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Disease-associated fibronectin matrix fragments trigger anoikis of human primary ligament cells: p53 and c-myc are suppressed

R. Dai, A. Iwama, S. Wang and Y. L. Kapila

Department of Stomatology, School of Dentistry, University of California, San Francisco, CA 94143-0512 USA (R. Dai, A. Iwama, S. Wang); Department of Periodontics, Prevention, and Geriatrics, School of Dentistry, University of Michigan, Ann Arbor, MI 48109-1078, USA (Y. L. Kapila)

Inflammation in periodontal disease is characterized by the breakdown of the extracellular matrix. This study shows that an inflammation-associated matrix breakdown fragment of fibronectin (FN) induces anoikis of human periodontal ligament (PDL) cells. This 40 kDa fragment was identified in human inflammatory crevicular fluid and is associated with disease status. Previously, we reported that a similar recombinant FN fragment triggered apoptosis of PDL cells by an alternate apoptotic signaling pathway that requires transcriptional downregulation of p53 and c-myc. Thus, to determine whether the physiologically relevant 40 kDa fragment triggers apoptosis in these cells, the 40 kDa fragment was generated and studied for its apoptotic properties. The 40 kDa fragment induces apoptosis of PDL cells, and preincubation of cells with intact vitronectin, FN, and to a limited extent collagen I, rescue this apoptotic phenotype. These data suggest that the 40 kDa fragment prevents PDL cell spreading, thereby inducing anoikis. The signaling pathway also involves a downregulation in p53 and c-myc, as determined by Western blotting and real time quantitative PCR. These data indicate that an altered FN matrix as is elaborated in inflammation induces anoikis of resident cells and thus may contribute to disease progression.

Keywords: Anoikis; Fibronectin Fragments; Inflammation.

Introduction

Altered FN matrices, as those elaborated during periodontal disease or inflammation compromise PDL cell or resident cell function. During the course of inflammation, bacterial and host-derived proteases cleave the extracellular matrix (ECM) and release fragments, including FN fragments, into the inflammatory milieu. We have shown that these fragments negatively influence PDL cell function by limiting their proliferative capacity and chemotaxis, and by inducing programmed cell death or apoptosis.[1–3] Specific FN fragments also induce the expression of matrix degrading enzymes in these cells that can further perpetuate the breakdown of the ECM components and contribute to disease progression.[4] Thus altered FN matrices trigger altered cellular responses that are not normally induced by intact FN. Exactly how these altered matrices of FN mediate such a diverse repertoire of cell functions is not fully understood. However, these effects likely depend on the different specific ligand-receptor interactions that take place in different cell types with different functional domains of FN, and on the subsequent complex signaling mechanisms induced by these receptor-mediated actions. Thus, in the present study we focused on examining the role of the 40 kDa inflammation-associated FN fragment in potentially altering PDL cell behavior and the potential mechanisms involved. The rationale for examining this 40 kDa fragment is that it is similar to a recombinant FN fragment (V+H−) that we previously showed induces apoptosis of PDL cells via a novel signaling pathway.[2,3] The similarity between the 40 kDa fragment and the V+H− fragment stems from the fact that they are both missing either part of the V region or have a nonfunctional heparin-binding domain of FN; both of which are critical for the regulation of cell survival.[2] Thus, the 40 kDa fragment only has the CS-1 site of the V region and the V+H− fragment has a mutated and non-functional heparin-binding domain.

Since the 40 kDa fragment (Figure 1) contains the heparin-binding domain and the CS-1 site of the alternatively spliced V region of FN, it can potentially bind to cells using the $\alpha 4\beta 1$ integrin and proteoglycan receptors as discussed below. There may also be cooperative interactions between these two classes of receptors. Indeed, there is evidence of interactions between integrins and cell surface chondroitin sulfate proteoglycan receptors.[5,6] We

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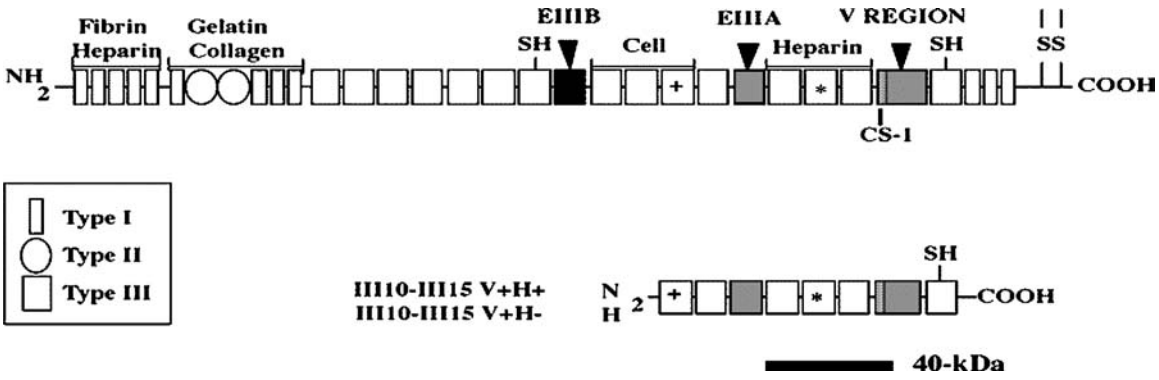
Correspondence to: Dr. Yvonne L. Kapila, DDS, PhD, University of Michigan, School of Dentistry, Department of Periodontics/Prevention/and Geriatrics, 1011 N. University Ave., Room 5213, Ann Arbor, MI 48109-1078, USA.

Figure 1. Intact FN monomer (top) and recombinant (middle) and proteolytic (bottom) FN proteins. Both recombinant FN proteins span repeats III10–15. They contain the V region (V+) and one protein also contains a functional heparin-binding domain (H+), whereas the other protein has a mutated heparin-binding domain (H−). The arginine, glycine, aspartic acid (RGD) cell-binding site (+) and the heparin-binding sequence (*) are indicated within the clear boxes. Alternatively spliced repeats (EIIIB, EIIIA, V) are shaded. Type I–III repeating modules are indicated in the key. The proteolytic, chymotryptic 40-kDa FN fragment is shown on the bottom. It contains the heparin-binding domain and the CS-1 site of the V region of FN.



have specifically identified the $\alpha 4, \alpha 5, \alpha v$, and $\beta 1$ integrin subunits on the surface of PDL cells, using fluorescence-activated cell sorting (FACS) analysis on at least five different PDL cell isolates.[1] The FACS data reveal that relatively more αv is 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. In the case of the V+H− fragment, $\alpha 5$, αv, $\alpha 4$, and $\beta 1$ can bind to the RGD site on repeat III-10,[7–9] and $\alpha 4$ and $\beta 1$ can further bind to the V region (REDV or LDV sites; 10) and the high-affinity heparin-binding domain (IADPS on repeat III-14; 11) on the V+H− protein. Indeed, our published studies (2) with the V+H− fragment suggest that integrins, and specifically, integrins $\alpha 4\beta 1$, may be mediating this apoptotic phenotype.

Evidence for the role of proteoglycans in mediating 40 kDa and V+H− fragment–mediated apoptosis is provided by our earlier studies.[2] These data demonstrate that treating cells with chondroitinase or chondroitin sulfate, but not other glycosaminoglycans reversed the apoptotic phenotype induced by the V+H− fragment. Candidate chondroitin sulfate proteoglycan receptors include CD44 (and its isoforms), which binds the high-affinity heparin-binding domain of FN on repeat III-14 (synthetic peptide FN-C/H II, 6), and a chondroitin sulfate/heparan sulfate proteoglycan, like the syndecans, which also bind the high-affinity heparin-binding domain of FN.[12] Another strong candidate is the NG2 chondroitin sulfate proteoglycan, which can cooperate with the $\alpha 4\beta 1$ integrin in binding to the V region and heparin-binding domain of FN.[13,14]

In our previous studies with the V+H− recombinant FN protein, the signaling mechanism by which this apoptotic mechanism is triggered is novel and requires the transcriptional downregulation of the tumor suppressor, p53 and oncogene, c-myc.[3] Upstream of p53, decreases in focal adhesion kinase phosphorylation and increases in c-Jun N-terminal kinase (JNK)1 phosphorylation further regulate this pathway.[15] JNKs are a group of MAP kinases that are activated by cytokines or environmental stress and are involved in various signaling pathways including those for apoptosis. JNK proteins are activated by phosphorylation and they directly associate with and regulate p53.[16–18] Thus, in this study we examined whether the physiologically relevant, inflammation-associated 40 kDa FN fragment possesses pro-apoptotic characteristics like the V+H− fragment and whether similar signaling mechanisms are involved.

Materials and methods

PDL cell culture

Primary PDL cells were harvested as previously described[4] and used from passages 2 to 6 for all experiments. Culture medium consisted of alpha-minimal essential medium supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin. For experimentation, cells were trypsinized, pelleted under centrifugation, washed twice with phosphate-buffered saline, and suspended in assay medium (serum-free alpha-minimal essential medium supplemented with 0.2% lactalbumin hydrolysate [Invitrogen Corporation, Carlsbad, CA] and 1% penicillin/streptomycin).

Generation and purification of the 40 kDa FN fragment

The 40 kDa FN fragment was prepared by digesting 400 μl of human plasma FN with 2 μl of α-chymotrypsin (Sigma-Aldrich, St. Louis, MO; 0.5% stock reconstituted in distilled water) at 37°C in a water

bath for 1 h and 10 min. The reaction was stopped by adding 2.3 mg/ml of Diphenylcarbamoyl Chloride (Sigma-Aldrich). The 40 kDa fragment was subsequently purified using heparin-sepharose affinity chromatography, gel filtration chromatography, and dialysis, and concentrated using lyophilization as described below. For heparin-sepharose chromatography, the digested 400 μl of plasma FN preparation and an equal volume of double distilled pyrogen-free water were loaded on the column and incubated at 4°C for 1 h with gentle shaking. After the flow through was collected, affinity-bound FN fragments were eluted with 0.01 M NaCl/50 mM Tris HCl (pH 7.0). Fourteen fractions were collected and analyzed by SDS-PAGE and Coomassie Blue stain, to select the fractions that contained a 40 kDa fragment. All the fractions that contained a 40 kDa fragment were further chromatographed on G-25 Sephadex gel filtration columns to remove excess salt and to further purify the 40 kDa fragment. After the flow through was collected, FN fragments were eluted from this column with a 1X phosphate buffered saline solution free of calcium and magnesium ions. Another fourteen fractions were collected and screened again by SDS-PAGE to select for those that contained a 40 kDa fragment. Fractions containing a 40 kDa fragment were then pooled and dialyzed against pyrogen-free water at 4°C overnight in a 10,000 molecular weight cutoff dialysis cassette (Slide-A-Lyzer, Pierce, Rockford, IL). The dialyzed sample was concentrated by lyophilization, and the protein concentration was determined using the BCA kit (Pierce). Positive identification of the 40 kDa fragment was performed by Western blotting as described below.

Western immunoblotting

Western immunoblotting was used to confirm the presence of the purified 40 kDa fragment protein. 5 μg of the 40 kDa fragment sample and media control were electrophoretically resolved on 10% SDS-PAGE gels and transferred to nitrocellulose membranes for Western immunoblotting. The membranes were probed with a mouse anti-human FN antibody against the CS-1 site (MAB 1939, Chemicon Intl., City; used at a dilution of 1:200) and a mouse anti-human FN antibody against the heparin binding-domain (MAB 1938, Chemicon Intl; used at a dilution of 1:200). Membranes were then probed with an anti-mouse horseradish peroxidase-conjugated secondary antibody (1:2000) for 45 min at room temperature followed by ECL detection (Amersham).

Recombinant FN fragment

Two recombinant FN protein fragments were tested in these experiments. These fragments, described elsewhere,[19] included (V^+) the alternatively spliced V region and contained either an unmutated (H^+) or a mutated, nonfunctional high-affinity heparin-binding domain (H^-).

Apoptosis assessment

Apoptosis in PDL cells was evaluated by three criteria: (1) cell rounding plus membrane blebbing, (2) nuclear disintegration, and (3) DNA fragmentation.

To evaluate cell rounding and membrane blebbing after incubation under test and control conditions, cells were visualized, counted, and photographed at 400X magnification using an inverted microscope equipped with an automatic camera.

To assess the quality of the nucleus, PDL cells were incubated with the 40 kDa protein fragment (1 mg/ml) and the recombinant FN protein, V+H− (0.1 μM) or with control assay medium for 14 h in 16-well glass chamber slides, and nuclear staining of DNA was used to detect nuclear disintegration. Specifically, after incubation under test and control conditions, 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-Aldrich) for 10 min, rinsed three times with calcium- and magnesium-free PBS, dried, and sealed with a coverslip, using mounting medium (Vectashield; Vector Laboratories, Burlingame, CA). Cells were counted and photographed at 400x magnification using a fluorescent photomicroscope equipped with a camera and filter for DAPI stain detection (Nikon E400 microscope). Condensed, brightly stained and disintegrated nuclei were counted as positive. An average of five fields from triplicate wells were counted.

Immobilized/coated and soluble FN fragments and ECM ligands

In experiments testing whether the effects of the 40 kDa fragment were dependent on its presentation as a soluble or immobilized matrix, the 40 kDa fragment (0.5 mg/ml), the V+H− fragment (0.1 μM), and a serum-free media control were coated overnight at 4°C on 16-well chamber slides to immobilize these molecules prior to treatment with PDL cells. Plates were then washed 3x with PBS/DPBS. Subsequently, cells were added to the wells and either not treated further (coated conditions) or treated with the 40 kDa fragment (1 mg/ml) and V+H− fragment (0.1 μM), and then cell shape and nuclear staining were examined after 14 h.

In experiments testing whether intact FN and other ECM ligands could rescue the apoptotic phenotype triggered by the 40 kDa fragment, intact human plasma FN (1 mg/ml; Calbiochem, San Diego, CA), human placenta collagen I (0.5 mg/ml; Chemicon International

Temecula, CA), human plasma vitronectin (0.4 mg/ml; Promega, Madison, WI) and an assay media control were coated overnight at 4°C on 16-well chamber slides as immobilized forms of ECM prior to treatment with PDL cells. Plates were then washed 3x with PBS/DPBS. Subsequently, cells were treated with the 40 kDa fragment (1 mg/ml) and cell shape and nuclear staining were examined after 14h. PDL cells were fixed, stained, and counted as described above.

DNA fragmentation assessment

For a quantitative assessment of cell apoptosis, DNA fragmentation in cell lysates and supernatants was assayed after a 14 h incubation with the 40 kDa fragment (0.5 mg/ml) and the recombinant FN protein, V+H− (0.1 μM) or with control assay medium using the cell death detection ELISA Plus Kit (Roche, 1774425, Indianapolis, IN). The ELISA, which measures cytoplasmic histone-associated DNA fragments after induced cell death, was performed according to the manufacturer's instructions. Colorimetric detection and quantification of the ELISA was performed on a Vmax kinetic microplate reader (Molecular Devices, Menlo Park, CA). Using relative units, total levels of DNA fragments were compared for all treatments.

Real-time quantitative PCR

Real time quantitative PCR was used to assess changes in p53 and c-myc levels triggered by the 40 kDa fragment. For this purpose, PDL cells were plated in 200 μl of assay medium at a density of 5.0×10^5 cells/well in a 24-well plate. Subsequently, cells were treated with the 40 kDa protein fragment (1 mg/ml) and the V+H− fragment (0.1 μM) or with control assay media for 7 h. Cells were then collected by scraping, washed twice in PBS, and lysed in RLT buffer (proprietary lysis buffer, Qiagen, Valencia, CA). Total RNA was extracted using the RNAeasy kit (Qiagen), according to the manufacturer's instructions. The concentration of RNA in the samples was ascertained by measuring optical density at 260 nm.

Total RNA from each sample was used to generate cDNA using an iScript cDNA synthesis kit (Bio-Rad, 170-8890, CA) according to the manufacturer's protocol. Briefly, 1 μg of total RNA was used as starting material for each sample, to which 4 μl of 5x iScript reaction mix, 1 μl of iScript reverse transcriptase, and nuclease-free water were added to a final volume of 20 μl. Reactions were carried out at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 mins.

The primers and probes used for the housekeeping gene, GAPDH, and human p53 genes were purchased from Biosource Inc. (Camarillo, CA). The primer and probe for c-myc were obtained from the UCSF Genome Analysis Core Facility (UCSF, Comprehensive Cancer Center) as a gift. The sequences of primer/probes are listed in Table 1.

All assays were performed using the ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA). Each assay was performed separately. In brief, 100 ng of extracted DNA, control DNA or water (with no template control), 5 μl of a 10x forward and reverse primer mixture, and 1 μl of 50x probe were added to a 1x master mix (5x TM buffer, 25 mM $MgCl_2$, 25 mM dNTP and water; master mix obtained from the UCSF Genome Analysis Core Facility of the UCSF Comprehensive Cancer Center) in a final volume of 50 μl. The RT-PCR reaction conditions used were 10 min at 95°C as an initial polymerase activation step, followed by 40 amplification cycles of 15 s at 95°C, and a cycle for 1 min at 60°C.

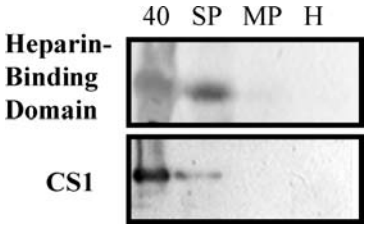
To quantitate the relative amount of gene expression for both the target and housekeeping gene, the comparative C_T (threshold cycle) method was used. The corresponding arithmetic formulas used are the following: $\Delta C_T = C_{T\text{target gene}} - C_{T\text{GAPDH}}$; and $C_{T\text{Linear}} = 2^{-(\Delta C_T\text{treated} - \Delta C_T\text{control})}$. $C_{T\text{Linear}}$ represents the fold change in mRNA expression between the control and treated groups, and assumes a doubling of target sequence with each PCR cycle. The mRNA transcript level was normalized against GAPDH at each dilution. Percentage expression of p53 and c-myc were normalized and compared with GAPDH. The comparative C_T method was used as outlined in User Bulletin #2 (Applied Biosystems).

Table 1. Primer/probe used in RT-PCR taqman assay

Primer/Probe	Fluorochrome	Sequence
F-c-myc	—	GGACGACGAGACCTTCATCAA
R-c-myc	—	CAGCTTCTCTGAGACGAGCTTG
Probe		
GAPDH	TET	5′-CAA GCT TCC CGT TCT CAG CC-3′
P53	FAM	5′-TTG GCT CTG ACT GTA CCA CCA TCC AC-3′
c-myc	FAM	5′-CCA GGA CTG TAT GTG GAG CGG CTT CT-3′

F: forward primer; R: reverse primer.

Figure 2. Western blots illustrate the presence of the 40 kDa fibronectin fragment present in human gingival crevicular fluid from healthy (H), moderate periodontitits (MP), and severe periodonitits (SP) sites. The same 40 kDa that induces apoptosis *in vitro* is found in the most diseased sites. Blots were developed with ECL Plus (Amersham). The 40-kDa chymotryptic fragment was loaded as a positive control (40). Blots were probed with antibodies to the heparin-binding domain of fibronectin (MAB 1938, Chemicon) in A and the CS-1 site of fibronectin (MAB 1939, Chemicon) in B.

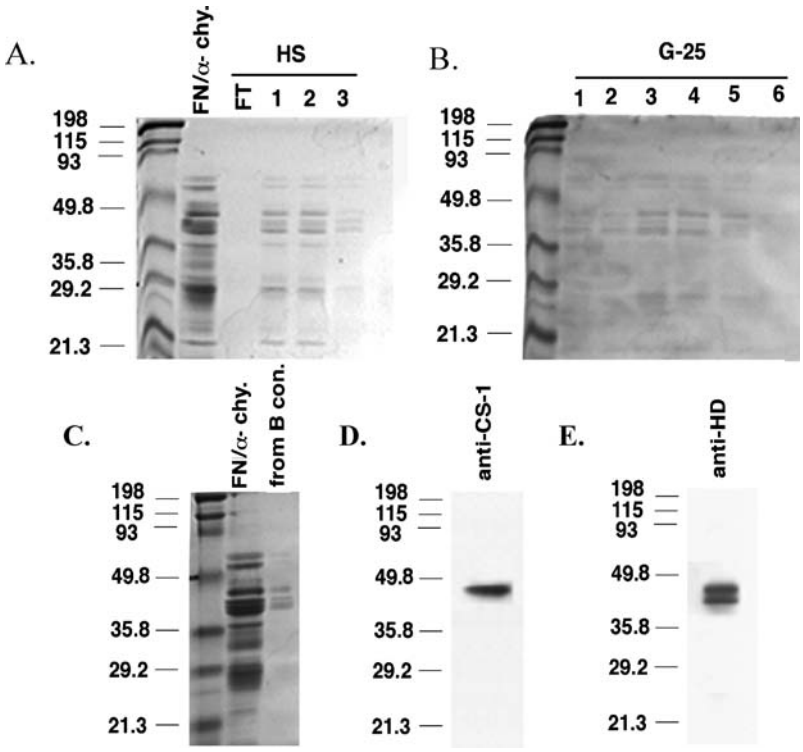


Briefly, this comparative C_T method involved averaging triplicate samples, which were taken as the C_T-values for p53, c-myc and GAPDH. The ΔC_T-value was obtained by subtracting the average GAPDH C_T-values from the average C_T-values of p53 and c-myc. The fold change was calculated according to the formula $2^{-(\Delta\Delta C_T)}$, where $\Delta\Delta C_T$ was the difference between ΔC_T and ΔC_T value. The level of gene expression for the control group was arbitrarily set at 1 to serve as a reference. Therefore, the expression of the target gene from experimental groups represents the fold-difference expression relative to the reference control gene.

Results

We previously showed that disease associated FN fragments, including the 40 kDa fragment are associated with periodontal disease status.[20] The 40 kDa fragment, which contains the N-terminal heparin-binding domain and CS-1 site of the V region of FN was identified at sites of severe periodontitis and it was found in high levels at these sites compared to sites with moderate disease or healthy sites (Figure 2). Furthermore, since, we previously determined that a recombinant FN protein with similar FN domains

Figure 3. These series of gels and Western blots illustrate the steps involved in the purification scheme for the 40 kDa FN fragment. A. This is a Coomassie Blue stained gel of plasma fibronectin (FN) digested with α- chymotrypsin and column fractions of this preparation after affinity chromatography on Heparin-Sepharose (HS) and elution with 0.01 M NaCl/50 mM Tris-HCl (pH 7.0). The starting material (second lane on the left) is plasma fibronectin digested with α- chymotrypsin (FN/α-chy). The flow through (FT) represents the unbound material. Fractions 1 through 3 represent the bound material eluted from the column. B. This is a Coomassie Blue stained gel of column fractions from a G-25 Sephadex gel filtration column containing the 40 kDa α-chymotryptic FN fragment. Column fractions 1 and 2 from the Heparin-Sepharose column in Figure A were used as the starting material. The column fractions were eluted with double-distilled pyrogen free water. C. This is a Coomassie Blue stained gel of the concentrated and dialyzed G-25 fractions 3 and 4 (in Figure B) containing the 40 kDa fragment. D. This Western immunoblot illustrates the presence of the 40 kDa fragment (CS-1 reactive band) in the purified and concentrated sample in Figure C (third lane on the right). E. This Western immunoblot illustrates the presence of the 40 kDa fragment (heparin-binding domain [HD] reactive band) in the purified and concentrated sample in Figure C.



as those in the 40 kDa fragment induces PDL cell apoptosis, we hypothesized that the physiologically relevant 40 kDa fragment might also trigger apoptosis in these cells. To examine this possibility, we first generated, purified, and positively identified the 40 kDa fragment from intact plasma FN (Figure 3) using various purification schemes that included heparin sepharose affinity chromatography, G-25 Sephadex gel filtration chromatography, dialysis, and lyophilization.

Having generated the 40 kDa fragment (Figure 3), the fragment was subsequently incubated with PDL cells to determine whether it had any proapoptotic properties. Indeed, similar to the V+H− recombinant FN fragment, the 40 kDa fragment induced PDL cell rounding, membrane blebbing, nuclear condensation, and high levels of DNA fragmenation; all characteristic of apoptosis (Figure 4). Cells incubated in control media or with the control recombinant FN fragment, V+H+, did not exhibit this phenotype, and instead were well spread with oval shaped nuclei, and had low levels of DNA fragmentation (Figure 4).

To determine whether the effects of the 40 kDa fragment were dependent on its presentation as a soluble matrix or whether an immobilized 40 kDa fragment could also induce apoptosis, cells were incubated with both soluble and immobilized forms of the 40 kDa fragment (Figure 5). Only the soluble form of the 40 kDa fragment induced the apoptotic phenotype in these cells. Similarly, only the soluble form of the positive control V+H− fragment induced the apoptotic phenotype, whereas the

Figure 4. Effect of the 40 kDa chymotryptic fragment of FN on PDL cell apoptosis. A. PDL cells plated in the presence of the 40 kDa fragment (0.5 mg/ml) or positive control fragments V+H− (0.1 μM) are rounded (Phase) and have DAPI-stained nuclei that are bright and condensed by 14 h after treatment. This is in contrast to the negative controls, PDL cells plated in media alone or with the V+H+ fragment, which spread (Phase) and have normal, oval-shaped nuclei (DAPI). B. ELISA for DNA fragments indicates high levels of DNA fragmentation in PDL cell lysates and supernatants from cells treated with the 40 kDa fragment (0.5 mg/ml) and the V+H− (0.1 μM) FN protein compared to media (c) and V+H+ (0.1 μM) controls.

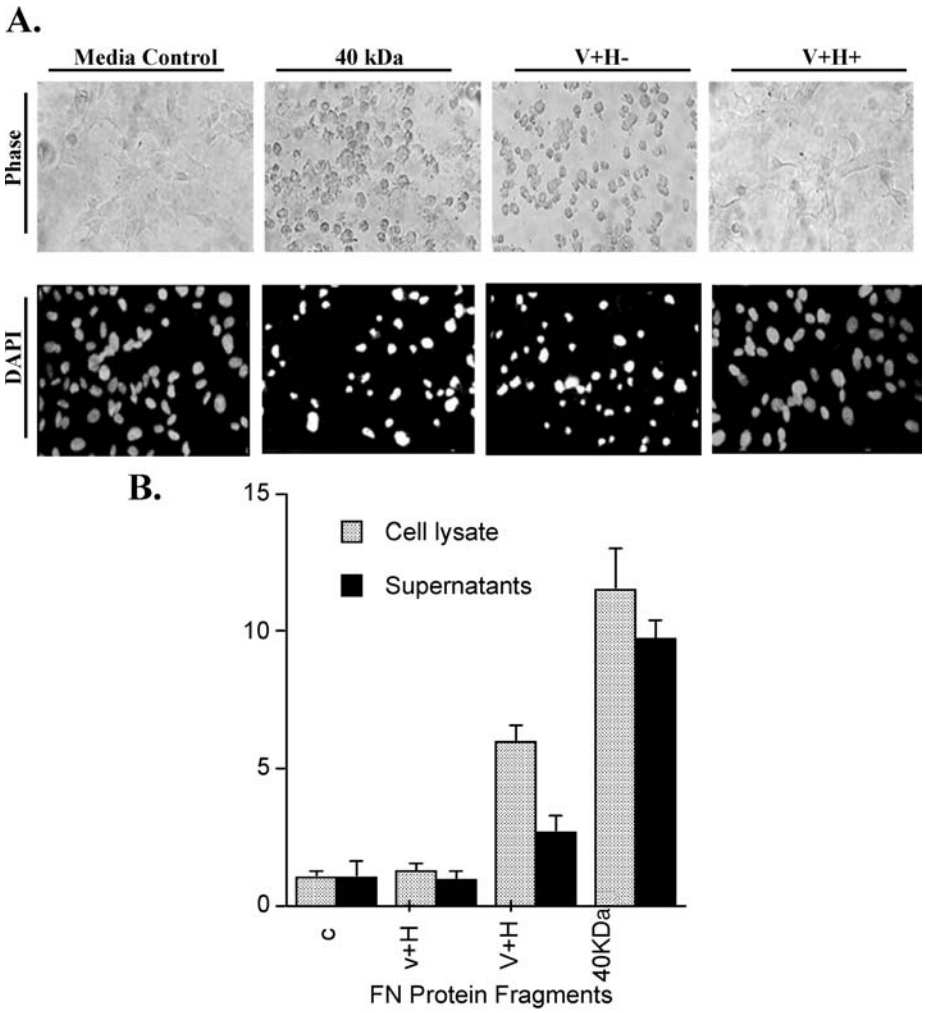
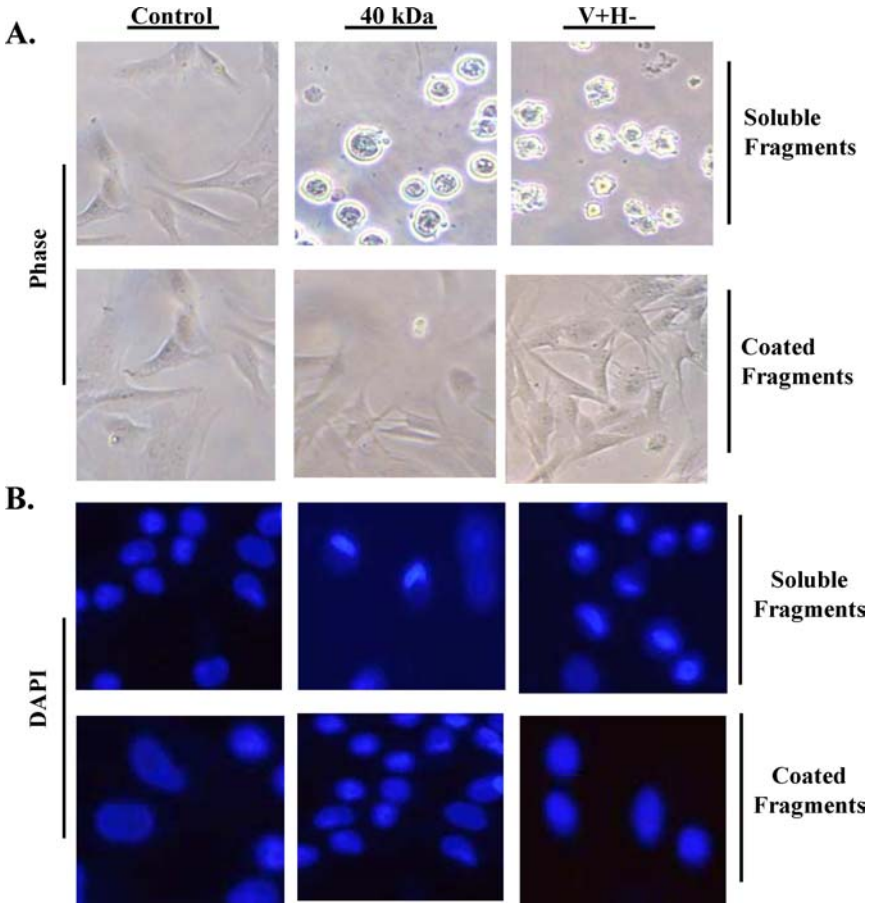


Figure 5. Effect of soluble and coated forms of the 40 kDa chymotryptic fragment of FN on PDL cell apoptosis. A. PDL cells plated in the presence of soluble forms of the 40 kDa fragment (0.5 mg/ml) or positive control fragments V+H− (0.1 μM) are rounded (Phase) and have DAPI-stained nuclei that are bright and condensed by 14 h after treatment. In contrast, cells plated in the presence of coated forms of the 40 kDa or V+H− fragment, spread (Phase) and have normal, oval shaped nuclei (DAPI), like the controls.



immobilized forms of the FN fragments did not. Control conditions mirrored those of the immobilized fragment-treated cells. Thus, these findings suggest that the 40 kDa fragment in solution prevents cell spreading and thereby triggers anoikis. Anoikis is defined as apoptosis triggered by detachment from the ECM or inability to attach to the ECM.

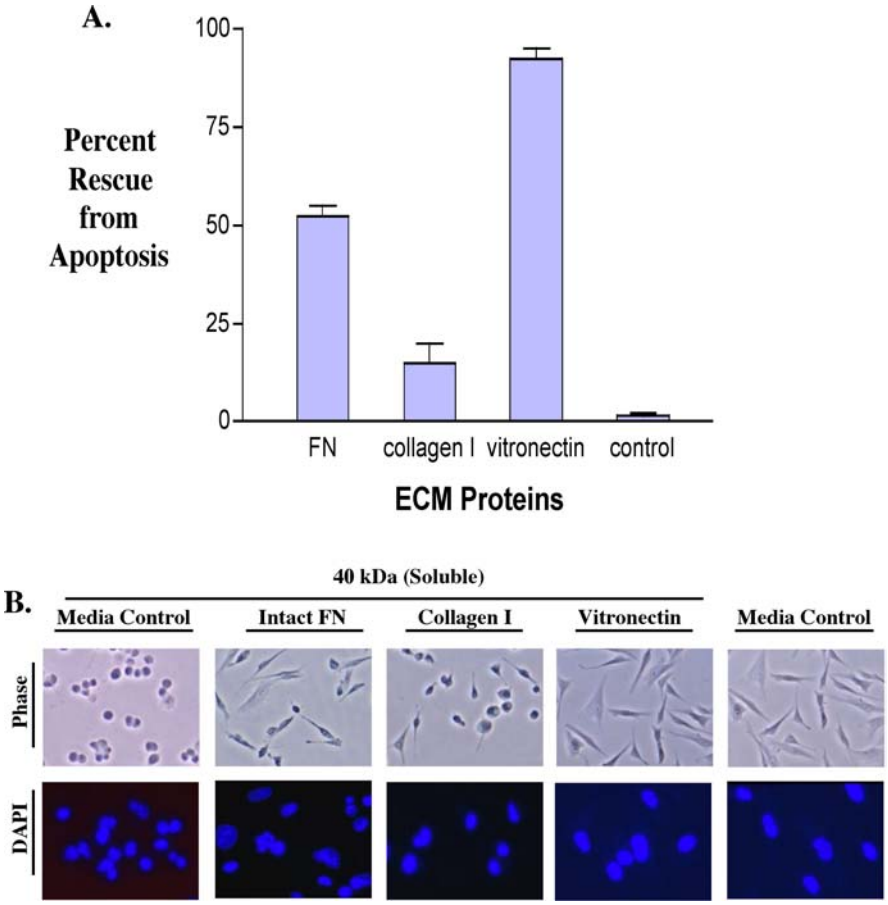
Given these findings, we next examined whether allowing cells to spread first in the presence of various intact ECM ligands would protect cells from apoptosis triggered by the soluble 40 kDa fragment. Indeed, the data demonstrate that cells allowed to spread on intact vitronectin were most resistant to apoptosis triggered by the 40 kDa fragment, followed by cells allowed to spread on intact FN and then by collagen I (Figure 6). These findings confirm that FN fragments induce anoikis in cells deprived of contacts with their ECM. In this context, since vitronectin provided the most rescue potential in these cells compared to the other ECM ligands examined, its receptors or associated survival signals may be key regulators in this mechanism.

The signaling mechanisms involved in this pathway of anoikis triggered by the 40 kDa fragment may be the same as those previously identified in our studies with the V+H− recombinant fragment.[2,3,21] These previous studies demonstrated that the V+H− FN fragment triggers apoptosis of PDL cells by transcriptionally downregulating p53 and c-myc and inducing increased JNK phosphorylation (pJNK). Thus, in the current study the levels of p53, c-myc, and pJNK were examined in 40 kDa fragment treated cells by Western blot analyses and quantitative real time PCR. These data show that p53 and c-myc levels are also downregulated and pJNK levels are upregulated by treatment with the 40 kDa fragment as in V+H− fragment treated cells (Figure 7).

Discussion

This study documents for the first time that disease associated matrix fragments of fibronectin induce anoikis in human primary cells. The mechanism by which this

Figure 6. Effect of different extracellular matrices on 40 kDa-mediated apoptosis. A. The graph illustrates the percent rescue from 40 kDa-mediated apoptosis by pretreatment with intact FN, collagen I, vitronectin, and control media. B. Images of cells under Phase microscopy and after DAPI staining illustrate the cell shape and nuclear profile of cells preincubated with intact FN, collagen I, vitronectin, and control media then treated with the 40 kDa fragment or treated with no fragment and incubated in media alone.

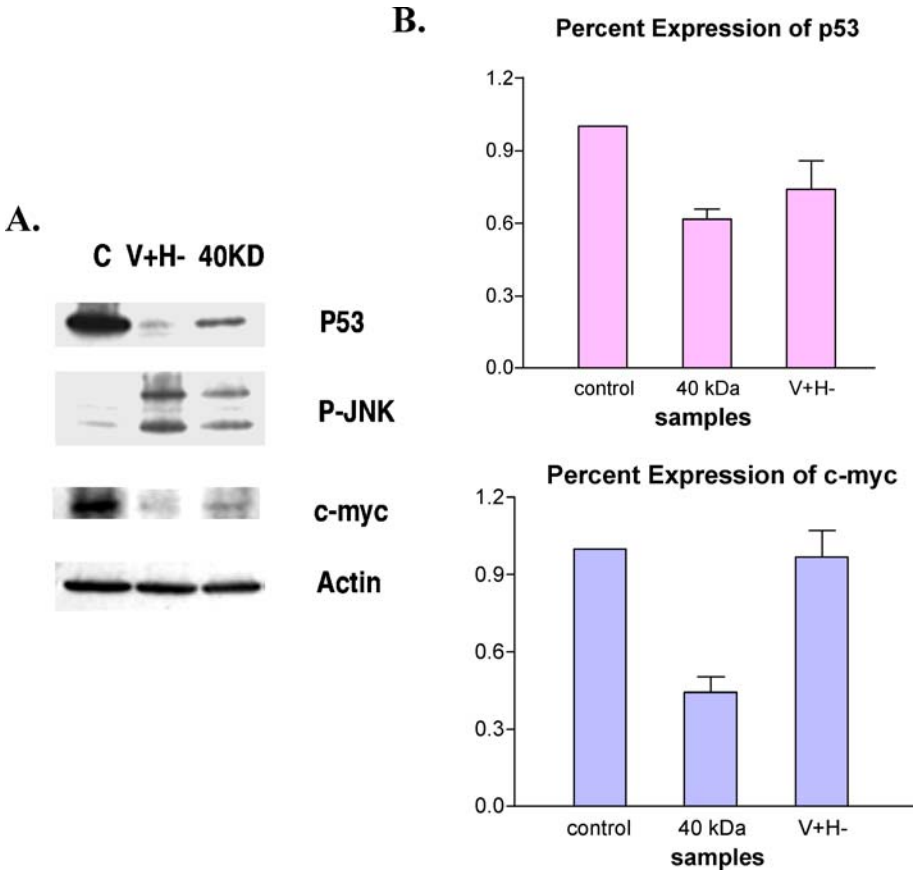


occurs appears to be comparable to that induced by a similar recombinant FN fragment that was previously shown to induce apoptosis of PDL cells by transcriptionally downregulating c-myc and p53[3] and increasing JNK phosphorylation.[15] The 40 kDa fragment also induced decreases in p53 and c-myc and increases in JNK phosphorylation. The implications from these findings is that during the course of inflammation, degradation of matrix molecules may further contribute to disease progression by induction of programmed cell death in resident cells.

Figure 4 indicates that the 40 kDa FN fragment induces higher levels of DNA fragmentation compared to the V+H− recombinant protein. The reason for this difference stems from the fact that the concentration of the 40 kDa fragment used (0.5 mg/ml) in this experiment is higher than that of the V+H− fragment (0.00825 mg/ml). The rationale for using these concentrations is that our initial work with the V+H− fragment indicated that this was the lowest concentration that induced apoptosis in cells. In contrast, a higher concentration of the 40 kDa fragment was required to induce apoptosis *in vitro*. Furthermore, *in vivo* data indicated that the physiologic concentration of the 40 kDa fragment in inflammatory fluids[20] was approximately equivalent to the 0.5 mg/ml tested *in vitro*. Thus, all subsequent experiments were performed with similar concentrations of these fragments to assess apoptosis. Based on these findings, the V+H− appears to be more efficient at inducing apoptosis than the 40 kDa fragment. The reason for this is not known although it may be related to structural differences in the proteolytically-generated 40 kDa fragment and the recombinantly derived V+H− protein. More specifically, these differences may be related to three-dimensional structural variations or sequence differences in these fragments that, in turn, interact differently with and have different binding affinities for the cell surface receptors that regulate this apoptotic mechanism and DNA fragmentation.

Finding that only the soluble forms of the FN fragments induced apoptosis in these cells suggests that the apoptotic effects of the fragments are related to their

Figure 7. Expression levels of p53, c-myc, and pJNK in PDL cells treated with FN fragments. A. These Western immunoblots illustrate the changes in p53, pJNK, and c-myc levels in PDL cells treated with the 40 kDa FN fragment, recombinant V+H− FN fragment, or media control. Actin was used as a loading control. B. Graphs illustrate the percent expression of p53 and c-myc in FN fragment treated cells as assessed by realtime quantitative PCR.



effects on cell spreading. The fragments may be inducing a unique form of apoptosis, called anoikis, which is defined as programmed cell death triggered by detachment from the extracellular matrix (ECM) or inability to attach to the ECM. Previous studies have shown that cells deprived of an ECM undergo anoikis.[22] In the current study, the soluble FN fragments may in their interactions with cells block the necessary receptors required for anchoring the cells to the ECM. However, the coated fragments allow cells to attach via other mechanisms that may include de novo secretion of their own matrix or only partial blocking of the receptors required for anchoring. Our previous studies indicate that receptors including integrins and a chondroitin sulfate proteoglycan may be mediating interactions with these fragments of FN[2] to regulate this apoptotic mechanism.

In further support of the idea that the 40 kDa fragment may be triggering anoikis in these cells, is the rescue data with intact ECM proteins, including vitronectin. These data show that allowing PDL cells to spread on ECM components first, prevents the induction of apoptosis by subsequent introduction of the soluble 40 kDa fragment. Thus, cells that have already spread are no longer responsive to the proapoptotic effects of the fragment.

Additionally, the rescue data with the intact ECM components shows that cells that spread on vitronectin are more resistant to the effects of the soluble 40 kDa fragment. These findings suggest that either the receptors used by the 40 kDa fragment are most like those for vitronectin, the alpha v beta 1 integrin for example, or that the survival signals generated by vitronectin are more complete that those induced by other intact ECM proteins, like fibronectin or collagen. The former presents an interesting possibility; that the alpha v beta 1 integrin is mediating interactions with the 40 kDa fragment of FN. However, the alpha v beta 1 integrin has been shown to interact only with the RGD site of FN[8], but not with the heparin-binding domain or CS-1 site of FN that are present on the 40 kDa fragment. Thus, further studies will focus on the interesting possibility that the alpha v beta 1 integrin also interacts with the heparin-binding domain and/or CS-1 site of FN.

The downregulation in p53 and c-myc mRNA levels induced by the 40 kDa fragment is more pronounced

than that induced by the V+H− recombinant protein, and this is in contrast to that noted for the downregulation in p53 and c-myc protein levels. One likely explanation for this difference is that the 40 kDa fragment may have more of an effect on transcriptional and translational control, thus explaining the greater effects on mRNA levels, and the V+H− protein may be exhibiting more post-translational effects (i.e. proteasomal degradation), thus also explaining its greater effects at the protein level.

Conclusion

Our results demonstrate that matrix breakdown fragments of FN that are produced during inflammation induce anoikis of primary resident PDL cells, and thereby may contribute to disease progression.

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