoptotic phenotype.

The apoptotic effect induced by the V'H' protein in primary fibroblasts does not affect all cell types and may be cell typespecific. We previously showed that this same FN fragment induces increased invasion by human oral squamous cell carcinoma cells in vitro but not the nuclear and cell shape changes typically seen with apoptosis, even though apoptosis was not directly monitored in that study (15). Possible explanations for these different effects may be the presence of different receptors on these two cell types or the fact that the squamous cell carcinoma cells are transformed and, thus, may have an altered gene expression that counteracts the effects of the V'H' protein.

Our results, like those of others (11, 13, 14, 24–26), suggest that FN protects cells from entering the apoptotic pathway. The rounded phenotype and nuclear changes of apoptosis observed in cells incubated with the V'H' protein were reversed after FN was added to the cells. Also, the fact that cells co-cultured with FN plus the V'H' protein exhibit increased spreading when compared with those cultured with the recombinant fragment alone suggests that FN provides a protective mechanism against apoptosis. However, the protection FN confers in co-culture is not complete, because not all the cells spread. This observation suggests that FN is competing with the V'H' fragment for binding sites on cells. Therefore, our study and others (11, 14, 20) suggest that FN provides protective cellular signals.

The cell rounding, nuclear disintegration, and DNA fragmentation triggered by the V'H' protein, together with the rescue experiments, suggest that this FN fragment, unlike the
The role of pp125FAK, its phosphorylation and activation in apoptotic signaling mechanisms and cell adhesion and spreading, has been demonstrated by several groups (10–13, 28–30). However, although the role of pp125FAK signaling in apoptosis is difficult to dissociate from possible secondary changes in pp125FAK levels as a result of cell shape changes, our data demonstrate that the decrease in pp125FAK levels triggered by V-HE precede the changes in cell shape, as might be expected in a signaling response. Similarly, the fact that V-HE triggers cell shape changes only when introduced in soluble form and not as an anchored substrate also supports the idea that V-HE is mediating apoptotic changes as a result of direct cell signaling and not of alterations in cell adhesion.

In addition, consistent with other studies in which ICE has been associated with loss of the extracellular matrix and inhibitors of ICE prevent apoptosis (31), our studies indicate that the ICE family of proteases is involved in V-HE-induced apoptosis. Furthermore, caspase-3 is also involved in this apoptotic pathway, since rescue from apoptosis was almost complete with the caspase-3 inhibitor. Therefore, these data suggest that there is a cascade of caspases that regulates V-HE–induced apoptosis in fibroblasts. Possibly these caspases mediate proteolytic processing of integrin and/or proteoglycan signaling molecules necessary for survival, and the caspase inhibitors block this processing and, thus, rescue cells from apoptosis. For example, caspase-3 has been shown to cleave pp125FAK (32, 33) as part of the mechanism that leads to apoptosis. Therefore, in the present system, V-HE may be inducing the proteolytic processing of pp125FAK by caspase-3, and inhibitors of this caspase may in turn be blocking this proteolysis and rescuing the apoptotic phenotype.

At least two classes of receptors could potentially mediate apoptosis triggered by the V-HE–protein; integrins and proteoglycans. We have specifically identified the α4, α5, αv, and β1 integrin subunits on the surface of these cells using FACS. All of these integrins could potentially interact with the V-HE–protein. For example, α5, αv, α4, and β1 can bind to the RGD site on repeat III-10 (34–36), and α4 and β1 can further bind to the V region (REDV or LDV sites (37)) and the high affinity heparin binding domain (IADPS on repeat III-14 (38)) on the V-HE–protein. However, since the V-HE–protein induces apoptosis, receptor-ligand interactions are likely altered. These altered interactions may be the result of altered binding between α4β1 and the mutated heparin binding domain, even though the α4β1 integrin binding site on repeat III-14 (IADPS) of the heparin binding domain is far removed from the area of the mutation (PPTTAR), which is on repeat III-13. One explanation for this altered binding is that the mutation on III-13 may be disrupting three-dimensional and/or structural interactions (the cationic cradle) within repeat III-13 itself or between repeats III-13 and III-14. This in turn may alter the ligand binding site for the α4β1 integrin on III-14 or for other as yet unidentified receptors that normally bind the mutated site.

Proteoglycans that could mediate apoptosis triggered by the V-HE–protein include a chondroitin sulfate proteoglycan receptor, like one of the CD44 isoforms, which binds the high affinity heparin binding domain of FN on repeat III-14 (synthetic peptide FN-C/H II (39, 40)), and a chondroitin sulfate/heparan sulfate proteoglycan, like the syndecans, which also bind the high affinity heparin binding domain of FN (41). However, at least for the CD44 family, these proteoglycan receptors also bind the high affinity heparin binding domain outside of the mutated area. Thus again, either the receptor binding site has been structurally altered by the mutation so that these proteoglycan receptors cannot bind, or there are other as yet uniden-

FIG. 9. Responses of cells incubated with the V-HE–protein and with blocking antibodies to α4, α5, and αv. Cells were incubated with control serum-free medium (control), the V-HE–protein, the V-HE–protein, and anti-α4, anti-α5, and anti-αv antibodies alone or together with V-HE– or V-HE–. Others tested, triggers unique signals that induce apoptosis. The data shown here further indicate that the high affinity heparin binding domain of FN appears to be critical to cell survival, since function-perturbing mutations of this domain induce apoptosis. Furthermore, since molecular modeling of this domain has shown that there is a complex cationic cradle involved in the heparin binding function of FN (27), the apoptotic signal may result from alteration of this unique three-dimensional structure in the FN molecule. This alteration could, in turn, disrupt normal cell-matrix interactions necessary for cell survival, presumably by interfering with FN-integrin/proteoglycan interactions and the corresponding signaling pathways, which likely involve pp125FAK activation.
tified receptors that normally bind to the unmutated site and in the absence of binding trigger apoptosis. One indication that there might be receptors that bind to our mutated heparin binding region comes from another study (42), which demonstrated that epithelial cells can interact with this exact same site via some cell surface protein, since a FN peptide containing the wild-type version of this same sequence promoted cell adhesion, spreading, and migration.

Our data demonstrate that the receptors mediating this mechanism of apoptosis are a chondroitin sulfate proteoglycan receptor and, likely, the α4β1 integrin receptor. In the case of the chondroitin sulfate proteoglycan, blocking reagents to this class of proteoglycans rescued the apoptotic cell shape changes triggered by the V'H' protein. The proteoglycan receptor involved may also have some heparin and/or heparan sulfate-type side chains, since blocking reagents to these species produced a minimal, yet noticeable, level of rescue from the apoptotic cell shape changes. In the case of the α4 blocking antibody, the fact that it induces the apoptotic cell shape changes on its own, which are rescued by the addition of the V'H' protein, suggests that α4 is also one of the receptors involved in this mechanism. However, since these antibody blocking experiments are limited, further experimentation would be required to definitively determine the involvement of α4 in this mechanism of apoptosis. Finally, the proteoglycan and integrin receptors may be functioning cooperatively, since they have been noted to do so in other receptor-mediated actions (39, 43).

The induction of apoptotic features by the V'H' protein appears to be a general finding for primary fibroblasts, since all five human fibroblast isolates and mouse 3T3 fibroblasts (data not shown) have shown similar results. In addition, these data further support the idea that intact FN has functions distinct from those of its fragments, which may have profound implications for wound-healing dynamics and inflammatory diseases. In periodontal disease, for example, inflammatory cytokines and bacterial products would stimulate fibroblasts of the periodontium to express greater amounts of matrix metalloproteinases (44), which in turn would degrade the extracellular matrix, thereby generating FN fragments (8, 9). From our own studies and those of others, we know that FN fragments can on their own also induce elevated matrix metalloproteinase expression (1, 2) and tissue destruction in vitro (3) and suppress cell proliferation and chemotaxis (45), functions not normally observed with intact FN. If V-containing FN fragments with an absent or nonfunctional heparin binding domain can also induce apoptosis, the challenge to the healing ability of the host would be compounded. These in vitro studies help lay the groundwork for in vivo investigations leading toward validation of this model of tissue destruction.

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REFERENCES

1. Huhtala, P., Humphries, M. J., McCarthy, J. B., Tremble, P. M., Werb, Z., and Damasky, C. H. (1995) J. Cell Biol. 129, 867–879
2. Kapila, Y. L., Kapila, S., and Johnson, P. W. (1996) Matrix Biol. 15, 251–261
3. Homandberg, G. A., Meyers, R., and Xie, D-L. (1992) J. Biol. Chem. 267, 3597–3604
4. Xie, D-L., Hui, F., Meyers, R., and Homandberg, G. A. (1994) Arch. Biochem. Biophys. 311, 205–212
5. Carson, S. Lavietes, B. B., Diamond, H. S., and Kinney, S. G. (1985) Arthritis Rheum. 28, 601–612
6. Griffiths, A. M., Herbert, K. E., Perrett, D., and Scott, D. L. (1989) Clin. Chim. Acta 184, 133–146
7. Xie, D-L., Hui, F., Meyers, R., and Homandberg, G. A. (1992) J. Rheumatol. 19, 1448–1452
8. Talampoika, J., Paunio, K., and Soderling, E. (1993) Mol. Biol. Cell 5, 439–453
9. Talampoika, J., Heino, J., Larjava, H., Hakkinen L., and Paunio, K. (1989) Scand. J. Dent. Res. 101, 373–381
10. Ilic, D., Almeida, G. A. C., Schlaepfer, D. D., Dazin, P., Aiizawa, S., and Damasky, C. H. (1998) J. Cell Biol. 143, 547–560
11. Crouch, D. H., Fincham, V. J., and Frame, M. C. (1996) Oncogene 12, 2689–2696
12. Frisch, S. M., Vuori, K., Ruslalati, E., and Chan-Hui, P. Y. (1996) J. Cell Biol. 134, 793–799
13. Hungerford, J. E., Compton, M. T., Matter, M. L., Hoffstrom, B. G., and Otey, C. A. (1996) J. Cell Biol. 124, 1383–1390
14. Zhang, Z., Vuori, K., Reed, J. C., and Ruslalati, E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6161–6165
15. Kapila, Y. L., Niu, J., and Johnson, P. W. (1997) J. Biol. Chem. 272, 18932–18938
16. Bober-Barkalow, P. J., and Schwarzauer, J. E. (1991) J. Biol. Chem. 266,
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