Assembly of exogenous fibronectin by fibronectin-null cells is dependent on the adhesive substrate.

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Summary

The role of endogenously synthesized fibronectin (FN) in assembly was studied with cells lacking or expressing FN. Cells were cultured as homogeneous or mixed populations on surfaces coated with different matrix proteins. Compared to FN-expressing cells, FN-null cells poorly assembled exogenous plasma FN (pFN) when adherent to vitronectin or the recombinant cell-binding domain (III\textsubscript{7-10}) of FN. Vitronectin had a suppressive effect that was overcome by co-adsorbed pFN or laminin-1 but not by soluble FN. In co-cultures of FN-expressing cells and FN-null cells, endogenous FN was preferentially assembled around FN-expressing cells regardless of the adhesive ligand. If the adhesive ligand was vitronectin, exogenous pFN assembled preferentially around cells expressing cellular FN or recombinant EDa- or EDa+ FN. In co-cultures on vitronectin of FN-null cells and β1 integrin subunit-null cells, fibrils of cellular FN and pFN were preferentially deposited by FN-null (β1-expressing) cells immediately adjacent to (FN-secreting) β1-null cells. In co-cultures on vitronectin of FN-null cells and β1-null cells expressing a chimera with the extracellular domain of β1 and cytoplasmic domain of β3, preferential assembly was by the chimera-expressing cells. These results indicate that the adhesive ligand is a determinant of FN assembly by cells not secreting endogenous FN—suppressive if vitronectin, non-suppressive but non-supportive if III\textsubscript{7-10}, supportive if pFN or laminin-1—and suggest that efficient interaction of freshly secreted cellular FN with a β1 integrin, presumably α5β1, substitutes for integrin-mediated adherence to a preformed matrix of laminin-1 or pFN to support assembly of FN.
Introduction

Deposition of fibronectin (FN) into extracellular matrix (ECM) is a dynamic process that is tightly regulated and controlled despite the presence of high concentrations of FN in plasma (200-600 µg/ml, 440-1320 nM) and other body fluids (1,2). FN is a disulfide-linked dimer of 230-250 kDa subunits. Each subunit is comprised mostly of 3 types of repeating modules—12 type I modules, 2 type II modules, and 15 to 17 type III modules depending on splicing— and a variable region (V0, V64, V89, V95, and V120) that is not homologous to other parts of FN. There are two general types of FN: plasma FN (pFN), which is secreted by hepatocytes; and cellular FN (cFN), which is expressed and secreted by fibroblasts and other cell types. There are several structural differences between pFN and cFN. Two type III modules (EDa and EDb) are missing completely in pFN, but variably present in cFN. The V region is also completely missing in one of its subunits in pFN, but present in both subunits of cFN (1,3). Cell adhesion to immobilized FN by α5β1 integrin is mediated by the RGD sequence in the 10th type III module (III\textsubscript{10}) (4-6).

FN matrix assembly is a cell-dependent process that takes place at specialized sites on cell surfaces (7). The N-terminal 70-kDa region of FN binds to these sites with high affinity in a reversible and saturable manner (8,9). Subsequent homophilic interactions among bound FNs are thought to promote polymerization of FN molecules into insoluble matrix (10-14). The receptors for the N-terminal 70-kDa region of FN are poorly characterized. Cross-linking studies with N-terminal 70-kDa fragment revealed molecules that migrated with apparent sizes of >3000 kDa in SDS gels, suggesting that the receptors for the N-terminal region are either of unprecedented size or resistant to
solubilization with SDS (15). Integrins are also implicated in FN assembly (16-22). Because integrins are key mediators of cell adhesion to immobilized ligands such as FN, however, sorting out the roles of integrins in assembly of FN is complicated.

Both pFN and cFN have the potential to be deposited into the ECM (23,24). Knock-out of FN in the mouse results in embryonic lethality (25), indicating that deposition of FN is necessary for early development. Normal skin-wound healing and hemostasis, however, were observed in adult mice with a conditional knock-out of pFN (26), suggesting that pFN has a minor role and cFN is sufficient for physiologically important assembly of FN. A recent study of effects of siRNAs to inhibit FN synthesis in organ culture indicated that expression of cFN by cleft epithelium directs branching morphogenesis of mouse salivary glands by a process that is inhibited by monoclonal antibodies against α5, α6, or β1 integrin subunit (2). When 0.125 to 8 mg/ml (0.25 to 16 µM) exogenous pFN was added to organ culture, branching of salivary glands was stimulated (2). These results indicated that small amounts of cFN have effects that can be replicated only by larger amounts of pFN.

Here, we compare assembly of FN by monolayers of cFN-expressing and FN-null cells studied as homogeneous or mixed cultures on surfaces coated with different matrix proteins. The nature of the surface coating influenced assembly of exogenous FN much more for FN-null cells than for cFN-expressing cells. FN-null cells poorly assembled exogenous FN when adherent to vitronectin (VN) or the recombinant cell-binding domain (III7-10) of FN. VN had a suppressive effect that was overcome by surface-adsorbed pFN or laminin-1 (LN), but not by exogenously added soluble pFN or recombinant EDa+ or EDa- FN, whereas III7-10 was simply unable to support FN
assembly by FN-null cells. cFN was preferentially assembled by cFN-expressing cells regardless of the adhesive ligand. If the adhesive ligand was VN, pFN was assembled preferentially by cFN-expressing cells or transfected FN-null cells expressing recombinant EDa+ or EDa- FN. In co-cultures on VN of FN-null cells and β1-null cells or β1-null cells expressing wild type β1_ or a chimeric β-integrin subunit with extracellular domain of β1 and cytoplasmic domain of β3, the deposition pattern of cFN and pFN was dependent upon re-expression of a β-integrin subunit with the extracellular domain of β1 in the cFN-expressing β1-null cells. We conclude that secreted FN assembles preferentially around cFN-expressing cells and such locally assembled cFN functions like surface-adsorbed pFN or LN to support assembly of soluble FN. This supporting effect is at least partially due to interaction of secreted FN with integrins containing the extracellular domain of β1.
Experimental procedures

Cells

The derivation of FN-/ mouse fibroblastic cells (FN-null cells) and FN +/ cells (cFN-expressing cells) from FN-/ or FN+/- mouse embryonic stem cells was described previously (27). β1-null GD25 cells and GD10 cells deficient in the integrin β1 subunit had been derived by a similar technique and transfected with the β1_A splice variant to give β1-expressing β1_A GD25 cells (19) or β1_A GD10 cells. β1_Aβ3 GD10 cells were generated by transfection of GD10 cells by a β1_A/β3 chimeric construct in which the cytoplasmic domain of β1_A was replaced with the cytoplasmic domain of β3 (28). GFP-expressing FN-null cells were generated by transfection of GFP followed by selection of a stable population with puromycin. A plasmid encoding GFP (pEGFP-N1, CLONTECH Laboratories, Inc., Palo Alto, CA) was digested with NheI and NotI, and the isolated NheI/NotI DNA fragment encoding GFP was then ligated to pIRESpuro2 (CLONTECH Laboratories, Inc., Palo Alto, CA) double digested with NheI and NotI. FN-null cells were transfected with the selectable GFP expression plasmid by liposome method (LipofectAmine™, Invitrogen, Carlsbad, CA).

Expression of GFP-fused FNs

pFH101 and pFH100 (29), which encode EDa+, EDb-, V89 human FN and EDa-, EDb-, V89 human FN, respectively, were gift from Dr. Alberto R. Kornblihtt (Buenos Aires, Argentina). The constructs were manipulated so that protein processing is mediated by the native prepro-sequence of human FN. A HindIII site, which had been
made in the leader sequence region during cloning of pFH101, was erased by substituting a DNA fragment generated by RT (reverse transcription)-PCR of total RNA from AH1F human foreskin dermal fibroblasts. The coding sequence of pFH101 was ligated to the NheI and NotI sites in pIRESpuro2 (CLONTECH Laboratories, Inc., Palo Alto, CA). GFP was introduced between the third and forth type III modules as pioneered by Ohashi et al. (30). Site-directed mutagenesis was performed to create a KpnI restriction enzyme site between the third and fourth type III modules into which the cDNA of GFP was inserted after amplification with primers that added KpnI sites at both ends. The sequence at the insertion site in FN is (III₃)TTGTMIEQ(gfp)DEFFGTPRSD(III₄). The GFP coding sequence is underlined. The cloning strategy resulted in the insertion of two amino acids (GT) at one end of the GFP. An EcoRI fragment (2530 bps) after EcoRI digestion of the cDNA encoding GFP-FN(EDa+) was replaced with the EcoRI fragment (2260 bps) of pFH100 to construct GFP-FN(EDa-). Plasmids encoding GFP-FN(EDa+) or GFP-FN(EDa-) were transfected into FN-null cells in monolayer culture by the liposome method. About 30 hours after transfection, cells were suspended by trypsinization and plated on cover slips. Due to a low transfection efficiency of about ~1%, most of the cells remained FN-null.

Preparations of insect cell medium containing human EDa+ or EDa- FN and of AH1F cell medium.

Recombinant baculovirus was generated by cotransfection of Baculogold linearized AcNPV viral DNA (BD Biosciences, San Jose, CA) and cDNA encoding mature human EDa+ FN or EDa- FN, which had been cloned in pCOCO transfer vector
Viruses were cloned and amplified as described (31). Human EDa+ FN and EDa-FN were expressed by infecting High Five insect cells (Invitrogen, Carlsbad, CA) in SF900II serum-free medium at 27 °C with pass 4 virus. Conditioned medium was collected ~ 65 hours postinfection, and concentrated to 1/8 of initial volume using Amicon® Ultra Centrifugal Filter Device (MWCO=10,000, Millipore, Bedford, MA) after cells were spun down and removed. The concentrated medium was dialyzed against PBS pH 7.4, and then dialyzed again against DMEM containing 0.2% BSA. For preparation of medium conditioned with cFN of AH1F human dermal fibroblasts, AH1F cells were incubated on VN-coated surface for 24 hours in serum-free medium (DMEM + 0.2% BSA), and the medium was centrifuged to save supernatant. FN present in AH1F cell medium and concentrated insect cell medium was quantified by Western blots.

Fluorescent labeling of pFN (Rx-pFN)

Human pFN, purified on DEAE-cellulose as described before (32), was labeled with Rhodamine Red™-X (FluoReporter Rhodamine Red™-X Protein Labeling Kit, Molecular Probes, Eugene, OR) according to the manufacturer’s instructions with the following slight modifications. Rhodamine Red™-X dissolved in DMSO was diluted in 0.5 M carbonate buffer (Na₂CO₃ and NaHCO₃, pH. 9.5) to 0.5 mg/ml, and pFN was diluted in 0.05M carbonate buffer (Na₂CO₃ and NaHCO₃, pH. 9.5) to 2 mg/ml for the conjugation reaction.

Cell culture
Cells were suspended with 0.05% trypsin/0.01% EDTA solution for ~5 min at 37 °C; trypsin was quenched by washing with 10% fetal bovine serum (FBS) in Dulbecco’s modification of Eagle’s medium (DMEM, Cellgro Mediatech, VA) followed by washing with PBS; and cells were cultured at 60~70% confluence for 4 or 18 hours in DMEM supplemented with 0.2% bovine serum albumin (BSA, Sigma, St. Louis, MO) on glass cover slips coated with pFN (3 µg/ml), LN extracted from Engelbreth-Holm-Swarm mouse tumor (10 µg/ml) (BD Biosciences, Bedford, MA), VN (3 µg/ml) (33) or III₇₋₁₀ (3 µg/ml) (34,35) unless indicated. The adhesive proteins were diluted to the indicated concentrations in PBS, pH 7.4, and incubated with the cover slips overnight at 4 °C. After blocking with 1% BSA for 30 min at 37 °C and washing with PBS, cover slips were used within 24 hours for cell culture. For some experiments, 2 µM lysophosphatidic acid (LPA, Avanti Polar Lipids, Alabaster, AL), 100 µg/ml heparin (Sigma, St. Louis, MO), or 15 µg/ml cyclo[Arg-Gly-Asp-D-Phe-Val] (cRGDfV, BIOMOL Research Laboratories Inc., Plymouth meeting, PA) was added to culture medium.

Fluorescence microscopy

Deposited FN was visualized with rabbit polyclonal antibodies and Rhodamine Red™-X conjugated AffiniPure donkey anti-rabbit antibody (Jackson ImmunoResearch laboratories, Inc., West Grove, PA). The rabbit polyclonal antibodies, although produced against human pFN, cross-reacted with mouse FN as demonstrated by Western blotting and ELISA. To visualize exogenous pFN only, Rx-pFN was added to the culture medium at 9 µg/ml. To detect EDa+ FN in the presence of pFN, the IST-9 monoclonal antibody to the EDa type III module of human FN (36) (Harlan Sera-lab Limited, UK) and Alexa
Fluor R 350 goat anti-mouse IgG (Molecular Probes, Inc., Eugene, OR) were used. Before staining cells with antibodies, cells were fixed with 3.7% paraformaldehyde for 15 min. For staining of intracellular proteins after paraformaldehyde fixation, cells were permeabilized with 0.2% Triton X-100 for 5 min. For staining of focal adhesion kinase (FAK), cells were fixed and permeabilized with methanol for 5 min. Monoclonal antibody against vinculin (hVIN-1) was from Sigma (St. Louis, MO), monoclonal antibody against integrin β1 (MB1.2) was from Chemicon (Temecula, CA), and monoclonal antibodies against paxillin (clone 349), FAK (clone77), and integrin β3 (2C9.G2) were from Pharmingen (San Diego, CA). Secondary antibodies, Rhodamine Red™-X conjugated AffiniPure donkey anti-mouse IgG, Rhodamine Red™-X conjugated AffiniPure donkey anti-rat IgG, and Rhodamine Red™-X conjugated AffiniPure goat anti-Armenian hamster IgG were from Jackson ImmunoResearch (West Grove, PA). After blocking with 1% BSA overnight at 4°C or for 10 min at 25°C, cells were stained with ~10 µg/ml of primary antibodies for 1 hour at room temperature, followed by washing with PBS. After staining cells with ~10 µg/ml of secondary antibodies for 40 min at room temperature and washing with PBS, cover slips were mounted on Vectashield (Vector laboratories, Inc., Burlingame, CA). Cells were viewed on an Olympus epifluorescence microscope (BX60, Olympus America Inc., Melville, NY). Pictures were taken with RT Slider digital camera (Spot Diagnostic Instruments, Inc., Sterling Heights, MI) and processed with Spot RT Software v3. and Adobe Photoshop version 5.0 (Adobe System Inc., San Jose, CA) for Mac OS.

Flow cytometry
Cells were harvested and suspended in PBS containing 1% FBS. Approximately 1.0 x 10⁶/ml of cells were incubated with ~ 0.5 μg/ml of primary antibody, and then incubated at 4° C with ~ 10 μg/ml of allophycocyanin (APC)-conjugated goat anti-rat secondary antibody (Pharmingen, San Diego, CA) or biotin-conjugated mouse anti-Armenian and Syrian hamster IgG monoclonal antibody and streptavidin-allophycocyanin conjugate (Pharmingen, San Diego, CA). Mouse β1 was detected with MB1.2. Monoclonal antibody MFR5 to mouse α5, H9.2B8 to mouse αV, 2C9.G2 to mouse β3, and GoH3 to mouse α6 were all from Pharmingen (San Diego, CA). For control samples, cells were incubated only with secondary antibody. Cells (at least 8,000 per sample) were analyzed in a FacsCalibor (Becton and Dickinson Co., San Jose, CA).

Assays of LN and FN

Cells (2 x 10⁵) were cultured at 37 °C in 2 ml of DMEM supplemented with 0.2% BSA in 6-well cell culture cluster plates (surface area per well: 10 cm², Corning Incorporated, Corning, NY) coated with VN (3 μg/ml). After 4 hours or 18 hours, cells were lysed with 300 µl extraction buffer [1.5% Triton X-100, 0.05M Tris-Cl, pH7.5, 0.3M NaCl, 1mM PMSF, protease inhibitor cocktail (Roche, Germany)]. Protease inhibitor cocktail and 1mM PMSF were added to the harvested culture medium. The cell extracts and culture medium were centrifuged at 12,000 rpm for 15 min at 4°C, and the supernatant was saved for Western blotting. For detection of FN and LN in Western blots, our anti-FN rabbit antibodies or anti-LN rabbit antibodies (Novus Biologicals, Inc.,
Littleton, Co) and peroxidase-conjugated AffiniPure donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) were used.

Cell adhesion assays

FN, VN, LN or BSA were coated at 2-10 µg/ml onto wells of a 96-well plate, and the wells were blocked with 1% BSA in PBS. Cells were incubated for 30 min at 37 °C in suspension of DMEM containing 0.2% BSA with or without 40 µg/ml of GoH3 anti-mouse α6 monoclonal antibody or 15-30 µg/ml cRGDfV. The cells were then allowed to attach to wells for 2 hours at 37 °C. Non-adherent cells were removed by washing, and adherent cells were quantified by colorimetric detection at 595 nm using microplate reader (Model EL340, BIO-TEK instruments, Inc. VT) and data were obtained with DELTA Soft II™ (BioMetallics, Inc. NJ).
Results

FN-null cells assemble exogenously added pFN when adherent to pFN or LN but not when adherent to VN or a recombinant FN protein that comprises 7th through 10th type III repeat.

FN-null cells allow experimental analysis of the contributions of the three sources of FN in cell culture—soluble exogenous FN, exogenous FN adherent to the substrate, and endogenous FN—on assembly of FN. We first studied the effects of adhesive proteins coated on the substrate on assembly of exogenously added FN. During a 4-hour culture in serum-free medium (DMEM + 0.2% BSA) containing 9 µg/ml (20 nM) Rhodamine Red™-X conjugated pFN (Rx-pFN), FN-null cells and cFN-expressing cells both assembled exogenously added Rx-pFN when adherent to pFN- or LN-coated coverslips (Fig. 1A). When adherent to VN, however, cFN-expressing cells assembled pFN better than FN-null cells did (Fig. 1A). Comparing substrates, cFN-expressing cells assembled pFN better when adherent to pFN or LN than when adherent to VN, but the difference was not as great as between FN-null cells cultured on the same substrates. These results indicate that substrate-coated VN is poorly supportive for assembly of exogenous pFN by FN-null cells whereas substrate-coated pFN or LN is supportive and that expression of endogenous cFN facilitates assembly of exogenous pFN.

The effect of VN on assembly of exogenous pFN by FN-null cells is suppressive and overcome by surface-adsorbed pFN or LN.

A number of experiments were performed to characterize further the different effects of adhesive proteins on the assembly of pFN by FN-null cells versus cFN-
expressing cells. Because α5β1 integrin is known to be strongly supportive for assembly of FN (16-18,37), we examined at whether differences in expression levels of α5 and β1 subunits accounted for defects in FN assembly by FN-null cells on VN. FN-null cells and cFN-expressing cells expressed similar levels of α5 and β1 integrin subunits as assessed by flow cytometry (Fig. 1B). Mean fluorescence intensities varied <1.7-fold. FN-null cells and cFN-expressing cells were also found to express equal amounts of α6β1, adhere equally well to LN, and respond to the GoH3 anti-mouse α6 monoclonal antibody by inhibited adhesion to LN (results not shown). Finally, FN-null cells and cFN-expressing cells were found to express similar levels of αV and β3 integrin subunits as tested by flow cytometry (results not shown). αVβ3 was the major receptor for adhesion of both FN-null cells and cFN-expressing cells on a VN-coated surface as assessed by inhibited adhesion upon incubation with cRGDfV, which interacts specifically with αVβ3 integrin (38) (results not shown).

Although FN and LN are major ligands for α5β1 and α6β1 integrins, respectively, ligation of β1 integrins is not enough to make FN-null cells competent to assemble exogenous pFN. When FN-null cells were cultured on cover slips coated with III7-10, which has the synergistic and RGD sites of FN for interaction with α5β1 (39-41), FN-null cells assembled exogenously added Rx-pFN poorly whereas cFN-expressing cells assembled exogenous FN robustly (Fig 1A).

Previously, β1-null GD25 cells were shown to be also defective in initial assembly of pFN when cultured on VN, and co-coating experiments indicated that the defective assembly is due to a suppressive effect of VN (35). Similar co-coating experiments with FN-null cells indicated that VN is also suppressive for FN-null cells.
Thus, a co-coat with 6-9 µg/ml pFN overcame the negative effects of a coat of 2 µg/ml VN on FN-null cells (Fig. 2A) as effectively as it did with control β1-null cells (results not shown). Coating with an increasing amount of LN also overcame the suppressive effects of VN, and an increasing amount of VN overcame the facilitating effect of LN (Fig. 2A). Interestingly, whereas a coat of 5 µg/ml LN poorly supported adhesion and assembly of pFN by FN-null cells, addition of an intermediate coat of 3 µg/ml VN enhanced the facilitating effect of LN (Fig. 2A). A coat with 10 µg/ml III7-10 did not overcome the negative effect of 2 µg/ml VN and did not suppress the facilitating effect of 2 µg/ml FN (Fig. 2B).

Because the co-coating experiments indicated that the presence of pFN or LN overcomes the suppressive effect of VN, we measured the amounts of pFN or LN in culture medium and cell layers of 2 x 10⁵ cells cultured in 2 ml medium on a surface area of 10 cm². Western blotting indicated that cFN secreted or deposited by cFN-expressing cells was ~2 ng over 4 hours and ~23 ng over 18 hours (results not shown). Greater than 80% of cFN was present in the medium. There was no detectable synthesis of cFN by FN-null cells. The amount of cell-associated LN was similar between cFN-expressing cells and FN-null cells: 125 and 250 ng LN over 4 and 18 hours, respectively (results not shown). There was little LN in medium. Thus, the concentrations of FN and LN present in monolayers of cells (0.2-2.3 ng/cm² for FN and 12.5-25 ng/cm² for LN) were low compared to the concentrations needed (1.5-2.75 µg/cm² for FN or LN) as a co-coat to overcome effects of VN.

FN-null cells were cultured on a VN-coated surface in the presence of high concentrations of soluble pFN to learn if the suppressive effect of VN can be overcome.
More pFN was assembled if the concentration of exogenous pFN was 200 µg/ml (440 nM), 600 µg/ml (1.3 µM), or 2 mg/ml (4.4 µM) than if the concentration of pFN was 9 µg/ml (20 nM) (Fig. 2C). At each of the higher concentrations of pFN, however, more pFN was assembled when FN-null cells were cultured on LN-coated coverslips than on VN-coated coverslips (Fig. 2C). These results are further evidenced that the nature of the cell adhesive ligand(s) is a major determinant of the ability of FN-null cells to assemble exogenous FN.

To learn if the suppressive effect of VN on the assembly of exogenous pFN by FN-null cells cultured on VN is due to induction of a distinctive cellular phenotype, adherent cells were stained for vinculin, paxillin, focal adhesion kinase (FAK), and β1 and β3 integrin subunits, which have been shown to be important for FN signaling and/or assembly (42-46). All the proteins were found in focal adhesions of cFN-expressing cells and FN-null cells that had been cultured on VN-coated coverslips for 4 hours (Fig 2D). The only apparent difference between cFN-expressing and FN-null cells was a more peripheral distribution of paxillin in FN-null cells on VN-coated coverslips. In FN-null cells cultured on pFN-coated coverslips, the distribution of paxillin was similar to cFN-expressing cells cultured on coverslips coated with either VN or pFN (results not shown).

**Endogenously synthesized cFN overcomes the negative effect of VN, but exogenously added cFN does not.**

The co-coating experiments shown in Fig. 2A indicate that coating at a concentration of pFN much greater than the 1-11.5 ng/ml present in conditioned medium of cFN-expressing cells is required to overcome the suppressive effect of a VN coating.
This conclusion suggests that endogenously produced and deposited cFN acts locally to favor pFN deposition by cFN-expressing cells cultured on VN. To evaluate this hypothesis, we examined deposition of cFN in mixed cultures of cFN-expressing cells and GFP-expressing FN-null cells to learn if cFN is locally deposited. The cells were plated at two ratios (20:1 or 1:20), and incubated for 18 hours in DMEM supplemented with 0.2% BSA on LN- or VN-coated cover slips. Like non-GFP-expressing FN-null cells, GFP-expressing FN-null cells expressed α5, β1, αv, β3, and α6 integrin subunits and poorly assembled exogenous FN on VN (results not shown). Deposited cFN was detected with anti-FN rabbit polyclonal antibody and Rhodamine Red™-X conjugated donkey anti-rabbit antibody. Under phase microscopy, GFP-expressing FN-null cells were less spread than cFN-expressing cells (Fig. 3A). When cFN-expressing cells were the dominating population, deposited cFN fibrils were observed throughout the culture (Fig. 3A). In contrast, preferential assembly of endogenous cFN was observed on cFN-expressing cells when GFP-expressing FN-null cells were the dominating population (Fig. 3A). Fibrils of cFN were also found on GFP-expressing FN-null cells adjacent to cFN-expressing cells (arrowhead in Fig. 3A). There was no assembly of cFN in a mixed culture of FN-null cells and GFP-expressing FN-null cells (results not shown).

We then explored whether exogenous pFN is locally deposited on cFN-expressing cells in the co-cultures of cFN-expressing cells and cytoplasmic GFP-expressing FN-null cells mixed at 1:20. The mix in DMEM containing 0.2% BSA and 9 µg/ml Rx-pFN were cultured for 18 hours at 37 °C on pFN-, LN-, or VN-coated cover slips (pFN+Rx-pFN, LN+Rx-pFN or VN+Rx-pFN, respectively). Preferential assembly of exogenous pFN was observed on and around the cFN-expressing cells when adherent to VN (Fig. 3B).
mixed cultures on pFN or LN, exogenous pFN was assembled by both cFN-expressing cells and GFP-expressing FN-null cells (Fig. 3B).

To corroborate the finding that endogenous cFN expression is associated with preferential deposition of exogenous pFN when cells are cultured on VN and test the need for EDa, GFP-tagged cFN splice variants, GFP-FN(EDa+) or GFP-FN(EDa-), were transiently expressed in FN-null cells (Fig. 4). The mix of rare transiently-transfected cells expressing GFP-FN and non-transfected FN-null cells in DMEM containing 0.2 % BSA and 9 µg/ml Rx-pFN were cultured at 37 °C for 18 hours on VN-coated cover slips. The EDa-specific monoclonal antibody IST-9 and Alexa Fluor R 350 goat anti-mouse IgG were used to detect GFP-FN(EDa+). Both GFP-FNs were detected in fibrillar patterns. The GFP signal colocalized with IST-9 around cells expressing GFP-FN(EDa+). Rx-pFN was found around cells expressing either GFP-FN(EDa+) or GFP-FN(EDa-) but did not always co-localize with fibrils of GFP-FN (arrows in Fig. 4). FN-null cells cultured for 18 hours in DMEM containing 0.2 % BSA and 9 µg/ml Rx-pFN on VN-coated coverslips poorly assembled Rx-pFN (Fig. 4C).

The results with endogenously synthesized GFP-FNs indicated that it is the location of synthesis rather than the presence of the alternatively spliced EDa that allows cFN-expressing cells to overcome the suppressive effect of adhesions to VN. To test this conclusion, serum-free culture medium collected after 24-hour incubation of AH1F human dermal fibroblast or insect cell medium that was conditioned with recombinant His-tagged human FNs with or without EDa was incubated with FN-null cells cultured on VN- or LN-coated cover slips. Deposited FN was detected with anti-FN polyclonal rabbit antibody (Fig. 5). The final concentrations of cFN in AH1F cell medium or EDa+ or
EDa- FNs in insect cell media were similar, about 2 µg/ml (results not shown). FN-null cells assembled all these sources of FN equally poorly when the cells were on VN-coated surface and equally well when the cells were on LN-coated surface (Fig. 5).

**Endogenously synthesized cFN interacts more efficiently with β1 integrins than β3 integrins when cells are adherent to VN.**

As described above, β1-null GD25 cells, which express endogenous cFN (35), and GFP-expressing FN-null cells, which express α5 and β1 integrin (Fig. 1B), are both defective in assembly of pFN when cultured on VN as homogenous cultures. To test whether the cells complement one another, the cells were cultured as a mix. Deposited endogenous cFN was detected with anti-FN rabbit polyclonal antibody and Rhodamine Red™-X conjugated donkey anti-rabbit antibody (Fig. 6A). Rhodamine red was present diffusely on the surface of β1-null GD25 cells (arrow in Fig. 6A). Long and thick fibrils of endogenous cFN were preferentially deposited on the neighboring GFP-expressing FN-null cells as compared to the β1-null GD25 cells (arrowhead in Fig. 6A). To visualize deposition of exogenous pFN, 9 µg/ml Rx-pFN was added to culture medium (Fig. 6B). Exogenous Rx-pFN was preferentially deposited at the interface between β1-null GD25 cells and GFP-expressing FN-null cells (arrowhead in Fig. 6B). On a surface coated with III_{7,10}, which supports assembly of FN by GD25 cells (35), GD25 cells preferentially assembled exogenously added Rx-pFN in the mixed culture with GFP-expressing FN-null cells (Fig. 6C). When mixed cultures of β1-null GD25 cells and GFP-expressing FN-null cells were adherent to FN-coated cover slips, long fibrils of pFN were found on both β1-null GD25 cells and GFP-expressing FN-null cells (results
not shown). In mixed cultures on VN substrate of GFP-expressing FN-null cells and β1<sub>A</sub>GD25 or β1<sub>A</sub>GD10 cells that express functional β1 integrin, preferential assembly of cFN and pFN was by the β1<sub>A</sub>GD25 or β1<sub>A</sub>GD10 cells (results not shown).

The negative effect of VN on assembly of exogenous pFN by β1-null GD25 cells was overcome by a coat of 6-9 µg/ml pFN (35), suggesting that ligation of β3 integrins via adhesion to FN rather than VN not only does not generate a suppressive effect but has a positive effect. In other words, β1 integrin-specific signaling is not necessary to overcome the negative effect of VN. In order to explain the observation that expression of functional β1 integrin in cFN-secteting GD25 cells releases the suppressive effect of VN (35), however, one must hypothesize that β1 integrin interacts with endogenous cFN more efficiently than β3 integrin when cells are adherent to VN.

We tested this hypothesis with β1-null GD10, β1<sub>A</sub>GD10 cells expressing wild type β1<sub>A</sub>, and β1<sub>A</sub>β3GD10 cells expressing a chimeric β1 subunit with the extracellular domain of β1 and the cytoplasmic domain of β3. Cells were incubated for 4 hours (Fig. 7A) or 18 hours (Fig. 7B) at 37°C in DMEM containing 0.2% BSA and 9 µg/ml Rx-pFN on FN-, LN- or VN-coated cover slips. GD10 cells adhered poorly on LN-coated cover slips and could not be tested for FN assembly. Assembly of exogenous FN on LN-coated cover slips was similar for β1<sub>A</sub>GD10 and β1<sub>A</sub>β3 GD10 cells. Comparing the three cell types at 4 and 18 hours, β1<sub>A</sub>β3GD10 cells on VN assembled exogenous FN less well than β1<sub>A</sub>GD10 cells but better than β1-null GD10 cells. The assembly of exogenously added FN on FN-coated cover slips was similar for all three cell types. These results indicate that the extracellular domain of β1 is more important than the cytoplasmic domain of β1 in supporting assembly of exogenous FN by cells cultured on VN.
Consistent with such a conclusion, in a mixed culture of $\beta_1\beta_3$GD10 cells and GFP-expressing FN-null cells, both cFN and pFN preferentially deposited on and around $\beta_1\beta_3$ GD10 cells rather than the FN-null cells after an 18-hour incubation in serum-free medium on VN-coated cover slips (results not shown).
Discussion

To explore the role of endogenously synthesized cFN in assembly of FN, we studied cells lacking and expressing cFN in monolayer culture as homogeneous or mixed populations. We confined our observations to the initial 4 or 18 hours of assembly. Initial assembly of pFN by FN-null cells was found to be dependent on the adherent substratum. FN-null cells adherent to VN or III$_{7,10}$ poorly assembled exogenous pFN, indicating that cell adherence via an integrin is not sufficient for FN assembly. FN-null cells were able to assemble pFN when adherent to pFN or LN. Adherence to type I collagen has been also shown to support assembly of pFN by FN-null cells (47,48). In contrast, cFN-expressing cells assembled pFN on all the adherent substrates that we tested. In the mixed culture on VN of cFN-expressing cells and GFP-expressing FN-null cells, cFN-expressing cells preferentially assembled both cFN and pFN, indicating that cFN acts locally to support assembly of itself and pFN. The conclusion that it is local secretion rather than the structure of cFN that is important for initial assembly was supported by experiments in which cFN of AH1F cells or EDa+ FN was added to medium. EDa-containing FNs behaved like pFN and was deposited preferentially around cFN expressing cells. Complementation experiments in which cFN-expressing β1-null GD25 cells or β1,β3 GD10 cells and β1-expressing FN-null cells were co-cultured on VN indicate that locally secreted cFN overcomes the suppressive effect of VN by efficient interaction of cFN with integrins containing the extracellular domain of β1.

The finding that FN-null cells are unable to assemble pFN when cultured on VN is consistent with the previous observation that the N-terminal 70-kDa fragment of FN, which mediates assembly of FN, does not become associated with cycloheximide-treated
cells adherent to a VN-coated surface (49,50). Analyses of mixed substrates of VN and pFN or LN revealed that the effect of VN is to suppress the ability of substrate-bound pFN or LN to support assembly of pFN and conversely the effect of substrate-bound pFN or LN is to overcome the suppressive effect of VN. In contrast, III$_{7,10}$ did not suppress the activity of FN when co-coated with FN, indicating that surface-adsorbed III$_{7,10}$ simply lacks assembly-promoting activity present in surface adsorbed intact FN, LN or collagen I. Marked suppression of FN assembly by substrate-bound VN has also been noted for β1-null GD25 cells (35).

The mechanism of the marked suppressive effect of substrate-bound VN on cells lacking FN is obscure. The effect could not be explained by detectable alterations in formation of focal adhesions and stress fibers in FN-null cells adherent to VN. Lysophosphatidic acid induces stress fibers concomitantly with enhancing FN assembly in normal fibroblasts (51) and induces stress fibers in FN-null cells plated on FN fragments lacking the heparin-binding domain (27). In agreement with the apparently normal stress fibers in FN-null cells adherent on VN, however, suppression of FN assembly could not be overcome by addition of 2 µM lysophosphatidic acid to culture medium (results not shown). In other exploratory experiments, we found that incubation of FN-null cells with cRGDfV, which inhibited cell adhesion to VN, did not overcome the effect of VN on assembly of pFN when cell are adherent to the mixed substrate of 2 µg/ml FN and 9 µg/ml VN and also that addition of heparin to block possible interactions of cells with the heparin-binding site on VN did not overcome the effect of VN (results not shown). Thus, the suppressive effect of VN is likely mediated by concerted interactions of substrate-adsorbed VN with several different cell surface receptors rather
than, e.g., just its interaction with αVβ3 or heparin sulfate proteoglycan. It will be of interest to learn whether certain other matrix components share the suppressive activity of VN.

The mechanism by which supportive matrix molecules such as FN and LN overcome the suppressive effects of VN is also obscure. β1Aβ3 GD10 cells assembled pFN on surface coated with pFN, LN or VN after an 18-hour incubation. These results indicate that β3 integrin-mediated signals can be supportive of assembly of pFN, i.e., signaling mediated by the β1 integrin cytoplasmic domain is not necessary to overcome the effect of VN. The mixed culture results with GFP-expressing FN-null cells and β1-null GD25 or GD10 cells or β1Aβ3 GD10 cells indicate that locally secreted cFN overcomes the suppressive effect of VN most efficiently by interaction with integrins containing the extracellular domain of β1. As with the suppressive effect of VN, the supportive effect of LN or intact FN is likely mediated by concerted interactions of the matrix molecule with several different cell surface receptors, including integrins, heparin sulfate proteoglycan, and yet-to-be identified molecules.

Although EDa+ FN, EDb+ FN, and V region-containing FN co-distribute in the mouse embryo, there are differences in the distribution of these splice variants in adult mice (52,53), suggesting that each spliced segment of FN may have unique function(s) in adult mice. Studies in which recombinant FN-splice variants were added to fibroblast cultures suggested that EDa+ or EDb+ FN is assembled more efficiently than FN lacking the extra type III modules (54). Mice engineered so as to be unable to express EDb+ FN lack a phenotype (55). In contrast, mice unable to express EDa+ FN have a diminished life span and abnormal wound healing (56). Our results, however, offered no indication
that the role of EDa is to regulate assembly. EDa- FN facilitated assembly of pFN on VN-coated surface when present as a coat of pFN or expressed endogenously as the recombinant protein. Interestingly, mice constitutively expressing EDa+ FN, like those unable to express EDa+ FN, also have a diminished life span and abnormal wound healing (56). These results indicate that it is advantageous to secrete cFN that is a mixture of EDa+ and EDa- splice variants.

The fibrils of exogenous FN found around cells expressing endogenous GFP-FN(EDa+) or GFP-FN(EDa-) did not co-localize exactly with locally deposited GFP-tagged FN. For this reason and because FN-null cells adherent to LN assemble pFN efficiently, it cannot be concluded that insolubilized FN, either adsorbed on tissue culture plastic or deposited after secretion from cells, supports deposition of exogenous pFN simply by serving as a template to which pFN binds. A likely explanation of how endogenous cFN is deposited efficiently by itself and supports efficient deposition of exogenous pFN is that newly secreted cFN acts like the surface-adsorbed pFN or LN to engage and activate components of the FN assembly machinery. One possibility is that FN moves from the Golgi to the cell surface pre-bound to membrane-intercalated molecules required for efficient assembly. However, the observation of assembly by adjacent FN-null cells in co-culture with β1-null cells indicates that secreted cFN is active in the immediate vicinity of the cFN-secreting cells. We suggest three possible explanations for this local effect that are not mutually exclusive of one another. First, freshly secreted cFN, whether EDa+ or EDa-, may transiently assume a conformation that is different from pFN in the circulation or the FNs in conditioned media. This conformation is hypothesized to mimic the conformation of pFN adherent to tissue
culture plastic and thus provide the same signal to cells as adherence to substrate-bound pFN. Second, molecules may be co-secreted with endogenous cFN to favor maintenance of the hypothesized conformation. Third, the concentration of freshly secreted endogenous cFN may locally exceed a threshold that favors interactions with cells and/or polymerization.

As described in the Introduction, a recent study of effects of siRNAs to inhibit FN synthesis in organ culture indicated that expression of cFN by cleft epithelium directs branching morphogenesis of mouse salivary glands by a process that is inhibited by monoclonal antibodies against α5, α6, or β1 integrin subunit (2). Attempts to overcome the effect of siRNAs by addition of pFN to organ culture medium indicated that the effects endogenous cFN can be replicated only by much larger amounts of pFN (2). Our demonstration that secretion of cFN is coupled to assembly of exogenous pFN when cells are adherent to a suppressive ligand such as VN or non-supportive ligand such as III7,10 offers insight into how FN assembly may be controlled in vivo. The mechanisms that favor local activity and deposition of cFN are likely to be operative and indeed more dominant in the three-dimensional environment of tissues than in the two-dimensional environment of monolayer cells in culture. Preferential deposition of cFN by cFN-expressing cells and cFN’s support for assembly of cFN thereby may allow regulated expression of cFN to be tightly coupled to assembly of FN in an environment not conducive to FN assembly but rich in pFN. Such local regulation of assembly of FN is presumably important in situations where a sharp boundary of FN matrix is necessary in development and healing (1,2,57,58).
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Acknowledgments – We thank Lara Johansson for help with cell culture, Douglas Annis and Dr. Xueping Shao for technical advice, and Drs. Bianca Tomasini-Johansson, Donna Peters, Francis Fogerty, and Mats Johansson for helpful discussions. This work was supported by National Institutes of Health Grant HL 21644.
Abbreviations List – cFN, cellular fibronectin; EDa, extra domain a; EDb, extra domain b; FAK, focal adhesion kinase; FN, fibronectin; GFP-FN(EDa+), green fluorescent protein fused fibronectin with EDa; GFP-FN(EDa-), green fluorescent protein fused fibronectin without EDa; LN, laminin-1; pFN, plasma fibronectin; Rx-pFN, Rhodamine Red™-X conjugated pFN; VN, vitronectin.
Figure legends

Figure 1. FN-null cells assemble exogenously added pFN when adherent to pFN or LN but not when adherent to VN or recombinant protein that comprises 7th through 10th type III repeat.

(A) Cells in DMEM containing 0.2% BSA and 9 µg/ml Rx-pFN were incubated for 4 hours at 37 °C on cover slips coated with pFN, LN, or VN. Deposited Rx-pFN and GFP was visualized by fluorescence microscopy after fixation with 3.7% paraformaldehyde. Bar = 20 µm. (B) Flow cytometric analysis for surface expression of α5 and β1 integrin subunits. Monoclonal antibody MFR5 to mouse α5 or MB1.2 to mouse β1 was detected with allophycocyanin (APC)-conjugated goat anti-rat Ig specific polyclonal antibody. Unshaded, negative control; Shaded, immune antibody.

Figure 2. The suppressive effect of VN on assembly of exogenous pFN by FN-null cells is overcome by surface-adsorbed pFN or LN.

(A) Cover slips were coated with 2 µg/ml VN with an increasing concentration of FN (VN/vFN) or with 2 µg/ml FN with an increasing concentration of VN (FN/vVN). For mixed substrate of VN and LN, cover slips were coated with 2 µg/ml VN with increasing concentration of LN (VN/vLN) or with 5 µg/ml LN with increasing concentration of VN (LN/vVN). FN-null cells in DMEM containing 0.2% BSA and 9 µg/ml Rx-pFN were incubated for 4 hours on cover slips pre-coated as described above (VN/vFN, vVN/FN, VN/vLN or vVN/LN). Deposited Rx-pFN was visualized by fluorescence microscopy after fixation with 3.7% paraformaldehyde. Numbers indicate the concentrations (µg/ml) of the adhesive proteins during coating of coverslips. (B) FN-null cells in DMEM
containing 0.2% BSA and 9 µg/ml Rx-pFN were incubated for 4 hours on cover slips pre-coated with 10 µg/ml rFNIII7-10, 2 µg/ml FN plus 10 µg/ml rFNIII7-10, or 2 µg/ml VN plus 10 µg/ml rFNIII7-10. Deposited Rx-pFN was visualized by fluorescence microscopy after fixation with 3.7% paraformaldehyde. Numbers indicate the concentrations (µg/ml) of the adhesive proteins during coating of cover slips. (C) FN-null cells in DMEM containing 0.2% BSA and 9 µg/ml, 200 µg/ml, 600 µg/ml or 2 mg/ml pFN were incubated for 4 hours on coverslips coated with VN or LN. Deposited FN was detected with anti-FN rabbit polyclonal antibodies and Rhodamine Red™-X conjugated donkey anti-rabbit antibodies after fixation with 3.7% paraformaldehyde. Cells were visualized for rhodamine red by fluorescence microscopy and by phase microscopy. (D) cFN-expressing cells and FN-null cells in DMEM containing 0.2% BSA were cultured for 4 hours at 37 °C. Cells were fixed by 3.7% paraformaldehyde or methanol and treated with by 0.2% Triton X-100 before staining for vinculin, paxillin, FAK, β1, and β3 integrins. Bar = 20 µm.

Figure 3. Locally deposited endogenously synthesized cFN overcomes the negative effect of VN.

(A) cFN-expressing cells and GFP-expressing FN-null cells mixed at ratios of 20:1 or 1:20 in DMEM containing 0.2% BSA were incubated at 37°C for 18 hours on LN-coated (LN) or VN-coated (VN) coverslips. Anti-FN rabbit polyclonal antibodies and Rhodamine Red™-X conjugated donkey anti-rabbit antibodies were used to detect deposited FN after fixation with 3.7% paraformaldehyde. Cells were visualized for rhodamine red and GFP by fluorescence microscopy and by phase microscopy. Merge
images of double fluorescence were generated by image processing. The fibrils of cFN in the 1:20 ratio experiment on VN are shown at higher magnification to demonstrate fibrils of cFN deposited on adjacent GFP-expressing FN-null cells (arrowhead). (B) cFN-expressing cells and GFP-expressing FN-null cells mixed at 1:20 in DMEM containing 0.2% BSA and 9 µg/ml Rx-pFN were incubated at 37°C for 18 hours on pFN-coated (FN), on LN-coated (LN), or on VN-coated (VN) coverslips. Deposited Rx-pFN and GFP was visualized as described for previous figures after fixation with 3.7% paraformaldehyde. Bar = 20 µm.

Figure 4. Locally deposited GFP-FN overcomes the negative effect of VN.

GFP-FN(EDa+) or GFP-FN(EDa-) were transiently expressed in FN-null cells. The efficiency of transfection was low, approximately ~ 1%, so most of the cells remained FN-null. Cells in DMEM containing 0.2% BSA and 9 µg/ml Rx-pFN were incubated at 37°C for 18 hours on VN-coated cover slips. After fixation with 3.7% paraformaldehyde, EDa-specific monoclonal antibody IST-9 and secondary antibody Alexa Fluor R 350 goat anti-mouse IgG were used to detect GFP-FN(EDa+). Arrow: Rx-pFN that does not colocalize with fibrillar GFP-FN. Arrowhead: Rx-pFN that colocalizes with fibrillar GFP-FN. Bar = 20 µm.

Figure 5. Soluble cFN or EDa+ FN does not overcome the negative effect of VN.

FN-null cells were incubated for 4 hour at 37°C in the medium containing approximately 2 µg/ml of cFN of AH1F cells, EDa+ FN, or EDa- FN on cover slips coated with VN or LN. Anti-FN rabbit polyclonal antibodies and Rhodamine Red™-X conjugated donkey
anti-rabbit antibodies were used to detect deposited FN after fixation with 3.7% paraformaldehyde. Cells were visualized as described for previous figures. Bar = 20 μm.

**Figure 6. Complementation of the defects in FN assembly by FN-null and β1-null cells cultured on VN.**

(A) β1-null GD25 cells and GFP-expressing FN-null cells in DMEM containing 0.2% BSA were incubated at 37°C for 18 hours on VN-coated cover slips. Anti-FN rabbit polyclonal antibodies and Rhodamine Red™-X conjugated donkey anti-rabbit antibodies were used to detect deposited cFN after fixation with 3.7% paraformaldehyde. Boxed areas are presented at higher magnification. (B and C) β1-null GD25 and GFP-expressing FN-null cells in DMEM containing 0.2% BSA and 9 μg/ml Rx-pFN were incubated at 37°C for 18 hours on cover slips coated with VN (B) or III7-10 (C). Deposited Rx-pFN and GFP was visualized after fixation with 3.7% paraformaldehyde as described for previous figures. **Arrow:** diffuse staining of cFN on β1-null GD25 cells. **Arrowhead:** fibrils of cFN or pFN deposited on GFP-expressing FN-null cells. Bar = 20 μm.

**Figure 7. A chimeric β1,β3 integrin subunit with a β3 cytoplasmic tail overcomes the suppressive effect of VN.**

GD10 cells, β1,β3GD10 cells, and β1,αGD10 cells in DMEM containing 0.2% BSA and 9 μg/ml Rx-pFN were incubated at 37°C for 4 hours (A) or for 18 hours (B) on VN, FN or LN-coated cover slips. Deposited Rx-pFN visualized after fixation with 3.7% paraformaldehyde as described for previous figures. Bar = 20 μm.
Figure 1

A

| A       | FN | LN | VN | III_{210} |
|---------|----|----|----|-----------|
| eFN-expressing cells | ![Image](image1) | ![Image](image2) | ![Image](image3) | ![Image](image4) |
| FN-null cells       | ![Image](image5) | ![Image](image6) | ![Image](image7) | ![Image](image8) |

B

- **α5**
  - eFN-expressing cells: ![Image](image9)
  - FN-null cells: ![Image](image10)

- **β1**
  - eFN-expressing cells: ![Image](image11)
  - FN-null cells: ![Image](image12)
Figure 2

A

2 / 0  2 / 3  2 / 6  2 / 9 (µg/ml)

VN+FN

FN+VN

LN+VN

2 / 0  2 / 3  2 / 6  2 / 9

5 / 0  5 / 3  5 / 6  5 / 9

B

10 III_7,10  2 FN + 10 III_5,10  2 VN + 10 III_5,10
Figure 2

C

9 µg/ml (20 nM) FN  200 µg/ml (440 nM) FN  600 µg/ml (1.3 µM) FN  2 mg/ml (4.4 µM) FN

VN

LN

D

Vinculin  Paxillin  FAK  Integrin β1  Integrin β3

eFN-expressing cells

FN-null cells

Downloaded from http://www.jbc.org/ by guest on March 24, 2020
Figure 3

B

|     | Rx-pFN | Merge | Phase |
|-----|--------|-------|-------|
| FN  | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| LN  | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |
| VN  | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) |
Figure 5

| VN | Medium containing EDA+FN | Medium containing EDA-FN | Medium containing eFN of AH1F cells |
|----|--------------------------|--------------------------|-----------------------------------|
|    |                          |                          |                                   |

| LN | Medium containing EDA+FN | Medium containing EDA-FN | Medium containing eFN of AH1F cells |
|----|--------------------------|--------------------------|-----------------------------------|
|    |                          |                          |                                   |
Figure 6
Figure 7

A

VNN

FN

GD1α cells

β1,3GlcNAc

β1,4GlcNAc
Figure 7

B VN FN LN

β-null CD10 cells

β1,3XOD10 cells

β1,6GD10 cells

β1,6GD10 cells
Assembly of exogenous fibronectin by fibronectin-null cells is dependent on the adhesive substrate
Eunyoung Bae, Takao Sakai and Deane F. Mosher

J. Biol. Chem. published online June 14, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406283200

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