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Proapoptotic fibronectin fragment induces the degradation of ubiquitinated p53 via proteasomes in periodontal ligament cells

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Background and Objective: The extracellular matrix (ECM) plays a key role in signaling necessary for tissue remodeling and cell survival. However, signals from the ECM altered by disease, e.g. inflammatory diseases such as periodontitis and arthritis, may lead to apoptosis or programmed cell death of resident cells. Previously, we found that a disease-associated fibronectin fragment triggers apoptosis of primary human periodontal ligament cells via a novel apoptotic pathway in which the tumor suppressor, p53, is transcriptionally downregulated.

Material and Methods: We used immunofluorescence, transfection assays, western blotting and ELISAs to show that p53 is degraded by a proteasomal pathway in response to a proapoptotic disease-associated fibronectin fragment.

Results: We found that in these apoptotic conditions, p53 is further downregulated by post-translational ubiquitination and subsequent targeting to proteasomes for degradation. Pretreatment of cells with the proteasomal inhibitors MG132 and lactacystin rescued the cells from apoptosis. The p53 levels in cells transfected with ubiquitin small interfering RNA were resistant to degradation induced by the proapoptotic fibronectin fragment, showing that ubiquitination is important for the proapoptotic fibronectin fragment-induced degradation of p53.

Conclusion: These data show that a proapoptotic fibronectin matrix induces ubiquitination and degradation of p53 in the proteasome as part of a novel mechanism of apoptosis associated with inflammatory diseases.

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The extracellular matrix (ECM) provides structural integrity to tissues and organs and is important for promoting cell adhesion, migration and survival. However, changes in the ECM due to infection, inflammation or wounding may disrupt the homeostasis of the extracellular environment. In such conditions, ECM proteins undergo proteolytic cleavage or alternative splicing, resulting in fragmented or altered forms (1–5). These alterations lead to aberrant signaling in surrounding cells and catalyze further degradation of the matrix, exacerbating the disease. For example, disease-associated fibronectin fragments can cause apoptosis and further tissue catabolism by inducing the expression of matrix metalloproteinases, nitric oxide and proinflammatory cytokines (6–14). The mechanisms by which ECM fragments elicit these adverse cellular effects may involve alterations in receptor regulation and signaling processes (13,15–18), such as altered regulation and signaling via p53 and

the c-Jun NH_2-terminal stress-related kinases (13,16,17,19).

The existence of fibronectin fragments has been documented in chronic inflammatory diseases and shown to be important in the pathogenesis of chronic inflammation, including arthritis and periodontal disease (1,2,5–7,14,15). We generated and characterized several recombinant counterparts of the fibronectin fragments that are found in *in vivo* conditions (9). One such fibronectin fragment is defective in binding to heparin. We found that while the native fibronectin molecule promotes growth and attachment of cells, the defective form of the fibronectin fragment triggered cell detachment and showed a proapoptotic effect, since it induced apoptotic cell shape changes and apoptotic cell signaling. Cells treated with this proapoptotic fibronectin fragment showed a decrease in p53 at both the transcriptional and the protein levels (2,11–13,15).

The transcription factor p53 is a tumor suppressor that plays a critical role in safeguarding the integrity of the genome (20) and controls cell-cycle arrest, apoptosis and cellular senescence (21). Its expression and activation are tightly controlled within the cell by post-translational modifications, including ubiquitination, acetylation and phosphorylation (22). Often, p53 regulatory networks are perturbed by stress signals, such as DNA damage, oncogene activation, hypoxia and nitric oxide production, resulting in ubiquitination of p53 (23) and its degradation in the proteasome (24,25).

As mentioned above, the reduction in p53 promoter activity and mRNA level in cells exposed to the proapoptotic fibronectin fragment was not sufficient to explain the significant loss of p53 protein. Thus, we hypothesized that the decrease in p53 levels in cells treated with the proapoptotic fibronectin fragment was also due to post-translational modifications. One of the prevalent modes of p53 downregulation is through its ubiquitination, which targets it to the proteasome for degradation (26). Currently, there is no literature describing the type of post-translational modification undergone by p53 that accentuates its degradation in response to inflammation/disease-associated extracellular matrix proteins or their fragments. Therefore, this study was initiated to investigate whether fibronectin fragments induce ubiquitination of p53 and its degradation through the proteasome.

Material and methods

Primary periodontal ligament (PDL) cell culture

Human primary PDL cells were isolated and cultured as described (12). The PDL cells were isolated from the teeth that were extracted from patients undergoing treatment at the School of Dentistry, University of Michigan, Ann Arbor, USA. The donors provided written consent and these were in accordance with the University of Michigan Health Sciences Institutional Review Board. Their use was approved by the University of Michigan Health Sciences Institutional Review Board. Cells were maintained in α-minimal essential medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (Hyclone, Logan, UT, USA) and penicillin (100 U/mL) and streptomycin (100 μg/mL).

Recombinant fibronectin proteins

For these studies, we used two previously described recombinant fibronectin fragments (10) that contain the alternatively spliced V region (V^+) and either an intact (H^+) or a mutated, non-functional (H^-) high-affinity heparin-binding domain. The control fibronectin fragment (CFn), with the intact H^+ domain, and the proapoptotic fragment (AFn), with the mutated H^- domain, were used at a concentration of 0.1 mM. The proapoptotic fragment is comparable to the disease-associated 40 kDa proteolytic fragment, also shown by our group to induce apoptosis in PDL cells.

Western blot analysis

For western blot analysis, cells were lysed in RIPA buffer (Sigma, St Louis, MO, USA) containing protease inhibitors (Sigma). Protein concentration was determined by the BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein were resolved by standard procedures using SDS-PAGE with 4–20% gels (Novex; Invitrogen) and electroblotted onto polyvinylidene membranes (PVDF; Immobilon-P; Millipore, Billerica, MA, USA) by semi-dry transfer blot (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The primary antibodies were mouse anti-human p53–horseradish peroxidase (DO-1), rabbit anti-human ubiquitin and goat anti-human actin (I-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Immunoprecipitation

For immunoprecipitation, cells treated with AFn or CFn fragments, or control serum-free medium, in the presence or absence of lactacystin, were lysed in RIPA buffer containing protease inhibitors. Equal amounts of protein from the lysates were precleared with protein G–agarose (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK), incubated with mouse anti-p53 overnight at 4°C and then with protein G–agarose beads for 2 h. After five washes with RIPA buffer, the beads were boiled in SDS-PAGE reducing sample buffer. Supernatants were resolved by SDS-PAGE with 4–20% gels and processed for western blot analysis. After incubation of the PVDF membranes with anti-ubiquitin antibodies, the blots were incubated with Clean Blot HRP reagent (Pierce), which recognized only the native primary antibodies and did not interact with the denatured primary antibodies (IgG) that were used to immunoprecipitate the p53 molecules.

Small interfering RNA (siRNA) transfection experiments

One day before transfection, cells were plated at 60% confluency in six-well dishes. Human ubiquitin UBA52 siRNA (Dharmacon, Thermo Fisher Scientific, Freemont, CA, USA; NM_003333) or the control siRNA (Dharmacon) were introduced into

cells using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. Thirty-six hours post-transfection, cells were treated with the fibronectin fragments in serum-free medium for 7 h and processed for western blot analysis.

Immunofluorescence

To immunolocalize p53, cells were fixed with 3% paraformaldehyde in phosphate-buffered saline, pH 7.2, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 20 min and incubated in 5% bovine serum albumin in Tris Buffered Saline Tween (TBST) (25 mM Tris, 150 mM NaCl, 0.05% Tween-20) for 1 h at room temperature. Cells were then incubated with mouse anti-p53 (DO-1) overnight at 4 °C, washed with TBST, treated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody for 1 h at room temperature and washed with TBST. Then cells were stained with 4′,6′-diamidino-2-phenylindole (DAPI; Sigma) for 10 min, rinsed with calcium- and magnesium-free phosphate-buffered saline, air dried, mounted on glass slides with Vectashield (Vector Laboratories, Burlingame, CA, USA), and examined with a Nikon TS100 photomicroscope.

Apoptosis ELISA assay

Cells were pretreated with lactacystin and then treated with proapoptotic or control fibronectin fragment or control media. Thereafter, the cells were lysed, and equal amounts of cell lysate protein were processed to quantify apoptosis using the Cell Death Detection ELISA^{PLUS} kit (Roche, Mannheim, Germany) as recommended by the manufacturer.

Other reagents

The proteasomal inhibitors, lactacystin and MG132, were from Calbiochem (La Jolla, CA, USA). All other chemicals were from Sigma unless mentioned otherwise.

Results

A fragmented fibronectin matrix triggers p53 degradation through a proteasomal pathway

In our previous studies, we observed that a recombinant fibronectin fragment, which is comparable to its *in vivo* proteolytic counterpart found in inflammatory gingival crevicular fluids, causes a decrease in p53 levels as part of a unique apoptosis pathway. However, only part of the reduction in p53 levels could be explained by transcriptional downregulation (11), suggesting that other protein degradation pathways, such as the lysosomal and proteasomal pathways (27,28), may be contributing to the elimination of p53 when cells are treated with the proapoptotic fibronectin fragment (AFn). First, we pre-incubated PDL cells with different lysosomal enzyme inhibitors before treating cells with the AFn and then examined the cell lysates for p53 protein levels by western blot analysis. As seen in Fig 1A, pretreatment of cells with lysosomal enzyme inhibitors failed to prevent p53 degradation in response to treatment with AFn (lanes 3–12), suggesting that p53 was not being degraded through the lysosomal pathway. Moreover, p53 was also degraded (Fig. 1A, lane 14) in cells pretreated with cycloheximide, a protein synthesis inhibitor, suggesting that new protein synthesis is not required for the degradation of p53 in this pathway.

In addition to the lysosome, the proteasome is an important component of the cell machinery that mediates protein degradation. Protein degradation is vital for initiating, terminating and regulating many cellular processes, and the proteasome is involved in this degradation process. One of the most important regulatory

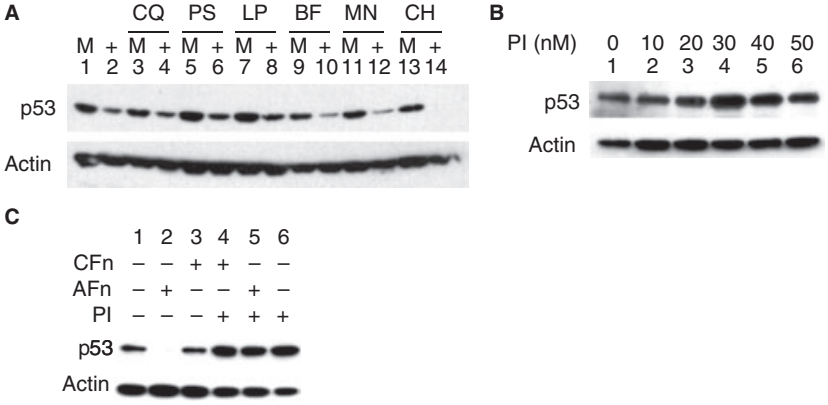

Fig. 1. Proapoptotic fibronectin induces p53 degradation through the proteasomal pathway. (A) Human primary periodontal ligament (PDL) cells were pre-incubated with serum-free medium, 10 μM chloroquine (CQ), 100 μM pepstatin A (PS), 100 μM leupeptin (LP), 50 nM bafilomycin A (BF), 10 μM monensin (MN) or 10 μM cycloheximide (CH), for 2 h and then treated with AFn (+) or control medium (M) in presence of the drugs for 7 h. Cells were then lysed, and the lysates were resolved by SDS-PAGE, immunoblotted with an anti-p53 antibody, and visualized by chemiluminescence. (B) Cells were treated with various concentrations of MG132 (PI) for 7 h, lysed, and analyzed by western immunoblotting to determine the concentration that resulted in the highest level of p53. (C) Cells were pretreated with 30 nM MG132 (PI) for 2 h and then with recombinant fibronectin fragment, AFn, or control fibronectin fragment, CFn, for 7 h. The cells were then lysed, and the p53 levels were determined by western blotting. Actin served as a loading control.

proteins in the cell is p53, and its level is tightly regulated by transcriptional and post-translational modifications (21). In many an instance, the degradation of p53 in cells is mediated by the proteasome (22). Therefore, to ascertain whether the degradation of p53 in response to AFn was mediated by the proteasome, we pretreated cells with MG132, a proteasome inhibitor, before treating the cells with AFn. First, we determined that 30 nM was the optimal concentration of MG132, which on its own did not induce cellular toxicity (Fig. 1B, lane 4). Thereafter, we pretreated cells with 30 nM MG132 for 2 h prior to treating them with AFn for a further 7 h. We observed that inhibiting the proteasome with MG132 prevented the degradation of endogenous p53 in response to AFn (Fig. 1C, lanes 2 and 5). Control medium or a control fibronectin fragment (CFn) did not alter the p53 levels (Fig. 1C, lanes 1 and 3). From these experiments, we concluded that the degradation of p53 in cells treated with the AFn is mediated through the proteasome.

In earlier studies (9, 11–13), we showed that primary PDL cells underwent apoptosis when treated with AFn. We observed that cells overexpressing p53 were less susceptible to apoptosis. Since, in this present study (Fig. 1) we observed that inhibition of the proteasome resulted in stabilization of p53, we tested whether this approach could be used as an alternative to overexpressing p53 to rescue the cells from apoptosis. Indeed, pretreatment of cells with MG132 prior to exposure of cells to the AFn prevented cell rounding, nuclear condensation and fragmentation, the hallmarks of apoptosis (11; Fig. 2A). There were also strong nuclear signals for p53 in cells that were pretreated with proteasome inhibitor and incubated with the AFn. This showed that primary cells could resist apoptosis induced by the AFn if the proteasomal degradation of p53 was halted. In fact, clinical trials testing proteasome inhibitors have shown that this may be a viable treatment for chronic inflammation (29–31).

To further show that proteasome inhibitors, by virtue of their ability to inhibit the degradation of endogenous p53, can rescue cells from apoptosis, we pretreated cells with lactacystin, another proteasomal inhibitor, and measured the fold change in apoptosis in cells exposed to AFn. As shown in Fig. 2B, apoptosis, measured by an ELISA assay, which measures cytoplasmic histone-associated DNA fragments, was substantially reduced by the pretreatment of cells with lactacystin.

Downregulation of p53 by a fragmented fibronectin matrix is mediated by ubiquitination

Prior to degradation, p53 undergoes ubiquitination (24), and in cells treated with proteasomal inhibitors, such as MG132 or lactacystin, there is an accumulation of multi-ubiquitinated p53 molecules. In further experiments,

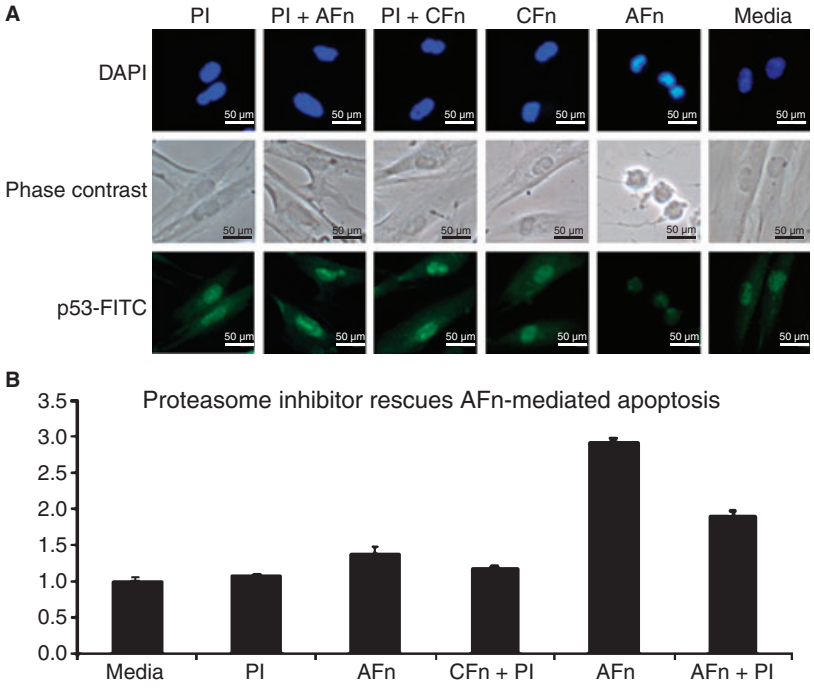

Fig. 2. A proteasome inhibitor rescues cells from apoptosis induced by proapoptotic fibronectin. (A) Human primary periodontal ligament cells (PDL) were pretreated for 2 h with 30 nM MG132 (PI) or control medium (Media) and then with the recombinant fibronectin fragment, AFn, or control fibronectin fragment, CFn, for 7 h. Subsequently, the cells were fixed, and p53 expression (FITC) and nuclear morphology (DAPI) were visualized by immunofluorescence and phase-contrast microscopy. Scale bar = 50 µm. (B) Human primary PDL cells were pretreated for 2 h with 10 µM lactacystin (PI) or control medium and then with the recombinant fibronectin fragments or control medium for 7 h. The cells were lysed, and cell death was determined using Cell Death Detection ELISA^{PLUS} (Roche) as outlined by the manufacturer.

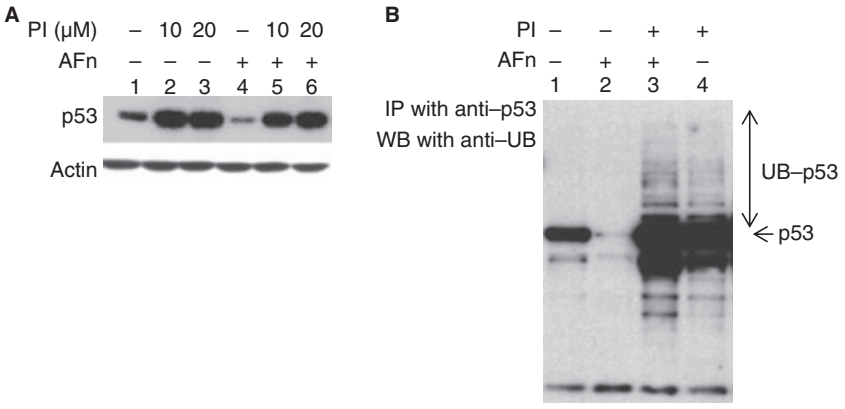

Fig. 3. A proteasomal inhibitor, lactacystin, inhibits ubiquitin-mediated p53 degradation induced by proapoptotic fibronectin**.** Human primary periodontal ligament cells were pretreated for 2 h with lactacystin (PI) or control medium, and treated with fibronectin fragment, AFn, or control medium for 7 h. (A) Cell lysates were analyzed by immunoblotting to determine the level of p53. Actin served as a loading control. (B) The lysates of lanes 1, 3, 4 and 6 in Fig. 3A were immunoprecipitated (IP) with an anti-p53 antibody and analyzed by immunoblotting with anti-ubiquitin.

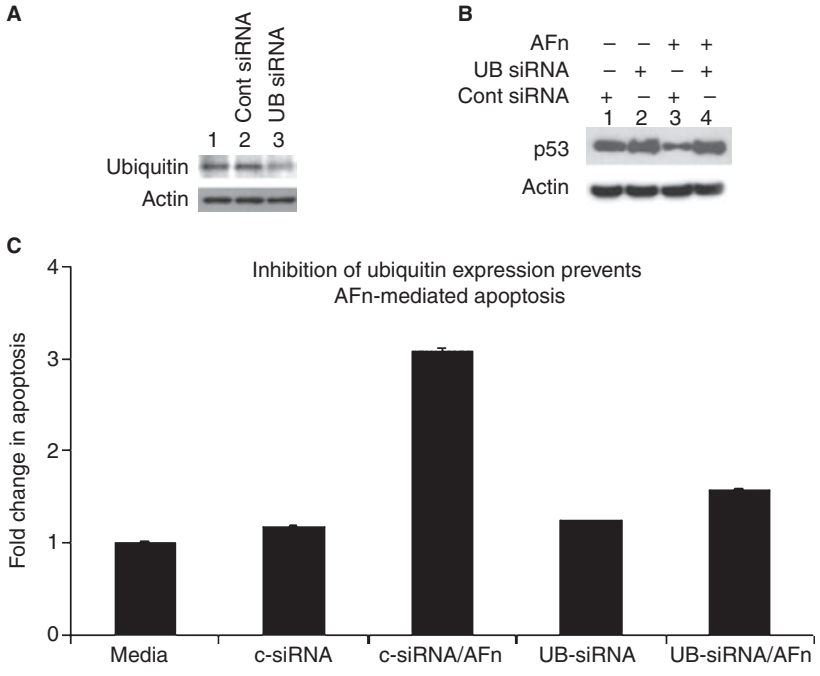

Fig. 4. Cells transfected with ubiquitin siRNA are resistant to proapoptotic fibronectin-induced p53 degradation. (A) Primary periodontal ligament (PDL) cells were transfected with ubiquitin (UB) siRNA to inhibit ubiquitin expression. The level of ubiquitin expression was analyzed by western blotting with anti-ubiquitin antibody 36 h after transfection. (B) Cells were transfected with ubiquitin siRNA or control siRNA for 36 h and then treated with AFn or control medium for 7 h. Cell lysates were prepared, and p53 expression was analyzed by western blotting with an anti-p53 antibody. Actin served as a loading control. (C) Primary PDL cells were transfected with ubiquitin or control siRNA as in (A) and then treated with AFn or control serum-free medium. Equal amounts of cell lysates (by protein) were used to determine cell death using Cell Death Detection ELISA^{PLUS} (Roche) as outlined by the manufacturer.

pretreatment of cells with the proteasome inhibitor lactacystin also stabilized p53 and prevented its degradation (Fig. 3A, lanes 4, 5 and 6). In the absence of lactacystin, cells treated with the AFn had lower levels of p53 (Fig. 3A, lane 4). Since treatment with a proteasomal inhibitor can result in the accumulation of multi-ubiquitinated p53 molecules, we examined this possibility in our system. We immunoprecipitated the proteins shown in Fig. 3A with an anti-p53 antibody and probed them with an antibody to ubiquitin. We observed a ladder of bands in cells pretreated with lactacystin, and these were, in fact, different ubiquitinated forms of p53, since they were recognized by an anti-ubiquitin antibody after immunoprecipitating them with an anti-p53 antibody. Furthermore, cells that were treated with AFn and lactacystin had more ubiquitinated p53 forms (Fig. 3B, lane 3) than the cells treated with lactacystin alone (Fig. 3B, lane 4).

From the preceding experiments, it was clear that the major site for the degradation of p53 in cells, which were exposed to AFn, was the proteasome (Figs. 1–3). When destined for degradation, p53 gets ubiquitinated, and ubiquitinated p53 is a substrate for the proteasome (20,22,32–34). We had also observed the presence of ubiquitinated p53 in cells that were treated with proteasome inhibitor and AFn. Therefore, to confirm the participation of ubiquitin in the degradation of p53 induced by AFn, we transfected cells with siRNA for ubiquitin. As seen in Fig. 4A, ubiquitin expression was significantly reduced in cells transfected with ubiquitin siRNA (Fig. 4A, lane 3). Next, cells were transfected with ubiquitin siRNA and then treated with the AFn (Fig. 4B). In cells transfected with ubiquitin siRNA, there was less ubiquitination of p53 and consequently higher levels of p53 (Fig. 4B, lane 2) compared with cells that were treated with the control siRNA (Fig. 4B, lane 1). Moreover, cells with suppressed levels of ubiquitin had higher levels of p53 even in the presence of AFn (Fig. 4B, lane 4) compared with cells transfected with a control siRNA (Fig. 4B, lane 3), indi-

cating that ubiquitination of p53 is important for its degradation in this process. In addition to mitigating the degradation of p53 mediated by AFn, inhibition of ubiquitin expression also blocked the apoptosis of cells initiated by AFn, as shown in Fig. 4C. The residual apoptosis seen in ubiquitin siRNA transfected cells in the presence of AFn might be attributed to the remaining low level of ubiquitin expression that was observed in these cells (Fig. 4A, lane 3).

Discussion

The ECM is composed of several macromolecules, which play an important role in development, differentiation and cell–cell communication and signaling events. Fibronectin, a major constituent of the ECM, is present in the matrix of a variety of connective tissues and its alternatively spliced forms are most commonly found during embryonic development and tissue remodeling. It usually exists as a dimer, consisting of similar but not necessarily identical chains, and each polypeptide chain is approximately 250 kDa in size. It has several domains, including the collagen/gelatin-, heparin- and central cell-binding domains, each of which has multiple functions (35). Proteolytic cleavage of fibronectin by proteases found in inflammatory fluids yields an array of fibronectin fragments. One such fibronectin fragment is comparable to AFn. Our present study shows that when PDL cells are treated with this fibronectin fragment, p53 gets ubiquitinated and degraded in the proteasome. Treatment with the proteasome inhibitor MG132 markedly inhibited the decrease in p53 protein levels induced by this fibronectin fragment and resulted in the accumulation of multiple species of ubiquitinated p53 molecules. The same was seen with lactacystin, an irreversible inhibitor of the proteasome. After treatment with AFn, p53 levels were higher in cells transfected with ubiquitin siRNA than in untransfected cells, demonstrating that ubiquitination is necessary for the degradation of p53 by this fibronectin fragment. Treatment of cells with a protein synthesis inhibitor or a lysosomal inhibitor did not prevent p53 degradation. Thus, the degradation did not involve the lysosomal pathway. Our immunofluorescence data further confirmed that pretreatment of cells with proteasome inhibitors can rescue the downregulation of p53 and consequent rounding and apoptosis of cells triggered by the fibronectin fragment. This was additionally confirmed by the ELISA data, which clearly showed that pretreating cells with a proteasome inhibitor greatly reduced the apoptosis of AFn-treated PDL cells. Slight apoptosis observed in control fibronectin fragment-treated cells is probably due to serum starvation of the cells.

Our results showing that proapoptotic fibronectin fragment-induced p53 degradation is ubiquitin dependent are in agreement with previous findings. In these reports, ubiquitination of p53 is necessary for its degradation through the proteasome (32–34). In conclusion, apoptosis mediated by a proapoptotic fibronectin fragment involves the association of p53 with ubiquitin and its subsequent degradation by the proteasome. Inhibiting either the proteolytic function of the proteasome or suppressing ubiquitin at its protein level prevented the degradation of p53 and subsequent apoptosis of these primary PDL cells. These findings provide potential therapeutic targets and strategies for addressing inflammatory diseases such as arthritis and periodontal disease that manifest alterations in the ECM.

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