The incorporation of fibrinogen into extracellular matrix is dependent on active assembly of a fibronectin matrix

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Summary

Fibrinogen is a soluble protein produced by hepatocytes and secreted into plasma, where it functions in hemostasis. During inflammation, the hepatic synthesis of fibrinogen is induced 2-10 fold. Recent studies demonstrate that after an inflammatory stimulus, *fibrinogen* gene expression and protein production is upregulated in lung epithelial cells, where it is secreted basolaterally and consequently deposited into the extracellular matrix in fibrils that extensively colocalize with fibronectin fibrils. In this study, we show that the deposition of fibrinogen into the matrix of fibroblasts occurred rapidly and in a Rho-dependent manner in response to serum or lysophosphatidic acid; RhoA GTPase signaling is also required for fibronectin matrix assembly. Using mouse embryonic fibronectin-null cells, we show that incorporation of exogenous fibrinogen into matrix fibrils occurred only in the presence of exogenous fibronectin, which is also assembled into matrix fibrils. Furthermore, treatment of fibroblasts and fibronectin-null cells with an antibody that inhibits fibronectin matrix assembly impaired incorporation of fibrinogen into matrix fibrils. Collectively, these data suggest that incorporation of fibrinogen into the extracellular matrix requires active fibronectin polymer elongation into matrix fibrils. From these data, we hypothesize that fibrinogen deposition rapidly changes the topology of the extracellular matrix to provide a surface for cell migration and matrix remodeling during tissue repair.

Key words: Fibrinogen, Fibronectin, Heparan sulfate proteoglycans, Extracellular matrix, Lysophasphatidic acid, RhoA GTPase, Wound repair

Introduction

Fibrinogen (FBG) is best known for its role as a bridging molecule for platelet aggregation and as the insoluble fibrin clot at the site of vessel injury in hemostasis. FBG is converted to fibrin through the proteolytic action of thrombin and release of fibrinopeptides A and B from the N-terminus of the Aα and Bβ chains, respectively. Soluble fibrin monomers polymerize and form the insoluble fibrin clot by factor $XIII_a$-mediated covalent crosslinking. In addition to its role in hemostasis, FBG plays an important role in homeostasis as it is one of several hepatic proteins whose plasma levels are upregulated during a systemic inflammatory response. In the acute phase response to inflammation, hepatic synthesis of FBG is upregulated 2-10 fold through the action of interleukin-6 and glucocorticoids (Heinrich et al., 1990; Huber et al., 1990; Otto et al., 1987). The increased levels of circulating FBG, and the resulting increase in fibrin levels, serve to restore homeostasis by augmenting the innate immune response to infection and tissue injury. FBG has also been implicated as a substratum, along with fibronectin (FN), of the provisional matrix for epithelial cell migration during wound repair (Clark et al., 1982; Donaldson et al., 1989). In addition, fibrin(ogen) and fibrillar FN stimulate cell proliferation (Francis et al., 1993; Sechler and Schwarzbauer, 1998; Sottile et al., 2000; Sottile et al., 1998).

The extracellular matrix (ECM) is composed of two main classes of macromolecules: proteoglycans and adhesive glycoproteins (Teti, 1992). Some common adhesive proteins found in the ECM or basement membrane include FN, collagen and laminin. Although these proteins differ in primary structure, they each display functional motifs that contribute to their adhesive properties for cells and other proteins, as well as to the ability to organize into fibrillar structures (Engel, 1991). The ECM provides structure and elasticity for tissues, compartmentalizes different cell types and serves as a reservoir for growth factors by sequestering and protecting them in the microenvironment (Mosher, 1992). It is clear that the functions of the ECM are not exclusively structural. The ECM is a dynamic environment that elicits distinct cellular phenotypes. ECM constituents interact with specific adhesion receptors on cell surfaces and regulate multiple cell functions, including adhesion, migration, proliferation and differentiation.

FBG is typically considered a soluble plasma protein produced by hepatocytes. However, extrahepatic synthesis of intact FBG occurs in epithelial cell lines from the intestine (Molmenti, 1993), the cervix (Lee et al., 1996), the lung (Simpson-Haidaris, 1997) as well as in lung alveolar epithelial cells (Simpson-Haidaris, 1998). We found that FBG expression is upregulated 5-10 fold in a lung epithelial cell line (A549)

following induction with the proinflammatory mediators of *FBG* gene expression during the acute phase response (Simpson-Haidaris, 1997). In addition, A549 cells synthesize and secrete FBG basolaterally (Guadiz et al., 1997a), which becomes incorporated into detergent-insoluble matrix fibrils, independently of either thrombin or plasmin enzymatic action (Guadiz et al., 1997b). FBG assembled into the ECM is conformationally altered to expose a cryptic epitope on the Bβ chain (Guadiz et al., 1997b). This epitope falls within residues β15-42 that constitutes the neo-N-terminus of the fibrin β chain, as well as the heparin binding domain (HBD), which is exposed after thrombin cleavage (Odrljin, 1996a; Odrljin et al., 1996b). FBG synthesized by lung epithelial cells or plasma FBG exogenously added to fibroblast monolayers is assembled into matrix fibrils that colocalize with FN, heparan sulfate proteoglycans (HSPG), collagen IV, and laminin (Guadiz et al., 1997b). Other matrix glycoproteins such as fibulin-1 and tenascin-C colocalize with FN in the ECM (Chung and Erickson, 1997; Chung et al., 1995; Godyna et al., 1995). Because a FN matrix is required for assembly of fibulin-1 and tenascin-C into the ECM (Chung and Erickson, 1997; Godyna et al., 1995), we hypothesized that the deposition of FBG into the ECM is also dependent on the presence of a FN matrix. In this study, we determine whether FN plays a role in mediating the assembly of FBG into matrix fibrils.

Materials and Methods

Cell culture

Human foreskin fibroblasts (HFF) were cultured in McCoy's 5A medium supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine (Life Technologies, Gaithersburg, MD) and 10% fetal bovine serum (FBS) (Intergen, Purchase, NY). HFF were cultured on glass coverslips (Electron Microscopy Sciences, Fort Washington, PA) precoated with 0.2% porcine gelatin and used in assays 2-3 days post-confluence. FN-null cells were cultured in a 1:1 mixture of Aim V (Life Technologies) and Cellgro (Fisher Scientific, Pittsburgh, PA) defined media (Sottile et al., 1998). FN-null cells were seeded at 6×10^3 cells/cm^2 onto 48 well plates (Costar, Cambridge, MA) or glass coverslips precoated at 4°C with 50 μg/ml type I collagen (Beckton Dickinson, Bedford, MA) or at 37°C with 5 μg/ml vitronectin (Life Technologies); cells were used at confluence.

Protein purification

Human plasma FN was purified as previously described (McKeown-Longo and Etzler, 1987). Human FBG was purchased from CalBiochem (San Diego, CA) and further purified by lysine-Sepharose affinity chromatography to remove contaminating plasminogen, followed by gelatin-Sepharose affinity chromatography in tandem with an anti-human FN-Sepharose affinity column to remove contaminating FN. Removal of detectable FN was confirmed by western blot and enzyme linked immunosorbent assay. FBG was labeled with the fluorophore Oregon-Green™ (Molecular Probes, Eugene, OR) as described (Odrljin et al., 2001); the resulting conjugate was designated FBG-Oregon Green.

Immunofluorescent detection

HFF and FN-null cells were seeded on round glass coverslips, grown to confluence and treated further as described in the figure legends. Rabbit anti-human FN antibody (Sigma, St. Louis, MO), which was affinity purified over FBG-Sepharose to remove contaminating antibodies to FBG, was used at 5-10 μg/ml. Rabbit anti-human FBG antibody (Dako Corp., Carpenteria, CA) was purified to remove contaminating antibodies to serum proteins and FN as previously described (Simpson-Haidaris, 1997) and used at 40 μg/ml. Monoclonal antibody (MoAb) against human FN (Sigma) was used at 70 μg/ml. MoAb against heparan sulfate (mouse IgM) (Seikagaku America, Falmouth, MA) was used at 50 μg/ml. Secondary antibodies were fluorescein- or rhodamine-conjugated goat anti-rabbit antibody (Cappel, Durham, NC) and rhodamine-conjugated goat anti-mouse polyvalent Ig antiserum (Chemicon, Temecula, CA). FBG-Oregon Green was detected by direct epifluorescence. Microscopy was carried out with a Nikon Eclipse E800 phase-contrast microscope equipped with single and dual band filters for epifluorescence. A cooled color digital camera, the Spot II from Diagnostic Instruments (Sterling Heights, MI) and a Hewlett Packard Pentium III computer with color monitor were used to capture images. Analysis was carried out using IP Lab image analysis software (Scanalytics Inc, Fairfax, VA).

Lysophosphatidic acid, phospholipase B and C3 transferase treatments

Lysophosphatidic acid (LPA), phospholipase B (PLB) and *Clostridium botulinum* C3 transferase were purchased from Sigma. HFF were grown to confluence on gelatin coated glass coverslips, and 24 hours prior to the addition of 200 or 500 nM LPA (Checovich and Mosher, 1993; Zhang et al., 1997) the cells were deprived of serum. After 24 hours of serum deprivation, medium containing LPA, but no FBS, or a range of 0.5% to 10% FBS was supplemented with 40 μg/ml FBG-Oregon Green then added to the cells and incubated for an additional 18 hours. Confluent HFF were serum-starved for 24 hours and then treated for an additional 24 hours with 1% serum-containing medium supplemented with 40 μg/ml FBG-Oregon Green in the absence or presence 0.1 U/ml PLB, which specifically hydrolyzes LPA (Checovich and Mosher, 1993; Zhang, 1997). To inhibit Rho-mediated signaling, C3 transferase (2 μg/ml) was incubated with LipofectAMINE (Life Technologies) (10 μg/ml) for 30 minutes at room temperature before application in serum-free medium to 24-hour serum starved HFF and incubated for one hour at 37°C (Wenk et al., 2000; Zhang et al., 1997; Zhong et al., 1998). After this, the HFF were incubated for 4 hours with 30 μg/ml FBG-Oregon Green in medium containing 1% serum to induce Rho activation.

MoAb 9D2 modulation of FBG and FN matrix assembly

Murine anti-human FN MoAb 9D2, which recognizes an epitope on FN's type III-1 module, was employed to inhibit FN-FN self association, a critical step in the assembly of a FN matrix (Chernousov et al., 1991). To determine whether inhibition of FN assembly in HFF-modulated deposition of FBG into the ECM, confluent HFF were treated with 9D2 (30 μg/ml) for 18 hours. This was followed by incubation for an additional 24 hours with 30 μg/ml FBG-Oregon Green in the continued presence of 9D2. To determine whether the inhibitory effect of 9D2 was reversible, after 18 hours, another set of coverslips were washed three times with medium to remove unbound 9D2 and incubated with FBG-Oregon Green for an additional 24 hours. FN-null cells were grown to confluence on vitronectin coated glass coverslips and incubated with FBG (20 μg/ml) and FN (25.8 μg/ml) in the presence of 35 μg/ml of either 9D2 or nonimmune mouse IgG_1 (Sigma) for 24 hours.

Cell binding assays

Iodination of FN and FBG was performed using Iodo-Gen® Reagent according to the manufacturer's protocol (Pierce, Rockford, IL). Specific activities of ^{125}I-FN and ^{125}I-FBG were 0.19 mCi/mg (4.2×10^8 cpm/mg) and 2.2 mCi/mg (48.9×10^8 cpm/mg), respectively. Binding studies were performed as previously described (Chernousov

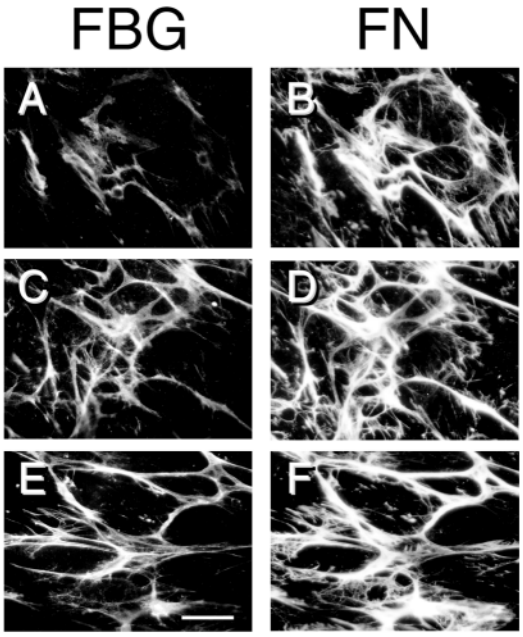

Fig. 1. Time course of FBG assembly into the ECM. Confluent human foreskin fibroblasts were incubated with 30 μg/ml Oregon-Green FBG for 1 (A,B), 6 (C,D) or 24 hours (E,F). Cells were washed, fixed and stained with affinity-purified (to remove contaminating antibodies to FBG) rabbit anti-FN IgG, followed by incubation with rhodamine-conjugated goat anti-rabbit IgG (B,D,F). Panels (A,C,E) represent FBG-Oregon-Green fluorescence. Bar in (E) represents 25 μm.

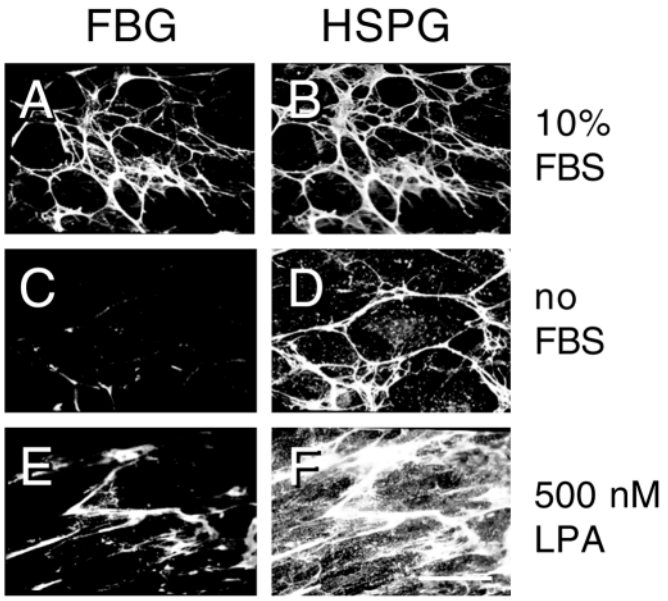

Fig. 2. The effect of serum starvation and LPA on FBG assembly in the ECM of fibroblasts. Confluent monolayers of human foreskin fibroblasts were serum-starved for 24 hours. Medium containing 10% FBS (A,B), no FBS (C,D) or 500 nM LPA (E,F) supplemented with 40 μg/ml FBG-Oregon Green was added to cells and incubated for an additional 18 hours. FBG is shown in (A,C,E), and HSPG staining is shown in (B,D,F). Bar in (F) represents 25 μm.

et al., 1991) on confluent monolayers of FN-null cells grown in 48-well plates. To determine the total amount of labeled ligand associated with the cells, the washed cell monolayers were solubilized in 1N NaOH. Nonspecific binding of ^{125}I-FN was measured in the presence of 500 μg/ml unlabeled FN. Nonspecific binding was subtracted from total binding to obtain specific binding. Scatchard analysis was performed using the software package Equilibrate version 1.2.16 available as freeware from http://equilibrate.homestead.com/files.

Results

Time course of FBG assembly into ECM

To assess the time required for FBG deposition into the matrix, confluent HFF were incubated with 30 μg/ml FBG-Oregon Green for 1, 6 and 24 hours. By 1 hour, FBG was already deposited and assembled into short matrix fibrils (Fig. 1A). Additional studies indicated that FBG fibrils were formed as early as 30 minutes (not shown). With increasing time, FBG matrix fibrils became more complex as denoted by thickness and length (Fig. 1C,E). At each time point, FBG matrix fibrils colocalized with FN matrix fibrils (Fig. 1B,D,F). Taken together, these data demonstrate that FBG is deposited rapidly into fibroblast matrix in a pattern coincident with that of FN. Furthermore, the incorporation of FBG into complex fibrils similar to that of FN was achieved by six hours.

FBG assembly into the ECM requires LPA

LPA, at least in part, regulates assembly of FN into ECM fibrils (Checovich and Mosher, 1993; Zhang et al., 1997). Therefore, we sought to determine whether LPA signaling was required to support FBG deposition into the ECM. Confluent monolayers of HFF were serum-starved for 24 hours then medium containing either 10% FBS (Fig. 2A,B), no FBS (Fig. 2C,D) or 500 nM LPA (Fig. 2E,F), each supplemented with 40 μg/ml FBG-Oregon-Green™, was added to cells and incubated for an additional 18 hours. The results show that in serum starved cells, very little deposition of FBG into ECM occurred (Fig. 2C) compared to the 10% serum condition (Fig. 2A). The FBG that was deposited in the matrix of starved cells appeared in short fibrils or in patches on cell surfaces (Fig. 2C). In the presence of 500 nM LPA in place of FBS, fibrillar FBG assembly was partially restored (Fig. 2E).

Because HSPG play a role in the assembly of FN in the ECM (Bultmann, 1998; Chung and Erickson, 1997; Hocking et al., 1999; Klass et al., 2000; Sottile et al., 2000), we analyzed the colocalization of FBG with HSPG in the ECM in the presence or absence of serum. The results show that FBG colocalized with HSPG matrix fibrils in the presence of 10% FBS (Fig. 2A compared with 2B). Although serum starvation significantly reduced assembly of FBG into matrix fibrils, significant levels of HSPG (Fig. 2C compared with 2D) that were pre-established into matrix fibrils over seven days of cell growth remained after serum deprivation. LPA treatment of serum starved HFF resulted in restoration of FBG assembly in thick matrix fibrils that colocalize with HSPG fibrils (Fig. 2E compared with 2F). Moreover, the data indicate that the presence of HSPG in the pre-established ECM was not sufficient to support assembly of FBG into matrix fibrils in the absence of serum stimulation.

PLB specifically hydrolyzes LPA, the component in serum that induces incorporation of FN into the ECM (Checovich and Mosher, 1993; Zhang et al., 1997). To confirm that LPA is one

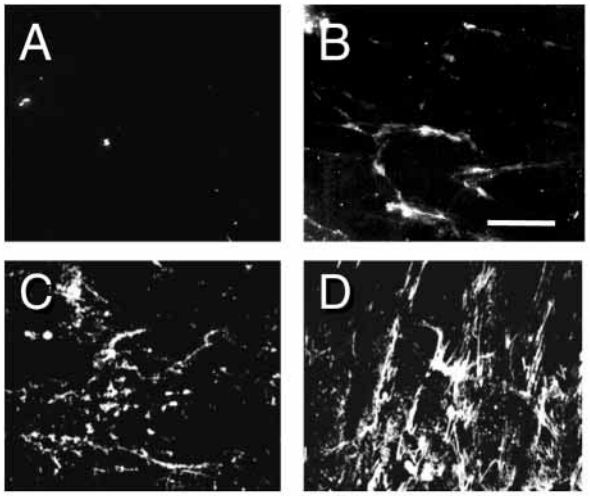

Fig. 3. FBG assembly into mature matrix fibrils is Rho dependent. Human foreskin fibroblasts were serum starved for 24 hours prior to treatment with 1% FBS-medium with (A) or without (B) PLB (0.1 U/ml) in the presence of FBG-Oregon Green (40 μg/ml). To inhibit RhoA activation, C3 transferase (2 μg/ml) was delivered to serum-starved HFF via LipofectAMINE (10 μg/ml) (C); control cells were treated only with LipofectAMINE (10 μg/ml) (D). FBG-Oregon Green (30 μg/ml) in 1% FBS-medium was added to cells and incubated for an additional 4 hours. Bar in (B) represents 25 μm.

of the major serum components responsible for signaling to HFF to assemble FBG in their matrix, confluent HFF were serum starved then treated with 1% FBS containing medium with or without 0.1 U/ml PLB during the subsequent 24 hour incubation with FBG-Oregon Green. FBG-Oregon Green in the presence of 1% serum assembled into fibrils in the ECM (Fig. 3B); however, in the presence of PBL-treated 1% serum containing medium, little or no incorporation of FBG-Oregon Green into matrix fibrils was observed (Fig. 3A). These data indicate that LPA is a major serum component responsible for outside-in signaling during FBG assembly into a fibrillar ECM.

Inhibition of Rho activation by C3 transferase reduced FBG assembly into matrix fibrils

LPA has dramatic effects on actin polymerization, stress fiber formation and focal adhesion assembly (Hall et al., 1993; Nobes and Hall, 1995). Activation of the small GTPases, Rho and Rac, by LPA plays a role in mediating these cellular events. C3 transferase, a bacterial toxin from *Clostridium botulinum*, is used to inhibit Rho-mediated signal transduction; C3 transferase treatment reduces FN binding to cell surfaces and subsequent assembly into a fibrillar matrix by inhibiting Rho-mediated cell contractility (Zhang et al., 1997; Zhong et al., 1998). Therefore, 24-hour serum starved cells were treated with C3 transferase for 1 hour to inhibit Rho activation to determine whether such treatment prevents or reduces the subsequent assembly of FBG into mature matrix fibrils in the presence of serum containing medium. The results show that treatment of HFF with C3 transferase (Fig. 3C) for 1 hour partially inhibited both the amount of FBG deposited and the extent of FBG fibril elongation achieved in the presence of 1% serum containing medium (Fig. 3D). In the presence of C3 transferase (Fig. 3C), the FBG appeared in patches, which suggests cell surface binding, and in short stitch-like fibers instead of the thicker and longer fibrils associated with mature matrix. A quantitative analysis of the relative fluorescence of matrix fibrils from three experiments revealed that 1 hour of C3 transferase treatment reduced the amount of FBG deposited from 1% serum-containing medium into the matrix by an average of 38.4±14%. Taken together, these data suggest that LPA is the major serum component that induces Rho-dependent signaling to permit assembly of FBG into the ECM.

Active FN matrix assembly is required for FBG incorporation into matrix

To determine whether active FN assembly plays a role in FBG assembly into the ECM, we examined whether inhibition of FN matrix assembly would alter FBG incorporation into the ECM. Deposition of FN into the ECM of HFF was inhibited by the anti-human FN MoAb 9D2, which inhibits assembly of FN into the matrix but not the initial binding of FN to the cell surface (Chernousov et al., 1991). Following pretreatment with 30 μg/ml 9D2 for 18 hours, confluent HFF were incubated with 30 μg/ml FBG-Oregon Green in the continued presence of 9D2 for 24 hours. At each time point analyzed in the presence of 9D2, matrix FN was predominantly organized into short, linear arrays (Fig. 4, panels B,D,F) compared with FN fibrils found over the same time course in the absence of 9D2 (Fig. 1B,D,F). These results suggested that extensive fibrillar formation was reduced by MoAb 9D2; the FN fibrils remaining are probably those pre-established in the matrix of HFF prior to 9D2 treatment. In the presence of 9D2, FBG incorporation into the ECM was negligible at all time points (Fig. 4A,C,E) compared to the amount of FBG assembled into matrix fibrils in the absence of 9D2 (Fig. 1A,C,E). The results suggest that 9D2 inhibition of ongoing FN matrix assembly prohibited the deposition of FBG into the matrix of HFF. To test whether removal of 9D2 would allow for recovery of FN and FBG deposition into the matrix, HFF, which were treated as described above, were washed to remove 9D2 from the medium and further incubated in the presence of FBG-Oregon Green for 24 hours. The restoration of both FN and FBG assembly into matrix after removing 9D2 from the medium was observed as changing from short stitch-like fibers at 1 hour (Fig. 4G,H) to thicker, longer and more branched fibrils by 24 hours (Fig. 4K,L). However, the restoration of FBG fibril formation was not as robust 24 hours after washing out 9D2 as at 24 hours in the absence of 9D2 (Fig. 4K compared with Fig. 1E). This is probably due to the residual 9D2 bound to FN at the surface of the cells. Nonetheless, the data suggest that cell-associated contractility mediated by ongoing FN polymerization (Hocking, 2000) is only transiently inhibited by the presence of 9D2.

To further test whether there is an absolute dependence on active FN matrix assembly for FBG incorporation into the ECM, the following experiments were performed using mouse embryonic FN-null cells. These cells synthesize and deposit HSPG into the ECM, which is required for assembly of a FN matrix (Sottile et al., 2000) but do not synthesize or secrete endogenous FN. Nonetheless, they assemble exogenously added human FN into mature matrix fibrils (Sottile et al., 1998). In the presence of added FN (Fig. 5A), FBG was

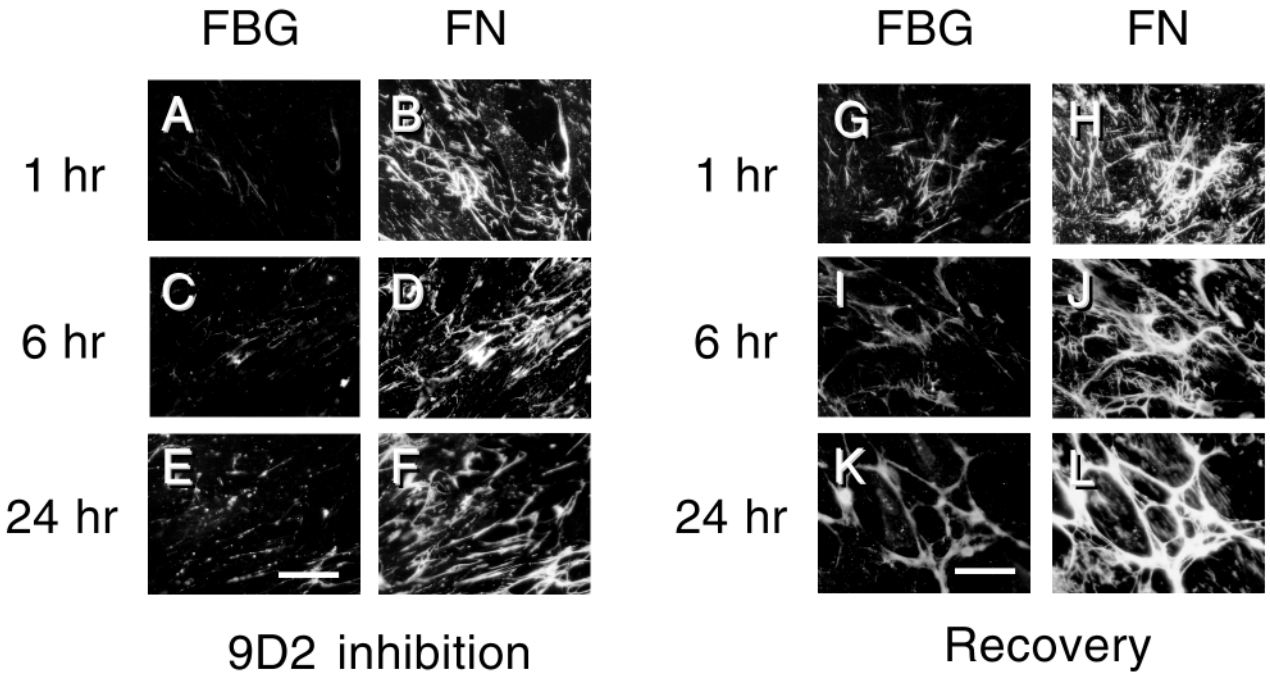

Fig. 4. Effect of 9D2 on FBG matrix deposition and recovery. Confluent human foreskin fibroblasts were treated with 30 μg/ml 9D2 for 18 hours. This was followed by incubation with 30 μg/ml FBG-Oregon-Green in the continued presence (A-F) or absence of 9D2 (G-L). MoAb 9D2 was removed (G-L) by washing the cells after the 18 hours treatment. Cells were washed, fixed and stained for FN (B,D,F,H,J,L). Bars in (E) and (K) represent 25 μm.

incorporated into matrix fibrils (Fig. 5B). However, in the absence of FN (Fig. 5D), FBG was not incorporated into the ECM of the FN-null cells (Fig. 5E). Phase contrast images (Fig. 5C,F) demonstrate that the FN-null cells remained as a confluent monolayer during the assembly of FN and FBG into the ECM. These data provide evidence that assembly of a FN matrix plays a critical role in the incorporation of FBG into matrix fibrils.

Kinetics of FN binding to FN-null cells

To determine whether human FN bound to mouse embryonic FN-null cells with the same affinity as human FN for human fibroblasts, we characterized the binding of ^{125}I-FN to the null cells. FN-null cells were incubated with increasing amounts of ^{125}I-FN with or without an excess of unlabeled FN for 1 hour at 37°C as described previously (Chernousov et al., 1991). The results indicate that specific binding of FN to the cell surface of FN-null cells was both dose dependent and saturable, implying a receptor-mediated binding event (Fig. 6). Scatchard analysis of the data indicates that human FN protomers bind to the mouse embryonic FN-null cells with an average K_d of 98 nM; human FN was shown previously to bind to human fibroblasts with an average K_d of 62 nM (McKeown-Longo and Mosher, 1989).

9D2 inhibition of FBG binding to FN-null cells

Previous studies show that MoAb 9D2 does not interfere with the initial binding of FN to the cell surface matrix assembly sites, instead 9D2 inhibits FN elaboration into a fibrillar matrix by blocking FN-FN homotypic binding interactions

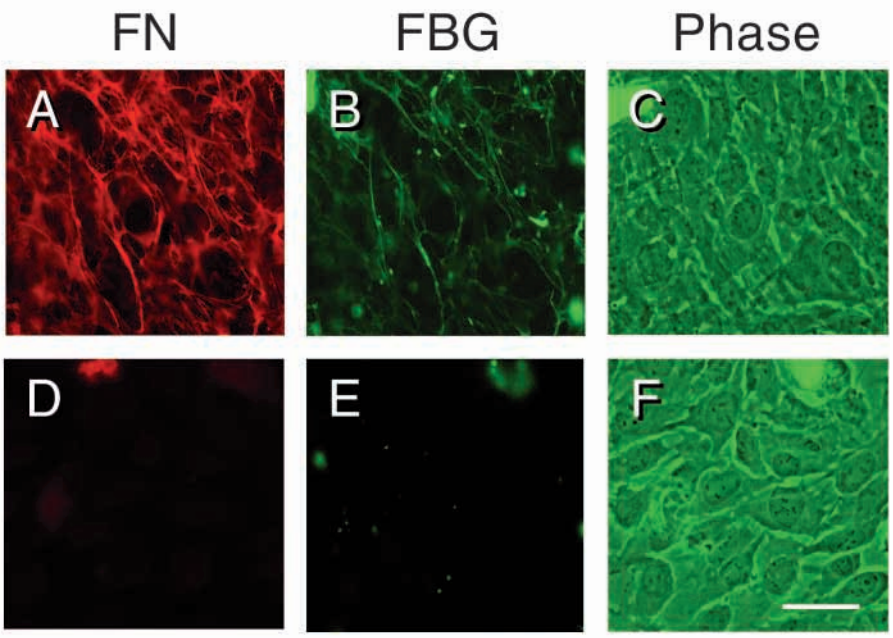

Fig. 5. FN and FBG incorporation into FN$^{-/-}$ cell matrix. Confluent FN$^{-/-}$ cells were incubated with 20 μg/ml FBG-Oregon Green in the presence (A-C) or absence (D-F) of 10 μg/ml FN. FN (A,D), FBG (B,E) and phase contrast (C,F) images are shown. Bar in (F) represents 25 μm.

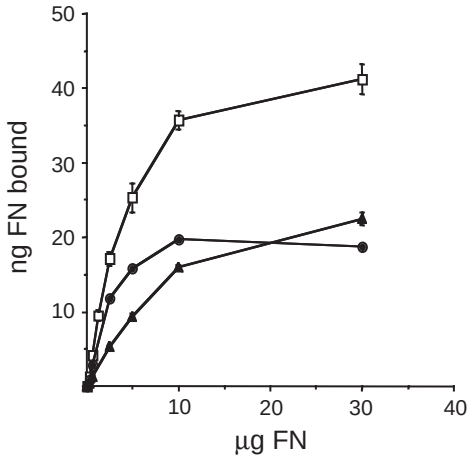

Fig. 6. Binding of ^{125}I-FN to FN$^{-/-}$ cell layers. Confluent FN$^{-/-}$ cell layers grown in 48 well plates were incubated for 1 hour at 37°C with 0.2 ml defined medium containing 0.2% BSA and increasing concentrations of ^{125}I-FN. Nonspecific binding (closed triangles) was determined by adding excess unlabeled FN (500 μg/ml) to the incubation medium. Specific binding (closed circles) was assessed by subtracting nonspecific binding from total binding (open squares). Each point represents the average of triplicate determinations from a representative experiment. Bars represent±s.e.m.

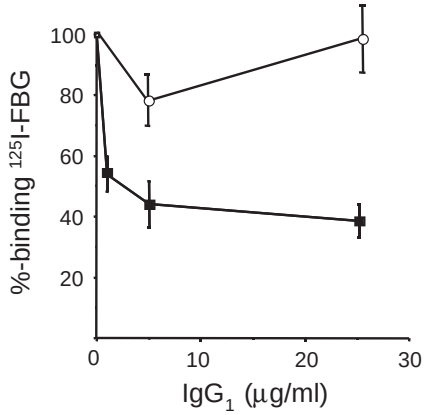

Fig. 7. Effect of MoAb 9D2 on ^{125}I-FBG binding to FN$^{-/-}$ cell layers. Confluent FN$^{-/-}$ cell layers grown in 48-well plates were incubated for 1 hour at 37°C with 0.2 ml defined medium containing 0.2% BSA, FN (25.8 μg/ml) and ^{125}I-FBG (20 μg/ml) in the presence of increasing concentrations of MoAb 9D2 (closed squares) or nonimmune IgG$_1$ (open circles). After 1 hour of binding, cell monolayers were washed, solubilized in 1N NaOH and radioactivity measured. Binding of ^{125}I-FBG is expressed as percentage of binding observed in the absence of 9D2. These data are representative of two experiments each done in triplicate at each IgG$_1$ concentration. Bars represent±s.e.m.

(Chernousov et al., 1991). To analyze whether FBG assembly into the ECM was dependent on the polymerization of FN, we utilized MoAb 9D2 to halt FN-FN homotypic self association. FN-null cells were treated with increasing concentrations of 9D2 in the presence of constant amounts of ^{125}I-FBG and nonlabeled FN. Exogenous FN was added to the cells to allow FN binding to the cell surface matrix assembly sites. Control experiments demonstrated that, in the presence of nonlabeled FBG, increasing concentrations of 9D2 did not significantly alter the binding of iodinated FN to the FN-null cells (not shown). In contrast, MoAb 9D2 inhibited, either directly or indirectly, the association of ^{125}I-FBG to FN-null cells by 60% (Fig. 7). Nonimmune mouse IgG$_1$ had no significant effect on the binding of ^{125}I-FBG to the FN-null cells (Fig. 7). These data indicate that FBG assembly into the ECM was dependent on FN-FN self-association for FN fibril elaboration, a step that is also dependent on Rho-mediated cell contractility (Zhang et al., 1997; Zhong et al., 1998).

To visualize the inhibitory effects of MoAb 9D2 on FN and FBG fibril elongation, FN-null cells were treated with and without 9D2 or nonimmune IgG$_1$ in the presence of exogenous FN and FBG for 24 hours. MoAb 9D2 effectively inhibited matrix fibril formation of FBG (Fig. 8D) and FN (Fig. 8E), as compared with cells that received no treatment (Fig. 8A,B) and those treated with nonimmune IgG$_1$ (Fig. 8G,H). In the presence of 9D2, both FBG and FN bound to the cell surfaces and between cells (Fig. 8D-F). The effects of 9D2 on fibril formation are shown most dramatically by the dual fluorescence images (Fig. 8C,F,I). In the control condition and with cells treated with nonimmune IgG1, significant colocalization of FBG and FN fibrils is demonstrated by the yellow-orange fluorescence (Fig. 8C,I, respectively). However, 9D2 treatment reduced FN and FBG 'cofibril' formation as denoted by the dramatic reduction of yellow-orange fluorescence; only a few short stitch-like fibrils of both FN and FBG are observed in this representative field (Fig. 8F). Furthermore, residual FBG staining is revealed in green fluorescence, whereas little to no red fluorescence, which is indicative of only FN staining, was observed. These data suggest that FBG binds to cell surface and cell-cell contact sites other than cell surface FN matrix assembly sites (Fig. 8F). Together, these data support the hypothesis that assembly of FBG into complex matrix fibrils is dependent on the active assembly of a fibrillar FN matrix and suggest further that FBG binds to cell surface sites distinct from those bound by FN.

Discussion

The assembly of FN into the ECM is a multi-step, cell-mediated process (Magnusson and Mosher, 1998; Mosher et al., 1992; Schwarzbauer and Sechler, 1999) involving various FN domains that bind to cells, to other FN molecules, and to constituents of the ECM (Mosher et al., 1992; Sechler et al., 1998; Wu, 1997). FN binding to the α5β1 integrin receptor via the RGD cell-binding domain located in the type III$_{10}$ repeat and the synergy site in the III$_9$ repeat leads to conformational changes in FN that are important for fibril formation. The N-terminal 70-kDa fragment of FN binds to cell surface receptors termed matrix assembly sites (McKeown-Longo and Mosher, 1985; Quade and McDonald, 1988). This binding event is thought to expose a cryptic site in the type III$_1$ domain that is involved in FN-FN self association (Erickson, 1994; Hocking et al., 1996; Hocking et al., 1994; Morla et al., 1994; Sechler et al., 1996). Over time, these homophilic binding interactions among or between FN molecules then convert detergent soluble, cell-associated FN into detergent-insoluble disulfide-stabilized matrix fibrils (McKeown-Longo and Mosher, 1984).

In this study, we present evidence that FBG assembly into the ECM of adherent cells requires the active assembly of a FN matrix. We pursued this line of investigation because our

earlier data indicated that the mechanisms of FBG assembly into the ECM showed striking similarities to that of FN. Extraction of confluent monolayers of cells with 1% deoxycholate will remove cells and soluble matrix proteins although it leaves behind the complex detergent-insoluble matrix containing FN and HSPG (McKeown-Longo and Mosher, 1983). We determined that this cell-free residual matrix produced by HFF was not sufficient to support the deposition of FBG-Oregon Green into matrix fibrils (unpublished data). Newly synthesized and secreted FBG binds to the alveolar epithelial cell surface in a saturable manner, suggesting a receptor-mediated binding event. This newly synthesized FBG remains cell and/or matrix-associated in a trypsin-sensitive fraction, which, over time, becomes incorporated into a deoxycholate-insoluble matrix fraction (Guadiz et al., 1997b) similar to FN (McKeown-Longo and Mosher, 1983). We have shown that, like FN (Chernousov et al., 1985; McDonald et al., 1987), FBG assembly into the ECM requires metabolically active cells but not new synthesis of a cell surface receptor or matrix constituent (Pereira and Simpson-Haidaris, 2001). Other similarities between FN and FBG incorporation into matrix were found. FBG secretion from alveolar epithelial cells (Guadiz et al., 1997a) and FN secretion from endothelial cells (Kowalczyk et al., 1990) is polarized to the basolateral face of the cells, directing these glycoproteins to the ECM. In addition, both cellular and plasma FN are known to incorporate into ECM of heterologous cell types (Peters et al., 1990). Similarly, lung-cell-derived FBG and purified plasma FBG incorporate into the ECM of fibroblasts and lung epithelial cells. The deposition of FBG into the ECM results in exposure of the β15-21 fibrin-specific epitope independently of thrombin or plasmin cleavage (Guadiz et al., 1997b). FN also undergoes conformational changes during assembly into the ECM exposing cryptic sites that are important for fibril elongation (Erickson, 1994; Hocking et al., 1996; Hocking et al., 1994; Morla et al., 1994; Sechler et al., 1996). Finally, FBG and FN are extensively colocalized in the ECM of HFF and lung epithelial cells (Guadiz et al., 1997b), suggesting a heterotypic association between FN and FBG in matrix fibrils. Collectively, these data led us to hypothesize that FN plays an essential role in FBG incorporation into the ECM.

Cultured cells rapidly assemble focal adhesions in response to serum components, such as LPA, which activate the Rho-family of small GTPases (Hall et al., 1993; Nobes and Hall, 1995) through a G-protein-coupled cell surface receptor (Ridley and Hall, 1992). In this report, we show that FBG assembly into the ECM of HFF is modulated by Rho-dependent signaling, a requirement for assembly of FN into the ECM as well (Zhong, 1998). Furthermore, the inhibition of Rho-mediated signaling with C3 transferase from *C. botulinum* inhibited the assembly of FBG into the ECM of fibroblasts, but left the pre-established matrix components largely intact. During wound healing, matrix deposition and remodeling create tensile forces that modulate integrin-mediated cell function (Bultmann, 1998; Hocking et al., 2000). Indeed, FN polymerization stimulates cell spreading and triggers a significant increase in cytoskeletal contractility in a Rho-dependent manner (Hocking et al., 2000). Taken together, these data indicate that the cell-dependent processes necessary for promoting assembly of FBG into the ECM probably involve the concerted action of FN polymerization and regulation of the actin cytoskeleton through Rho-dependent pathways.

The FN-specific MoAb 9D2 inhibits FN polymer elongation into matrix fibrils without inhibiting the initial binding of FN to the cell surface (Chernousov et al., 1991). To determine whether MoAb 9D2 inhibition of FN assembly would also inhibit FBG assembly into the ECM, we treated HFF with 9D2. Over time, 9D2 inhibited the formation of long and thick fibrils of FN, which was accompanied by a striking change in the pattern and decrease in the amount of FBG assembled into the ECM. Inhibition of both FN and FBG assembly into the matrix of HFF was reversible, as removal of 9D2 allowed recovery of the cells' ability to assemble a mature matrix composed of extensive fibrils of both FN and FBG. Because HFF produce and secrete endogenous FN, experiments were conducted using FN-null cells to clarify the role of FN in mediating the incorporation of FBG into the ECM. In the complete absence of FN, exogenously added FBG was unable to assemble into the ECM of FN-null cells. However, when FBG was added concomitantly with FN, extensive FBG matrix fibrils, colocalizing with those of FN, were present in the ECM of FN-null cells. Similar to HFF, MoAb 9D2 significantly inhibited assembly of FN and FBG into matrix fibrils in the ECM of FN-null cells. Human FN bound to the mouse embryonic FN-null cells with comparable affinity to that of human FN binding to HFF (McKeown-Longo and Mosher, 1983). Furthermore, FN binding to the FN-null cell monolayers was concentration-dependent and saturable, indicating the involvement of specific cell surface receptors. We conclude from these data that the incorporation of FBG into the ECM is dependent on the active assembly of a FN matrix.

Although understanding the functional significance of FBG as a matrix protein remains a focus of our continued investigations, we do not know the mechanism of FBG interactions with FN that are essential for assembly of FBG into complex matrix fibrils. There are two fibrin-binding sites on each FN subunit that may play a role in the assembly of the conformationally altered FBG in matrix (McKeown-Longo and Mosher, 1989). Previously, we found that the MoAb T2G1, which has always been considered a fibrin-specific antibody (Kudryk et al., 1984), reacts with intact FBG assembled into the ECM of HFF (Guadiz et al., 1997b). FBG recovered from HFF ECM showed no evidence of thrombin or plasmin enzymatic action, confirming that FBG, not fibrin, is assembled into the matrix. Therefore, upon incorporation into the matrix, FBG undergoes conformational changes exposing a cryptic epitope on the Bβ chain normally only exposed after FBG is proteolytically converted to fibrin. Importantly, thrombin-generated exposure of the β15-42 region promotes cell spreading and enhances cell proliferation on a fibrin matrix (Francis et al., 1993). In addition, the β15-42 HBD of fibrin monomers binds to the surface of endothelial cells in a heparin-dependent manner (Odrljin et al., 1996a). We hypothesize that exposure of the HBD in matrix-associated FBG may be important for modulating cellular responses to matrix FBG.

The data obtained from both HFF and FN-null cells suggest that FBG assembly into the ECM is dependent on active FN polymerization and not on the presence of a pre-established matrix or the initial binding of FN to the cell surface. The results support the hypothesis that FBG assembly into matrix fibrils requires FN-FBG heterotypic-association. FBG deposition into

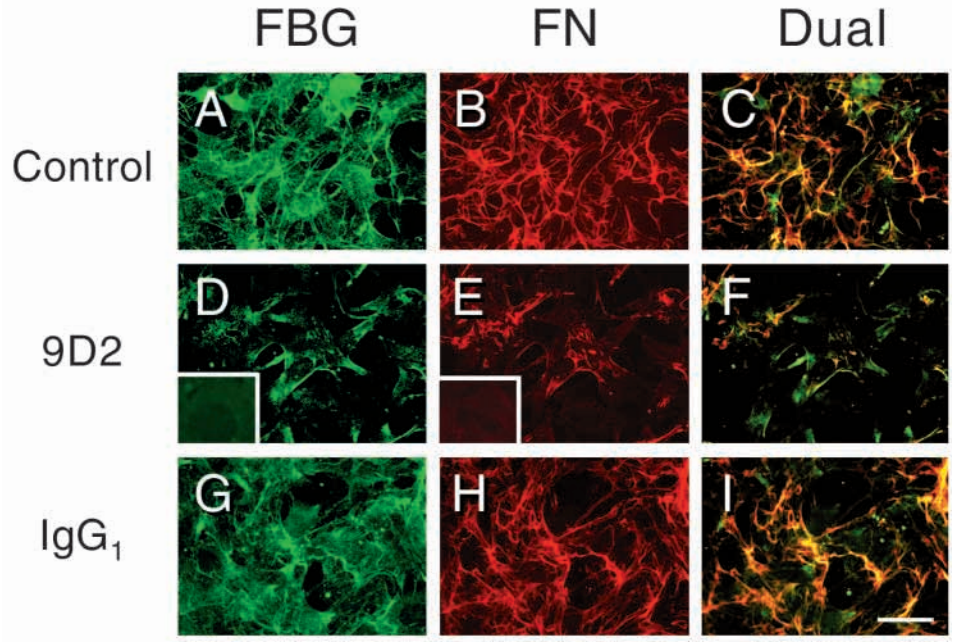

Fig. 8. Effect of 9D2 on FBG and FN incorporation into ECM. Confluent $FN^{-/-}$ cells were treated with FN (25.8 μg/ml) and FBG (20 μg/ml) in the absence (A-C) or presence (D-F) of 35 μg/ml MoAb 9D2 or nonimmune mouse IgG_1 (G-I). Cells were washed, fixed and stained for FBG in green fluorescence (A,D,G) and FN in red fluorescence (B,E,H). Insets represent background staining for green (D) and red (E) fluorescence. Composite images shown in (C,F,I) indicate colocalization of FBG and FN in yellow-orange fluorescence. Bar in panel I represents 25 μm.

the ECM occurs in the absence of covalent crosslinking to itself or other matrix constituents. Furthermore, non-reducing SDS-PAGE analysis of ^{125}I-FBG recovered from ECM of HFF and A549 cells indicates that this FBG is not multimerized by new disulfide bond formation to itself or any other matrix molecules. Soluble FBG does not self polymerize; however, thrombin cleavage of FBG results in conformational changes in the fibrin monomer that lead to lateral self association and fibrin polymers that are stabilized by factor $XIII_a$-mediated covalent crosslinking. Thrombin cleavage of FBG also enhances exposure of the HBD, which comprises residues β15-42 (Guadiz, 1997b). Because exposure of β15-42 by thrombin cleavage is accompanied by fibrin monomer self association, we cannot rule out the possibility that exposure of β15-42 during deposition of FBG into the ECM promotes FBG-FBG homotypic interactions.

The role of fibrin in both hemostasis and homeostasis is well documented. Following vascular injury, FBG plays a role in controlling blood loss by promoting platelet aggregation as well as forming an insoluble fibrin clot. Although fibrin is the predominant protein, other adhesive glycoproteins such as FN are constituents of the provisional clot matrix (Clark et al., 1982). The crosslinking of FN to the fibrin clot enhances the stability of the clot, and both fibrin and FN act in concert to promote cell migration into the clot and modulate gene expression of cells within the clot (Knox et al., 1986). Altering the composition of a fibrin clot with FN promotes matrix composition-specific modulation of cellular responses (Corbett et al., 1996). We hypothesize that FBG deposition rapidly changes the topology of the ECM to provide a surface/conduit for cell migration during tissue repair. Furthermore, the exposure of new epitopes on matrix FBG probably signals to cells to alter their morphology in response to changes in the microenvironment while potentially masking epitopes on matrix FN or other matrix components to modulate signal-transduction pathways and ultimately target gene expression.

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