Stimulatory effects of a three-dimensional microenvironment on cell-mediated fibronectin fibrillogenesis

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Summary

The assembly of fibronectin into a fibrillar matrix is a regulated step-wise process that involves binding to integrin receptors and interactions between fibronectin molecules. This process has been studied extensively using cells in two-dimensional (2D) monolayer culture. In most situations in vivo, however, matrix assembly occurs within existing three-dimensional (3D) extracellular matrix networks. In an attempt to mimic this environment, we analyzed matrix assembly by fibroblasts cultured on a pre-assembled 3D fibronectin matrix and found significant stimulation of fibronectin fibril assembly compared to cells in 2D culture. Lower amounts of fibronectin were needed to initiate the assembly process, fibrils accumulated to higher density, and the 3D fibril organization played a key role in the stimulatory effect. Moreover, cells expressing activation-dependent integrins were able to assemble fibronectin matrix without exogenous stimulation, suggesting regulatory effects of the 3D fibronectin matrix on integrin activity. These results provide evidence for an additional level of control of fibronectin deposition through cell interactions with the local microenvironment.

Key words: Extracellular matrix, Integrins, Fibronectin, Matrix assembly, Fibroblasts

Introduction

Fibronectin is an abundant component of the extracellular matrix (ECM) playing an important role in cell growth, differentiation, survival and oncogenic transformation (Hynes, 1990). Many cell types make fibronectin and assemble it into a branched, fibrillar network that provides environmental information to the cells that encounter it. Fibronectin matrix assembly is a cell-mediated step-wise process. Dimeric fibronectin binds to cell surface integrin receptors and becomes activated to participate in interactions with other cell-associated fibronectin dimers. Through these fibronectin-fibronectin interactions, short deoxycholate (DOC)-soluble fibrils are formed which are gradually converted into a DOC-insoluble network (McKeown-Longo and Mosher, 1983; Schwarzbauer and Sechler, 1999; Wierzbicka-Patynowski and Schwarzbauer, 2003).

Heterodimeric integrin receptors and intracellular factors play important roles throughout assembly. Fibril formation is mediated primarily by α5β1 integrin binding to the RGD cell binding sequence and the adjacent synergy site in the cell binding domain of fibronectin (Fogerty et al., 1990; McDonald et al., 1987; Sechler et al., 1997; Sechler et al., 1996). Other integrins including αvβ3, αIIbβ3 and α4β1 can mediate matrix assembly when appropriately activated (Sechler et al., 2000; Wennerberg et al., 1996; Wu et al., 1996; Wu et al., 1995) and αvβ1 has been shown to temporarily replace α5β1 during embryogenesis of α5-null mice (Yang and Hynes, 1996). On the cytoplasmic side, connections from integrins to the actin cytoskeleton directly affect the fibronectin matrix assembly process (Hynes, 1990; Wu et al., 1995). In addition, signaling proteins like focal adhesion kinase (FAK), Src and Rho GTPase regulate assembly (Ilic et al., 2004; Wierzbicka-Patynowski and Schwarzbauer, 2002; Zhang et al., 1994).

Within the 3D environment of tissues, cells are affected by signals from the ECM and neighboring cells. Furthermore, cell responses to 3D matrices differ from cells grown on 2D surfaces (Cukierman et al., 2001; Sechler et al., 1998; Tamariz and Grinnell, 2002; Vlodavsky et al., 1980; Wenk et al., 2000). To test the requirements for fibronectin assembly in three dimensions, we followed fibril formation by cells grown on a cell-free 3D fibronectin matrix. We found that α5β1-mediated matrix assembly was stimulated in cells on this 3D matrix. The rate of accumulation of detergent-insoluble matrix was increased, the threshold concentration of fibronectin needed for fibril formation was reduced, and the stimulatory effect was dependent on the 3D fibrillar architecture. Furthermore, tumor cells expressing α5β1 integrins with reduced activity and CHO cells expressing activation-dependent αvβ3 were also stimulated to assemble fibronectin fibrils. Our results show that a 3D ECM network contains regulatory cues that enhance fibronectin matrix assembly.

Materials and Methods

Cell culture and reagents

Human lung fibroblast WI-38, SV40-transformed WI-38 (VA13) subline 2RA [WI-38 (VA13)], HT1080 cells and human dermal fibroblasts were obtained from ATCC. All cells were cultured in

DMEM supplemented with 10% FBS (Hyclone). NIH3T3 cells were cultured in DMEM supplemented with 10% BCS. CHO(B2) cells (Schreiner et al., 1989) were kindly provided by Yoshi Takada (The Scripps Research Institute, CA). CHO(B2) cells transfected to stably express human α5 integrin [CHO(B2)α5] or β3 integrin [CHO(B2)αvβ3] were kindly provided by Siobhan A. Corbett (Robert Wood Johnson Medical School, NJ) (Ly et al., 2003). CHO(B2)α5 and CHO(B2)αvβ3 cells were maintained in DMEM containing 1 mM glutamine, 1 mM non-essential amino acids, 10% fetal clone II serum and G418 or Zeocin, respectively. Sulfo-NHS-biotin (EZ-link) was purchased from Pierce. The rat-specific anti-fibronectin monoclonal antibody IC3 has been described previously (Sechler et al., 1996). HFN7.1 hybridoma cells were purchased from ATCC. Polyclonal antiserum R457 was raised against the N-terminal 70 kDa fragment of rat fibronectin (Aguirre et al., 1994). Function-blocking monoclonal antibodies to human integrin α5 were CBL497 (clone SAM-1) purchased from Cymbus Biotechnology (Eastleigh, UK) and BIIG2 kindly provided by Caroline H. Damsky (University of California, San Francisco, CA). Function-blocking antibody MAB1976Z (clone LM609) for integrin αvβ3 was purchased from Chemicon International. The monoclonal antibody 9D2 was kindly provided by Deane Mosher (University of Wisconsin, Madison). Anti-GAPDH (ab9484) antibody was purchased from Abcam (Cambridge, MA). Fluorescein-conjugated anti-mouse IgG and rhodamine-conjugated streptavidin were purchased from Molecular Probes (Eugene, OR). Growth Factor Reduced Matrigel™ was from BD Biosciences (San Jose, CA).

Preparation of 3D fibronectin matrix

The procedure for preparing cell-free 3D fibronectin matrix is modified from a published protocol (Chen et al., 1978). Briefly, NIH3T3 or WI-38 (VA13) cells were cultured in wells of a 24-well plate until highly confluent. Cells were washed twice with 1 ml PBS followed by two washes with 1 ml of wash buffer I (100 mM Na_2HPO_4, pH 9.6, 2 mM $MgCl_2$, 2 mM EGTA). 1 ml lysis buffer (8 mM Na_2HPO_4, pH 9.6, 1% NP-40) was added to each well and incubated at 37°C for 15 minutes; this was then removed and replaced with 1 ml fresh lysis buffer and incubation was continued for 40-60 minutes. Matrices were washed twice with 1 ml wash buffer II (300 mM KCl, 10 mM Na_2HPO_4, pH 7.5) and four times with 1 ml dH_2O. Matrix could be stored in PBS at 4°C for a few weeks. To examine matrix by confocal microscopy, human 3D fibronectin matrices on glass coverslips were fixed and immunostained with anti-human fibronectin antibody (HFN7.1) and fluorescein-conjugated goat anti-mouse IgG. Images were collected with a Ziess 510 confocal microscope. For SDS-PAGE, a confluent cell monolayer and a 3D matrix were solubilized in parallel with 2× SDS sample buffer (100 mM Tris-HCl, pH 6.8, 30% glycerol, 4 mM EDTA, 6% SDS, 0.01% Bromophenol Blue, 10% β-mercaptoethanol). Equal proportions of the two samples were electrophoresed on a 6% polyacrylamide gel containing SDS and proteins were visualized by silver staining.

2D substrates were prepared by coating wells with a 10 μg/ml solution of rat or human plasma fibronectin in PBS at 4°C overnight at the indicated concentrations. Total amounts of fibronectin in 3D and 2D substrates were comparable as determined by immunoblotting of solubilized substrate proteins (data not shown).

Analysis of matrix assembly by deoxycholate (DOC)-lysis

For experiments with human cells (WI-38, dermal fibroblasts), the 3D fibronectin matrix was prepared from NIH3T3 cell cultures. For all other cell types, WI-38 (VA13) cell cultures were used. 3D fibronectin matrix and 2D-coated substrates were blocked with 1% BSA in PBS at room temperature for 1 hour. Subconfluent cells were trypsinized, washed and 2×10^5 cells were seeded per well in DMEM supplemented with 2% fibronectin-depleted FBS. For cells that make limited amounts of their own fibronectin (CHO and HT1080), 2.5×10^5 cells were plated and 10 μg/ml pFN was added to medium along with 5% fibronectin-depleted serum, except where indicated. For some experiments with HT1080 cells, 10 μg/ml aprotinin was added during assembly.

At each time point, medium was removed and cells were washed with PBS three times. Matrix was solubilized using 200 μl DOC lysis buffer (Sechler et al., 1996). After centrifugation, the DOC-insoluble pellet was solubilized in 25 μl of 2% SDS, 20 mM Tris-HCl, pH 8.8, 2 mM PMSF, 2 mM iodoacetic acid, 2 mM N-ethylmaleimide and 2 mM EDTA. Equal volumes of DOC-insoluble samples were analyzed by SDS-PAGE using 5% polyacrylamide gels. Samples were immunoblotted with HFN7.1 concentrated hybridoma culture supernatant diluted 1:1000 (Brenner et al., 2000) or IC3 ascites diluted 1:10,000 (Sechler et al., 1996). DOC-soluble samples were immunoblotted in parallel with antibodies against GAPDH to ensure equal sample loading. Immunoblots were developed with SuperSignal West Pico Chemiluminescent substrate (Pierce Chemical), or for quantification, ECL plus Western Blotting Detection System (Amersham Biosciences) was used. With the ECL plus system, band intensities were measured with a Molecular Dynamics STORM system and ImageQuant software.

For all experiments, 3D matrix was incubated with 10 μg/ml rat pFN in the absence of cells to serve as a background control. For antibody blocking experiments, cells were seeded and allowed to attach for 2 hours. Rat pFN and function-blocking monoclonal antibodies against α5 (1 μg/ml SAM-1 or 4.8 μg/ml BIIG2) or αvβ3 (10 μg/ml LM609) were added to the culture medium and incubated for 14-16 hours. DOC lysates were prepared and analyzed as described above. 70 kDa fragment blocking was performed by plating 3×10^5 CHO(B2)α5 cells on 3D substrates with 10 μg/ml rat pFN and 125 μg/ml 70 kDa fragment or the same volume of buffer. After incubation for 7 hours, DOC-soluble and DOC-insoluble samples were prepared and analyzed.

To monitor assembly by metabolic labeling, 2×10^5 cells [WI-38 (VA13) or NIH3T3] were seeded on 3D matrix prepared from NIH3T3 cells or fibronectin-coated surfaces in methionine-minus DMEM containing 40 μCi/ml [^{35}S]methionine (ICN) and 5% fibronectin-depleted FBS. After incubation for 6 hours, media were collected and DOC-soluble and DOC-insoluble fractions were prepared. Fibronectin was isolated from the medium by gelatin-Sepharose affinity chromatography (Schwarzbauer, 1991) and from lysates by immunoprecipitation with anti-fibronectin antibody R457 (Aguirre et al., 1994). Immunoprecipitates from DOC-insoluble and DOC-soluble fractions and culture media were analyzed by SDS-PAGE. Dried gels were exposed to a phosphoimager screen, data were collected by scanning with a STORM system and band intensities were quantified using ImageQuant software. Total fibronectin was calculated by summing total counts in DOC-soluble material, DOC-insoluble material and culture media.

For preparation of resuspended matrix, conditioned medium (CM) or cell lysates, WI-38(VA13) cells were grown until highly confluent. CM was collected from the cells and centrifuged to remove floating cells. Cells were then lysed in lysis buffer and lysates were incubated with fibronectin-coated 2D surfaces at 4°C overnight. 3D fibronectin matrix was prepared as described above and resuspended by scraping the 3D matrix off the surface into 1 ml DMEM plus 5% fibronectin-depleted FBS using a rubber policeman. 2.5×10^5 CHO(B2)α5 cells were plated on 3D matrix, 2D fibronectin substrate or on 2D fibronectin substrate pre-incubated with cell lysate. In some experiments, the medium was replaced after 1 hour with CM or resuspended matrix plus 10 μg/ml rat fibronectin. After incubation for 16 hours, the DOC-insoluble and soluble fractions were prepared.

3D fibronectin matrix prepared from WI-38(VA13) cells was compressed following a procedure described (Cukierman et al., 2001). Growth Factor Reduced Matrigel™ (BD Biosciences) was diluted 1:3 in DMEM on ice. 55 μl diluted Matrigel or Matrigel containing 60

μg/ml plasma fibronectin were allowed to gel in the wells of a 24-well plate at 37°C for 15 minutes. Polymerized Matrigel was blocked with 1% BSA in PBS before use. To fix the 3D fibronectin matrix, 1 ml of 4% paraformaldehyde in PBS was added to 3D fibronectin matrix and incubated at room temperature for 30 minutes. The fixed matrix was washed four times with 1 ml PBS followed by blocking with 1% BSA in PBS. 2.5×10^5 CHO(B2)α5 cells were plated onto these 3D substrates and DOC lysis was performed as described above.

To block the cryptic fibronectin-binding site I_9-III_1, 100 μg/ml 9D2 monoclonal antibody was added to CHO(B2)α5 cells plated on 3D fibronectin matrix along with 10 μg/ml rat pFN. After 6 hours, the DOC-insoluble fraction was prepared as described above.

Indirect immunofluorescence

2×10^5 cells were seeded on 3D matrix or fibronectin-coated 12-mm coverslips with or without 4 μg/ml exogenous fibronectin and incubated for various times. Samples were fixed and stained with the appropriate species-specific antibodies (either HFN7.1 or IC3) followed by fluorescein-conjugated goat anti-mouse IgG (Sechler et al., 1996). Images were captured using a Nikon Eclipse TE2000-U microscope and IPLab software. To analyze distributions of 3D human fibronectin matrix and exogenous rat pFN matrix, 1.5×10^5 CHO(B2)α5 cells were plated in medium with 5% fibronectin-depleted FBS on coverslips with either 3D human fibronectin matrix or coated with human pFN. After allowing 2 hours for cell attachment, 4 μg/ml rat fibronectin was added and cells were incubated for 15 hours. Samples were fixed in 3.7% formaldehyde in PBS followed by sequential antibody incubations. First incubation was with IC3 ascites (1:1000) at 37°C for 30 minutes followed by fluorescein-conjugated goat anti-mouse IgG for 30 minutes. Samples were rinsed thoroughly and a second incubation with IC3 ascites (1:1000) at room temperature for 10 minutes was performed to saturate any remaining anti-mouse IgG. After rinsing, samples were incubated with biotin-conjugated HFN7.1 antibody (biotinylated according to instructions of Pierce Chemical) at 37°C for 30 minutes followed by staining with rhodamine-conjugated streptavidin. Cells were analyzed using Ziess 510 confocal microscope or Nikon Eclipse TE2000-U microscope.

Results

Preparation and characterization of a 3D fibronectin matrix

A 3D fibronectin matrix was prepared using a modification of a published extraction procedure (Chen et al., 1978). Highly confluent WI-38(VA13) cells, surrounded by a dense network of fibronectin fibrils (Fig. 1B), were removed by lysis with a low-salt detergent buffer leaving a 3D cell-free matrix attached to the surface. Confocal microscopy showed that the fibrillar organization of fibronectin was maintained in the cell-free matrix (Fig. 1A) and that the three-dimensionality of the matrix was preserved with an average thickness of about 8 μm compared to 10 μm before extraction (Fig. 1A,B). Analysis of solubilized cell-free matrix by SDS-PAGE showed a major fibronectin band in contrast to multiple protein bands before extraction (Fig. 1C). Given the abundance of fibronectin, we refer to it as 3D fibronectin matrix.

Increased fibril assembly by cells on 3D fibronectin matrix

Matrix assembly by cells on the 3D fibronectin matrix was compared to that by cells plated on a 2D substrate prepared by coating plastic or glass surfaces with a 10 μg/ml solution of

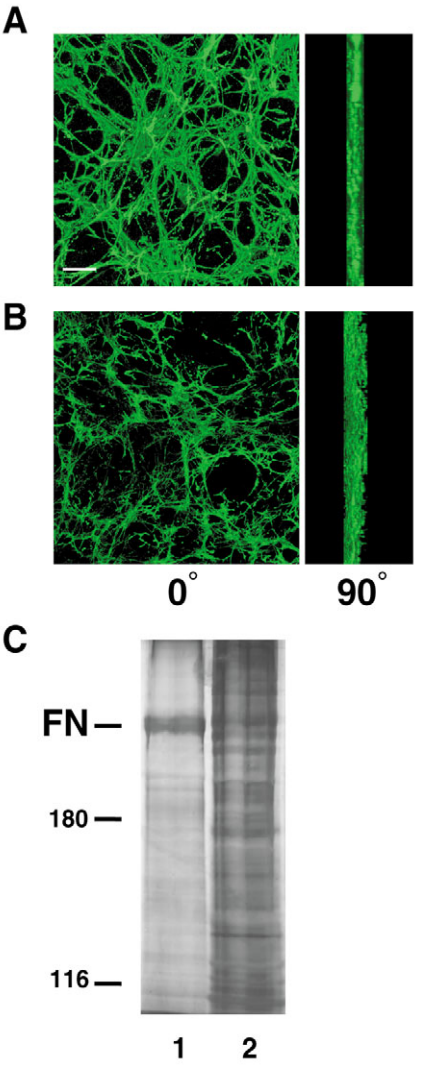

Fig. 1. Three-dimensionality of cell-free 3D fibronectin matrix. 3D matrix prepared from WI-38 (VA13) cells (A) and a monolayer of WI-38(VA13) cells (B) were immunostained with anti-human fibronectin antibody HFN7.1 and analyzed by confocal microscopy. 0° is the projected image and 90° the 3D image rotated through 90 degrees to show thickness. The basal cell surfaces are to the left. (C) Equivalent proportions of NIH3T3 3D matrix (lane 1) and confluent cell culture (lane 2) solubilized with SDS sample buffer were compared by SDS-PAGE using 6% polyacrylamide and silver staining. The major bands in lanes 1 and 2 were confirmed as fibronectin by immunoblotting (not shown). The location of fibronectin at 250 kDa and molecular mass markers (180 kDa and 116 kDa) are indicated.

plasma fibronectin (pFN). Cells and matrix from different species were used to differentiate cell-assembled matrix from the 3D fibronectin matrix. ELISA with monoclonal antibody HFN7.1, which inhibits cell binding to human fibronectin, showed that 2D-coated surfaces had more accessible cell binding sites than 3D matrices at all fibronectin concentrations used in this study (data not shown). WI-38 (VA-13)-transformed human lung fibroblasts on a 3D mouse fibronectin matrix assembled significantly more fibronectin fibrils. Comparison of deoxycholate (DOC) detergent-insoluble

matrix formed on 2D compared to 3D substrates showed time-dependent (Fig. 2A) and cell density-dependent (Fig. 2B) differences. By indirect immunofluorescence, it was clear that these cells assembled a more extensive network when grown on the 3D fibronectin matrix (Fig. 2C, left panel) in contrast to the short fibrils around peripheries of cells growing on the 2D substrate (Fig. 2C, right panel). To verify that the increased assembly is not unique to WI-38 (VA13) cells, several other fibroblast cell lines were tested. Primary cell lines WI-38 and human dermal fibroblasts also assembled more DOC-insoluble matrix on a 3D substrate (Fig. 2D). To quantify the levels of fibronectin expression and matrix deposition on the two substrates, assembly by cells labeled with [^{35}S]methionine was monitored. WI-38 (VA13) and NIH3T3 cells on 3D substrates assembled more than threefold the amount of DOC-insoluble [^{35}S]fibronectin than on 2D substrates (Fig. 3A) with percentage incorporation increasing from 0.7% on 2D substrate to 3.3% on 3D matrix for WI-38 (VA-13) cells and from 0.7% to 2.2% for NIH3T3 cells. Total production of fibronectin by these cell lines did not differ significantly on 3D compared to 2D substrates (Fig. 3B). Together these results show that fibroblasts within a 3D fibronectin matrix environment are stimulated to assemble fibronectin matrix.

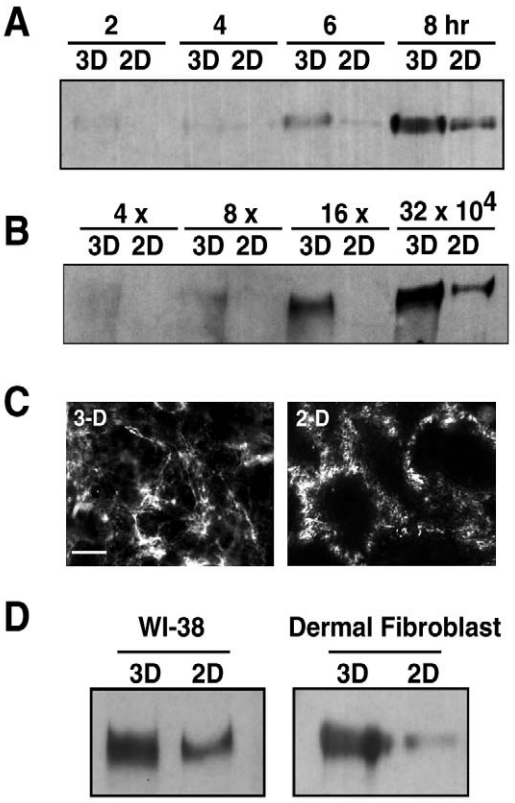

Fig. 2. Increased fibronectin matrix assembly by WI-38 (VA13) cells on a 3D fibronectin matrix. WI-38(VA13) cells were seeded on 3D matrix prepared from NIH3T3 cells (3D) or on surfaces coated with 10 μg/ml rat pFN (2D). (A) DOC lysates were prepared at the indicated times. DOC-insoluble samples were analyzed on 5% polyacrylamide-SDS gels and immunoblotted with HFN7.1. (B) Cells were seeded in 24-well dishes at the indicated cell densities. After incubation for 6 hours, DOC-insoluble samples were prepared and analyzed as described in A. (C) WI-38 (VA13) cells seeded for 8 hours on 3D matrix (3D, left) or fibronectin-coated coverslips (2D, right) were fixed and immunostained with HFN7.1 followed by fluorescein goat anti-mouse IgG. (D) DOC-insoluble fibronectin matrix formed by WI-38 human lung fibroblasts (left) and human dermal fibroblasts (right) was analyzed at 8 hours as in A. Bar, 20 μm.

Partial overlap of newly assembled fibrils and 3D matrix

To control the amount and availability of fibronectin during matrix assembly, we analyzed formation of exogenous rat pFN matrix by CHO(B2)α5 cells that produce very little endogenous fibronectin. Incorporation of exogenous rat pFN by CHO(B2)α5 cells on a 3D human fibronectin matrix was significantly increased compared to these cells on a 2D human pFN substrate. DOC-insoluble matrix was detectable at all time points and accumulated to high levels in a time-dependent fashion (Fig. 4A). Minimal background binding of exogenous fibronectin to the 3D matrix was observed over this time course. It has been previously shown that interactions involving the N-terminal assembly domain of fibronectin are essential for fibril formation by cells in monolayer culture (McDonald et al., 1987; McKeown-Longo and Mosher, 1985; Schwarzbauer, 1991). Inclusion of excess 70 kDa fibronectin fragment, which contains the assembly domain, inhibited assembly of DOC-

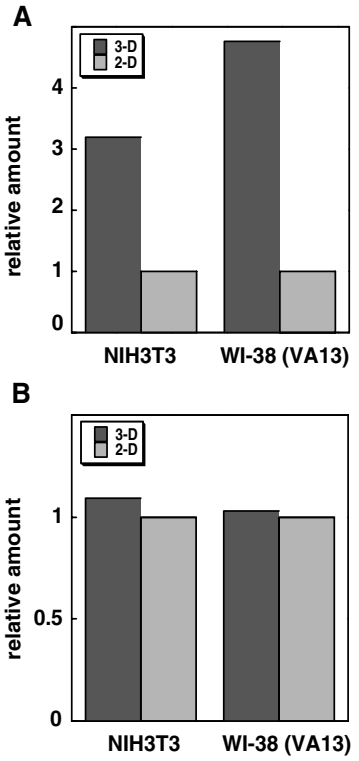

Fig. 3. Quantification of matrix levels. WI-38 (VA13) or NIH3T3 cells were seeded on 3D or 2D substrates in [^{35}S]methionine labeling medium for 6 hours. Fibronectin was isolated from DOC fractions and culture media, resolved by SDS-PAGE, and quantified by analysis with a phosphorimager. Phosphorimager counts for 3D samples were normalized to 2D samples, which were set to 1 for each sample pair. (A) Relative amounts of DOC-insoluble fibronectin compared to total fibronectin synthesized. (B) Relative amounts of total fibronectin (sum of DOC-soluble, DOC-insoluble and secreted).

insoluble matrix on 3D fibronectin matrix (Fig. 4B). Therefore, assembly on 2D and 3D substrates follows similar mechanisms. Fibronectin fibril formation was distinctly different on 2D and 3D substrates. CHO(B2)α5 cells elaborated a network of extended fibrils that stretched across and between adjacent cells (Fig. 4C, left panel). In contrast, fibrils formed by cells on the 2D substrate were shorter, fewer in number, and mainly extended between cell and substrate (Fig. 4C, right panel). In these experiments, exogenous fibronectin was provided in the medium at 4 μg/ml, a concentration significantly lower than the concentrations routinely used in matrix assembly experiments, typically 50 μg/ml or more (Sechler et al., 1996; Wu et al., 1995). With higher concentrations of fibronectin, cells on 2D substrates usually assemble a more extensive network over this time period. Thus, in addition to increasing the rate, 3D matrix also lowers the threshold concentration needed for assembly of mature matrix.

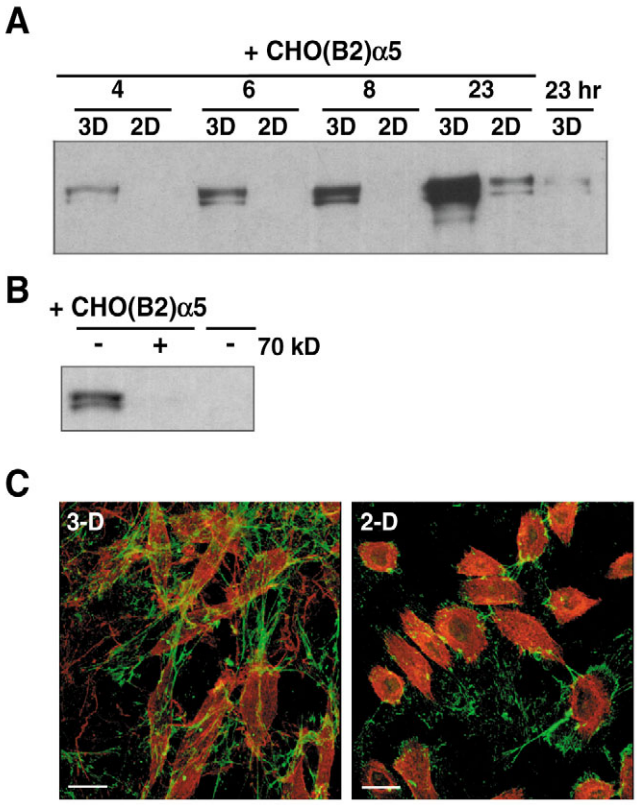

Fig. 4. Matrix incorporation of exogenous fibronectin on 2D and 3D fibronectin substrates. (A) CHO(B2)α5 cells were plated on 2D and 3D substrates in the presence of 10 μg/ml rat pFN. Fibronectin levels in DOC-insoluble matrix were detected with IC3 anti-rat fibronectin antibody at the indicated times. Exogenous fibronectin was also incubated with 3D matrix in the absence of cells and background binding was analyzed at 23 hours (far right-hand lane). (B) 3×10^5 CHO(B2)α5 cells were plated on substrates with 10 μg/ml rat pFN with or without 125 μg/ml 70 kDa fibronectin fragment. DOC-insoluble samples were collected and analyzed by immunoblotting after 7 hours incubation. The right lane shows no background fibronectin binding in the absence of cells. (C) CHO(B2)α5 cells plated with 4 μg/ml exogenous rat pFN on 3D (left) and 2D (right) substrates for 15 hours were fixed and stained with IC3 antibody and fluorescein-goat anti-mouse IgG plus rhodamine-conjugated phalloidin. Bars, 20 μm.

Double staining of newly assembled fibrils (rat pFN) and 3D matrix fibrils (human fibronectin) showed only partial co-alignment and there were areas where rat fibronectin matrix was much denser than the 3D fibronectin matrix substrate (Fig. 5A). To determine the extent of colocalization, fluorescence intensity profiles were calculated for four areas in the merged image. Fibrils containing varying amounts of both human 3D matrix and rat fibronectin fibrils are shown under lines 1 and 2 (Fig. 5B). Some fibrils are primarily composed of newly assembled rat fibronectin (green lines). However, other regions show coincidence of rat fibronectin and human 3D matrix (red lines) suggesting that the rat fibronectin fibrils extend from the 3D matrix. Relatively homogeneous fibrils were also observed; line 3 indicates fibrils composed primarily of rat fibronectin and devoid of 3D matrix whereas line 4 crosses mostly human 3D matrix. These data indicate that the newly assembled fibrils are not simply deposited onto the 3D matrix.

Dependence on 3D matrix architecture for stimulation of fibronectin assembly

To evaluate the importance of 3D matrix structure in stimulating fibronectin assembly, we disrupted the organization of the 3D fibronectin matrix by scraping it off the dish into cell culture medium. Exogenous rat fibronectin was added to the resuspended human fibronectin matrix and this mixture was incubated with CHO(B2)α5 cells growing on a 2D fibronectin substrate. After 16 hours, the cells had assembled somewhat more matrix than cells growing on a 2D fibronectin-coated surface (Fig. 6A). Other molecules released by cells as they assemble the 3D fibronectin matrix may contribute to the stimulatory effects. This possibility was tested using cell conditioned medium (CM) collected from WI-38(VA13) cells at the time of preparation of the 3D fibronectin matrix. CHO(B2)α5 cells on a 2D fibronectin substrate were incubated with CM plus exogenous rat fibronectin. As with resuspended matrix, there was some stimulation of assembly with CM (Fig. 6A). However, stimulatory effects of CM and resuspended matrix were significantly less than the effect of the 3D fibronectin matrix. Thus the presence of these matrix components in a different form did not have the same effect as a fibrillar 3D matrix. Cellular proteins released by cell lysis during 3D fibronectin matrix preparation might adsorb onto the matrix and could potentially affect subsequent matrix assembly. However, no significant stimulation of assembly was observed with cells on a 2D fibronectin substrate pre-incubated with cell lysate compared to cells on 3D fibronectin matrix (Fig. 6B).

In a further test of the requirement for a 3D nature of the substrate, the 3D fibronectin matrix was compressed to reduce its thickness (Cukierman et al., 2001). The amount of newly assembled fibronectin was quantified and significantly less fibronectin was assembled on the compressed matrix than on 3D fibronectin matrix by 7 hours, with a relative level of 0.4 (Fig. 6C). However, by 18 hours, little difference was observed between these two substrates. This change over time correlates with the level of compression of the matrix. Confocal microscopy of these matrices revealed that the compression was transient showing a thickness of 3 μm shortly after compression and a restoration to 9 μm after 18 hours (data not shown). These results show a correlation between 3D matrix

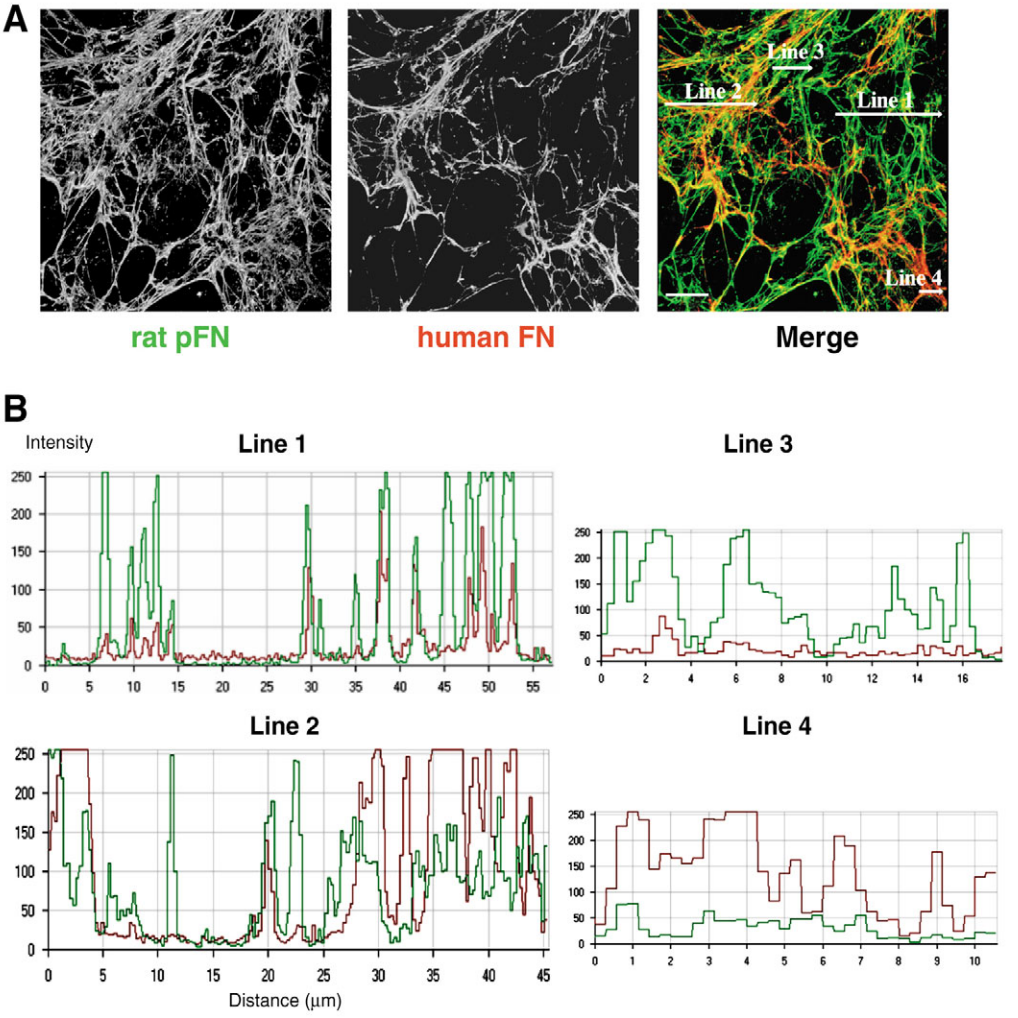

Fig. 5. Distribution of newly assembled and pre-existing fibrils formed on a 3D matrix. CHO(B2)α5 cells were incubated in medium with 4 μg/ml rat pFN on coverslips with 3D human fibronectin matrix. After incubation for 15 hours, samples were fixed and stained sequentially with IC3 and fluorescein-goat anti-mouse IgG to detect rat fibronectin fibrils followed by biotinylated HFN7.1 and rhodamine-streptavidin to detect human fibronectin 3D matrix. (A) Projected confocal images of newly assembled rat pFN fibrils (left), 3D human fibronectin matrix (middle) and the merged image (right). (B) Fluorescence intensity profiles of four areas (lines 1-4 in merged image of A) were analyzed using laser-scanning microscopy LSM 510 version 3.2 software (Zeiss).

thickness and the amount of fibronectin that is assembled. Taken together, these control experiments support the idea that the 3D nature of the matrix plays a key role in enhancing fibronectin matrix assembly.

Fibronectin matrix assembly is affected by cell contractility (Zhang et al., 1994; Zhong et al., 1998). A matrix more pliable than a 2D fibronectin-coated surface may change contractility and stimulate assembly. To examine this possibility, matrix assembly was analyzed in cells growing on growth factor-reduced Matrigel matrices with or without added fibronectin. There was some stimulation of fibronectin assembly in cells on Matrigel matrices compared to cells on 2D fibronectin substrates but much less than cells on 3D fibronectin matrix (Fig. 7A). Furthermore, inclusion of fibronectin in the Matrigel matrix had no additional effect over Matrigel alone. To reduce the pliability of the 3D fibronectin matrix, it was crosslinked with 4% paraformaldehyde. Solubilized fixed 3D fibronectin matrix migrated as high molecular weight protein complexes in a 5% polyacrylamide SDS gel indicating covalent crosslinking of the matrix (data not shown). In addition, time-lapse video microscopy of cells migrating on these matrices showed significant flexibility of the 3D matrix fibrils that was reduced after fixation (data not shown). Even so, fibronectin matrix assembly on fixed 3D matrix was stimulated to a comparable degree as on native unfixed matrix (Fig. 7B).

We also tested whether matrix assembly was stimulated by availability of additional sites for fibronectin binding that might be generated by cell-mediated reorganization of the 3D fibronectin matrix. The 9D2 monoclonal antibody binds within a cryptic fibronectin-binding site in human fibronectin and inhibits fibronectin matrix assembly (Chernousov et al., 1991). We have not detected 9D2 inhibition of rat fibronectin assembly in monolayer culture, which allowed us to use this antibody to block sites in the human 3D matrix while following formation of rat fibronectin fibrils. If the stimulatory effect depends on increased availability of fibronectin binding sites in the 3D matrix, then 9D2 antibody binding to these sites should reduce fibronectin assembly. However, the addition of 9D2 did not diminish the assembly of rat fibronectin fibrils (Fig. 7C), making it unlikely that cell interactions with the 3D matrix led to exposure of additional 9D2-sensitive matrix assembly sites.

Stimulatory effects of 3D matrix on integrin-dependent assembly

Integrin α5β1 is primarily responsible for fibroblast matrix

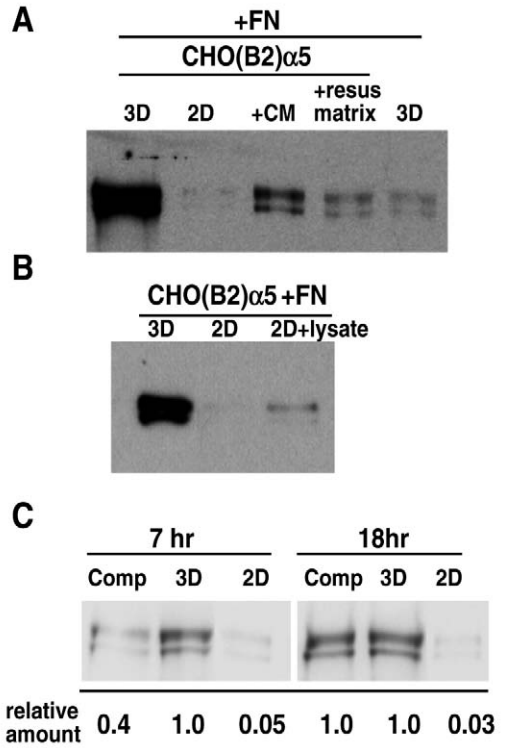

Fig. 6. 3D architecture plays a key role in stimulating fibronectin matrix assembly. (A) CHO(B2)α5 cells in medium supplemented with rat fibronectin (FN) were plated on 3D matrix or on 2D fibronectin-coated substrates with no other additions (3D, 2D) or with conditioned medium (+CM) or resuspended matrix added (+resus matrix). 3D matrix was incubated with rat fibronectin but without cells (3D, right lane). DOC-insoluble material was prepared after 16 hours and fibronectin was detected with IC3 antibody. (B) Confluent WI-38(VA13) cells were lysed and incubated with a 2D fibronectin substrate. CHO(B2)α5 cells were plated on 3D, 2D and 2D+lysate substrates. DOC-insoluble fractions were prepared and analyzed as in A. (C) CHO(B2)α5 cells were plated on 3D fibronectin matrix (3D), on compressed matrix (Comp) or on 2D substrate in the presence of 5 μg/ml rat pFN. After incubation for 7 hours and 18 hours, DOC-insoluble fractions were prepared and analyzed. The immunoblot was developed with ECL plus western blotting detection system and band intensities were quantified. Numbers below each lane represent relative amounts of fibronectin normalized to 3D samples, which were set to 1 for each time point.

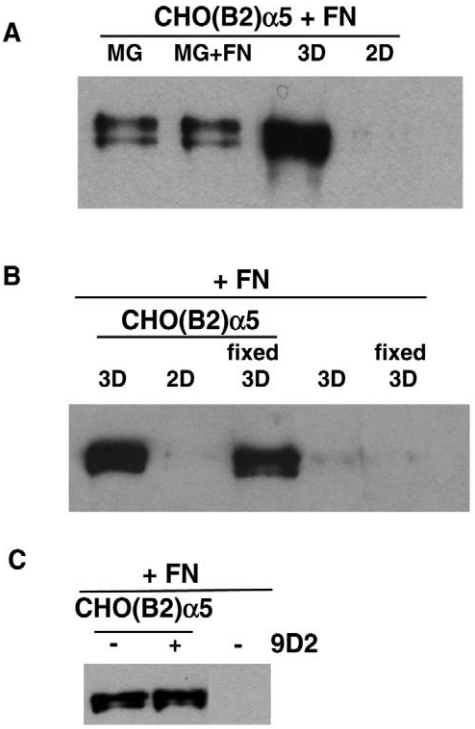

Fig. 7. Controls for the effects of 3D matrix pliability. (A) CHO(B2)α5 cells were plated in medium supplemented with rat fibronectin on Matrigel (MG), Matrigel containing 60 μg/ml fibronectin (MG+FN), 3D fibronectin matrix (3D) or 2D fibronectin substrate (2D). (B) Cells were plated in medium supplemented with rat fibronectin (FN) on 3D fibronectin matrix (3D), 2D fibronectin substrate (2D), or 3D fibronectin matrix that had been fixed in 4% paraformaldehyde (fixed 3D). 3D and fixed 3D (right lanes) were incubated with fibronectin but without cells. (C) Cells were seeded on 3D substrates in the absence (–) and presence (+) of 100 μg/ml 9D2 antibody for 7 hours. DOC-insoluble fractions were analyzed by immunoblotting with IC3.

assembly. Although this integrin does not require exogenous stimulation to mediate assembly, the amount of α5β1-dependent fibronectin matrix can be increased by treatment of cells with activating agents such as Mn^{2+} (Sechler et al., 1997) or by overexpressing α5β1 integrin (Giancotti and Ruoslahti, 1990). Cell surface levels of α5β1 on 2D and 3D substrates were not significantly different (data not shown). To test the idea that integrin activity is modulated in the presence of 3D matrix, we used the human fibrosarcoma cell line HT1080, which is dependent on stimulation with transcriptional activators or exogenous integrin-activators such as Mn^{2+} in order to assemble fibronectin matrix (Brenner et al., 2000; McKeown-Longo and Etzler, 1987). There was significant accumulation of exogenous rat fibronectin matrix assembled by these cells on 3D matrix in the absence of stimulants (Fig. 8A). DOC-insoluble material was detected on 2D substrate only upon treatment with 1 mM Mn^{2+}. These results show that the presence of a 3D fibronectin matrix stimulates integrin-mediated assembly by these tumor cells.

Integrins other than α5β1 are dependent on activation in order to carry out matrix assembly. We used cells expressing αvβ3 integrin, which can be stimulated to form fibronectin fibrils in monolayer culture (Wu et al., 1996). CHO(B2)αvβ3 cells on 3D fibronectin matrix assembled detectable DOC-insoluble material when provided with concentrations of fibronectin as low as 5 μg/ml (Fig. 8B). In contrast, on a 2D substrate these cells did not form detectable matrix unless provided with a higher dose of fibronectin and longer incubation time combined with Mn^{2+} stimulation of the integrin (Fig. 8C). Assembly was dependent on integrins, as parental CHO(B2) cells which lack these receptors did not accumulate any detectable fibronectin (Fig. 8B). Function-blocking antibodies SAM-1 against α5β1 or LM609 against αvβ3 caused a dramatic reduction in matrix deposition (Fig. 8D). Similar inhibition of CHO(B2)α5-mediated assembly was observed with the BIIG2 anti-α5β1 antibody but not with LM609 antibody (not shown), demonstrating integrin

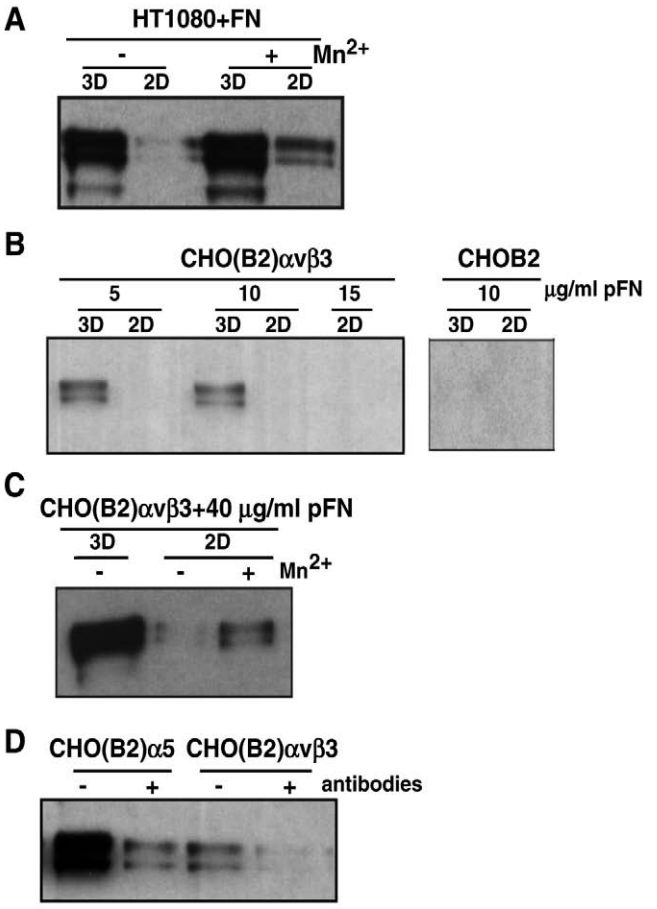

Fig. 8. Stimulatory effects of 3D matrix on integrin activities. (A) HT1080 cells were plated on substrates in the presence of 20 μg/ml rat pFN. After 15 hours, 1 mM $MnCl_2$ was added to one set of cells (+). After an additional 4-hour incubation, DOC-insoluble samples were prepared and analyzed by immunoblotting with IC3. (B) CHO(B2)αvβ3 or CHO(B2) cells were allowed to attach for 2 hours on substrates prepared from WI-38 (VA13) cells (3D) or coated with human pFN (2D). Rat pFN was added at the indicated concentrations followed by incubation for another 15 hours. The DOC-insoluble fractions were analyzed by immunoblotting with IC3 antibody against rat fibronectin. (C) CHO(B2)αvβ3 cells were plated as described in B but with 40 μg/ml rat pFN for 27 hours followed by an additional 4 hours with 1 mM $MnCl_2$. The DOC-insoluble samples were prepared as in B. (D) CHO(B2)α5 and CHO(B2)αvβ3 cells were allowed to attach to 3D fibronectin matrices for 1 hour. 1 μg/ml SAM-1 anti-α5 function-blocking antibody or 10 μg/ml LM609 anti-αvβ3 function-blocking antibody were then added to the cells with 10 μg/ml rat fibronectin (+). After incubation for 16 hours, the DOC-insoluble samples were collected and analyzed by immunoblotting with IC3 antibody.

specificity for the assembly process. Together with the results from HT1080 cells, these findings suggest that a 3D matrix might stimulate fibril formation through effects on integrin activity. Furthermore, in at least two different situations, with tumor cells and with an activation-dependent integrin, a 3D microenvironment has dramatic stimulatory effects on integrin-mediated fibronectin assembly.

Discussion

The process of fibronectin matrix assembly has been extensively studied using various culture systems to determine the molecular requirements for initiation and progression of fibril formation. In most situations in vivo, however, matrix assembly proceeds within an already assembled 3D ECM. Therefore, we analyzed fibronectin matrix assembly by cells cultured on a pre-assembled 3D fibronectin matrix. Our results show an increased rate of assembly and greater amounts of accumulated matrix assembled by cells in a process that is dependent on the fibrillar organization of the 3D fibronectin matrix. Newly formed fibrils extended beyond the existing matrix indicating that assembled fibrils were not simply deposited on the 3D network. Surprisingly, detergent-insoluble fibrils were assembled on 3D matrix under conditions that require integrin activation in 2D culture. Thus the number of situations and cell types involved in assembling fibronectin fibrils in vivo may be significantly more extensive than previously thought.

Substantial evidence shows that fibronectin matrix assembly is dependent on productive integrin-fibronectin interactions (Bae et al., 2004; Mosher, 1993; Schwarzbauer and Sechler, 1999). This process is usually mediated by α5β1 integrin binding to RGD and synergy sites in the central cell binding domain of fibronectin. Several other integrins can assemble fibronectin but only when they are appropriately stimulated to bind to fibronectin (Sechler et al., 2000; Wu et al., 1996; Wu et al., 1995) or by introducing activating mutations in the integrin cytoplasmic domains (Hughes et al., 1996). Clearly there are specific molecular requirements for productive interactions between integrins and fibronectin in order for assembly to proceed.

Here we have shown that integrin stimulation with exogenous activators is not required for fibril assembly by cells on a 3D matrix. This was true for tumor cells expressing activation-dependent α5β1 as well as CHO transfectants expressing αvβ3. Furthermore, the amount of fibronectin needed for integrin-mediated assembly was significantly lower on a 3D matrix than for cells grown on a protein-coated surface. Our results indicate that the 3D organization of the matrix fibrils plays an essential role in stimulating cell-mediated fibronectin assembly. Significant enhancement of assembly was lost when the 3D structure was disrupted or compressed or when fibronectin was presented in a non-fibrillar 3D form. We also did not detect differences in ERK/MAP kinase activation in CHO(B2)α5, HT1080 or WI-38(VA13) cells on 2D substrates or 3D matrices (unpublished observations) suggesting that these substrates did not induce detectably different levels of growth factor signaling through this pathway. Perhaps our native 3D network acts similarly to an in vitro-generated polymeric form of fibronectin, superfibronectin, that has improved adhesive activity owing to the combined effects of integrins and other non-integrin cell adhesion receptors (Morla et al., 1994).

We expect that the effect of 3D matrix on fibronectin assembly will extend to the tissue microenvironment. In fact, our findings may help to explain the compensatory activity of αvβ1 integrin in α5-null mice, which allowed the embryos to survive to day E10, 1.5 days longer than embryos lacking fibronectin, the ligand for α5β1 (Yang and Hynes, 1996; Yang

et al., 1993). When expressed in CHO(B2) cells grown in monolayer culture, αvβ1 was unable to support fibronectin matrix assembly (Zhang et al., 1993). Embryonic tissue might induce αv integrin functions in a manner similar to the activation we observed in cells on 3D fibronectin matrix.

Dimeric fibronectin in solution has a compact structure that becomes extended during fibril formation (Baneyx et al., 2002; Johnson et al., 1999; Schwarzbauer and Sechler, 1999). In a 3D matrix, dimeric fibronectin localized to the cell surface by integrin binding would also be in contact with matrix fibronectin and this interaction could lead to an activating conformational change in the fibronectin. Newly exposed fibronectin binding sites would then promote fibronectin-fibronectin interactions and increase the rate of fibril formation. In support of this mechanism, treatments and mutations that alter fibronectin conformation have been shown to increase fibronectin incorporation into matrix in monolayer culture (McKeown-Longo and Mosher, 1985; Morla et al., 1994; Sechler et al., 1996). Furthermore, local effects of fibronectin conformation have recently been proposed to explain the increased efficiency of exogenous fibronectin assembly by cells growing on vitronectin (Bae et al., 2004). However, fixed 3D fibronectin matrix maintained the ability to stimulate assembly and 9D2 antibody binding to the 3D fibronectin matrix did not detectably block assembly. These results suggest that major rearrangements of fibronectin organization within the 3D matrix do not account for increased fibronectin matrix assembly.

Mechanistically, the effects of a 3D network on fibronectin assembly are probably due to presentation of organized ligand binding sites. Integrins in solution undergo conformational changes when treated with ligand mimetics, activating agents or physiological ligands (Adair et al., 2005; Mould et al., 2003; Takagi and Springer, 2002; Takagi et al., 2003; Xiong et al., 2001; Xiong et al., 2002). Such conformational changes in integrins may affect both the specificity and affinity of ligand recognition (Frelinger et al., 1991), perhaps through formation of regulatory cytoplasmic complexes. In fact, talin binding to the β integrin cytoplasmic domain has recently been shown to stimulate inactive integrins (Tadokoro et al., 2003) and talin is found in 3D ECM adhesion complexes (Cukierman et al., 2001). Initial formation of activating cytoplasmic complexes may be sufficient to maintain increased integrin activity in cells surrounded by existing fibronectin matrix. Mn^{2+} treatment stimulated matrix assembly by HT1080 cells on 2D substrates but only had a minimal effect on cells in 3D fibronectin matrix. This observation suggests that, within a 3D substrate, integrins may already be in an active form.

Stimulatory effects of a 3D matrix provide additional opportunities for fibronectin assembly in vivo and this could have a profound impact on cell behavior. For example, some tumor cells have lost their ability to efficiently assemble their own matrix in culture owing to reduced expression of integrins and fibronectin or reduced integrin activity after oncogenic transformation (Brenner et al., 2000; Hynes, 1990; Plantefaber and Hynes, 1989; Ruoslahti, 1999). Our results suggest that when such cells are placed in the appropriate physiological environment, they might be stimulated to incorporate fibronectin into matrix. This effect could contribute to the growth and spread of tumors in vivo. Understanding the regulatory effects of 3D matrix on fibronectin assembly may shed light on the modulation of cell function in specific in vivo situations.

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