Cell Adhesion to Fibrillin-1 Molecules and Microfibrils Is Mediated by \( \alpha_5\beta_1 \) and \( \alpha_v\beta_3 \) Integrins*

Received for publication, March 27, 2003, and in revised form, June 3, 2003
Published, JBC Papers in Press, June 13, 2003, DOI 10.1074/jbc.M303159200

Daniel. V. Bax‡§§, Sarah E. Bernard§§, Amanda Lomas‡‡, Amanda Morgan§§, Jon Humphries§§, C. Adrian Shuttleworth§§, Martin J. Humphries§§, and Cay M. Kiely§§‡‡‡

From the §United Kingdom Centre for Tissue Engineering, the Wellcome Trust Centre for Cell-Matrix Research, the §School of Biological Sciences, and **School of Medicine, 2.205 Stopford Bldg., University of Manchester, Manchester M13 9PT, United Kingdom

Fibrillins are the major glycoprotein components of microfibrils that form a template for tropoelastin during elastic fibrillogenesis. We have examined cell adhesion to assembled purified microfibrils, and its molecular basis. Human dermal fibroblasts exhibited Arg-Gly-Asp and cation-dependent adhesion to microfibrils and recombinant fibrillin-1 protein fragments. Strong integrin \( \alpha_5\beta_1 \) interactions with fibrillin ligands were identified, but integrin \( \alpha_v\beta_3 \) also contributed to cell adhesion. Fluorescence-activated cell sorting analysis confirmed the presence of abundant \( \alpha_5\beta_1 \) and some \( \alpha_v\beta_3 \) receptors on these cells. Adhesion to microfibrils and to Arg-Gly-Asp-containing fibrillin-1 protein fragments induced signaling events that led to cell spreading, altered cytoskeletal organization, and enhanced extracellular fibrillin-1 deposition. Differences in cell shape when plated on fibrillin or fibronectin implied substrate-specific \( \alpha_5\beta_1 \)-mediated cellular responses. An Arg-Gly-Asp-independent cell adhesion sequence was also identified within fibrillin-1. Adhesion and spreading of smooth muscle cells on fibrillin ligands was enhanced by antibody-induced \( \beta_1 \) integrin activation. A375-SM melanoma cells bound Arg-Gly-Asp-containing fibrillin-1 protein fragments mainly through \( \alpha_5\beta_1 \)A, whereas HT1080 cells used mainly \( \alpha_v\beta_3 \). This study has shown that fibrillin microfibrils mediate cell adhesion, that \( \alpha_5\beta_1 \) and \( \alpha_v\beta_3 \) are both important but cell-specific fibrillin-1 receptors, and that cellular interactions with fibrillin-1 influence cell behavior.

Fibrillins are large cysteine-rich glycoproteins and the major constituents of fibrillin-rich microfibrils of the extracellular matrix (ECM)\(^1\). They are multidomain molecules, containing 47 epidermal growth factor (EGF)-like domains and 8-cysteine (TB) modules. Fibrillin-1 and fibrillin-2 are both expressed during fetal development, but fibrillin-1 isoform is by far the most abundant isoform in adult tissues (3, 4). The possibility that fibrillins interact with cell receptors was suggested by electron microscopy of vascular and other tissues showing juxtaposition of extracellular microfibrils and cellular dense plaques (5) and by the discovery that fibrillins contain the Arg-Gly-Asp (RGD) putative cell attachment motif (6). Integrins are \( \alpha\beta \) heterodimeric transmembrane receptors that mediate cell adhesion to the ECM, usually through the characteristic RGD motif, and have widespread essential functions in development, tissue organization, and the immune system (7). Integrin-mediated cell interactions regulate cell adhesion and migration and initiate signaling pathways that lead to reorganization of actin cytoskeleton and cellular proliferative and secretory responses.

Fibrillin-1 contains one RGD sequence in the fourth TB module (6). Fibrillin-2 contains two RGD sequences, one in the same position as the fibrillin-1 RGD sequence in the fourth TB module and the other in the third TB module where it is surrounded by hydrophobic amino acids (8). Fibrillin-3 also has two RGD sequences, one in the 19th cbEGF repeat and a second in the fourth TB module (9). The RGD motifs located in the fourth TB modules are surrounded by polar and charged amino acid residues, suggesting that the motif is solvent-exposed. This RGD tripeptide is located in the middle of a 13- to 20-amino acid sequence that is flanked on both ends by cysteine residues. By analogy with the homologous sixth TB module of fibrillin-1 (10), disulfide bonding of these cysteines in the fourth module would produce a finger-like loop structure with the RGD near the end of the loop and available for cellular interactions. The RGD in the third TB motif in fibrillin-2 does not mediate cell adhesion, probably due to its inaccessibility (11).

A cell adhesion role for the RGD motif in the fourth TB module of fibrillin-1 and fibrillin-2 has been described using recombinant fibrillin-1 peptides (12, 13), or synthetic fibrillin-1 RGD peptides and fibrillin molecules purified from tissues using a reducing denaturing protocol (11). These studies identified the integrin receptor \( \alpha_5\beta_3 \) as the major receptor mediating adhesion to these molecular fibrillin-1 ligands. One group found that purified \( \alpha_5\beta_3 \) integrins, when immobilized to wells, did not bind recombinant fibrillin-1 peptides (12). The \( \beta_3 \) integrin subunit bound a recombinant fibrillin-1 TB4 module expressed in bacteria (13). Fetal bovine chondrocytes were stimulated to bind fibrillin-1 using \( \alpha_5\beta_1 \) after antibody activation (11).

In early studies, we showed that isolated fibrillin-rich microfibrils support attachment of vascular smooth muscle cells (14). Here we have investigated the molecular basis of integrin-

---

* This work was funded by the Medical Research Council, British Heart Foundation, Biotechnology and Biological Sciences Research Council, and Engineering and Physical Sciences Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Both authors contributed equally to this work.

‡‡ To whom correspondence should be addressed. Tel.: 44-161-275-5739; Fax: 44-161-275-5082; E-mail: cay.kiely@man.ac.uk.

§ The abbreviations used are: ECM, extracellular matrix; EGF, epidermal growth factor; TB, 8-cysteine module similar to TGF-β binding module; cbEGF, calcium-binding epidermal growth factor-like domain; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; HDF, human dermal fibroblast; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; MES, 4-morpholineethanesulfonic acid; FACS, fluorescence-activated cell sorting; PBS, phosphate-buffered saline.
mediated adhesion to fibrillin microfibrils and molecules using human dermal fibroblasts, smooth muscle cells, HT1080 fibrosarcoma cells, and A375-SM melanoma cells. We have demonstrated that αβ1 and αβ6 are both important receptors for fibrillin ligands, and that RGD-dependent cell adhesion to fibrillin-1 influences cell shape and migration, focal complex formation, signaling, and ECM deposition. We have also identified a cell adhesion site within fibrillin-1 exons 41–52 that mediates cell adhesion in a non-RGD, non-cation, and non-heparin-sulfate-dependent manner. These findings have provided new insights into the molecular basis of fibrillin interactions with cells and suggest a key role for microfibrils in regulating cell behavior in connective tissues.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Inhibitory rat anti-human α1 integrin subunit mAb 16, inhibitory rat anti-human β1 integrin subunit mAb 13, and rat anti-human fibronectin mAb 333 were gifts from Ken Yamada (NIDCR, National Institutes of Health, Bethesda, MD). The activating mouse anti-human mAb TS2/16 directed against the β3 integrin subunit was a gift of John Werb (University of California, San Francisco, CA, CSIC, Madrid, Spain). The monoclonal αα1 integrin subunit antibody (JA218), and a recombinant 50-kDa fibronectin peptide comprising type III repeats 6–10 were produced as described previously (15, 16). A non-functional mouse anti-human β1 monoclonal antibody (K20) was obtained from Invitrogen (Paisley, Scotland). A mouse anti-human α1 monoclonal antibody (JF11) was obtained from Serotec (Oxford, UK). A monoclonal anti-αβ1 integrin antibody (LM609) was obtained from Chemicon International (Temecula, CA). A monoclonal antibody to human serum fibronectin (15T4) was obtained from Sigma Chemical Co. (Dorset, UK). A polyclonal antibody PF2 to a peptide fragment of fibrillin-1 was supplied by Dr. R. Glanville (Portland, OR). Primary human dermal fibroblasts (HDFs) and human corneal fibroblasts were obtained from BioWhittaker (Berkshire, UK). Low passage cells (p4–10) were used in these studies. HT1080 fibrosarcoma cells and A375-SM melanoma cells, which have both αβ1 and αβ6 receptors but predominantly use αβ1 or αβ6, respectively (17, 18), were also used in cell adhesion assays.

**Recombinant Fibrillin-1 Protein Fragments**—Recombinant human fibrillin-1 protein fragments PF9 (amino acids 1528–1807), PF10 (amino acids 1689–2126), and PF11 (amino acids 1528–2126) (6) were expressed and purified using the mammalian expression system pCEPpu/AC7 (obtained from Dr. R. Timpl, Munich, Germany) and 293-EBNA cells (Fig. 1A). The pCEP-pu/AC7 vector had been modified by incorporation of N-terminal His6 following the signal peptide, which facilitated rapid peptide purification by nickel chromatography. Site-directed mutagenesis was also used to mutate the Arg-Gly-Asp (RGD) sequence in PF9 to RDG. Following sequencing, this mutant (designated RDG PF9) was expressed and purified. The protein fragments were all N-glycosylated, had the correct molecular mass as judged by SDS-PAGE in the presence or absence of 10 mM dithiothreitol, size fractionation on Superdex 200 10×100 cm columns, and laser light scattering, and they bound calcium, because electrophoretic shifts were apparent following EDTA treatment, as previously shown (19). Fibrillin-1 protein fragments, in a similar system, were correctly folded, as confirmed by circular dichroism. Fibrillin-1 was isolated in the void volume of a Sepharose CL-2B column or a Sephadex G-25 column and then purified using a CaCl2 density gradient protocol. Fractions were extensively dialyzed into buffer (0.15 M NaCl, 0.05 mM Tris-HCl, pH 7.4, 2 mM CaCl2) at 4 °C for 18 h. Western blot analysis of CaCl2 fractions, prepared as previously described (24), showed that fibrillin-1 was present in the CaCl2 density gradient fractions 9 and 10 (Fig. 1C). Microfibril purity of these fractions was, in all preparations, confirmed by electron microscopy (Fig. 1D) or by tapping mode atomic force microscopy in air using a Multimode AFM with a Nanoscope IIIa controller at a resonant frequency of 250–300 kHz. Each fibrillin-1 protein fragment, following EDTA treatment, as previously shown (19). Fibrillin-1 protein fragments, in a similar system, were correctly folded, as confirmed by circular dichroism. Fibrillin-1 was isolated in the void volume of a Sepharose CL-2B column or a Sephadex G-25 column and then purified using a CaCl2 density gradient protocol. Fractions were extensively dialyzed into buffer (0.15 M NaCl, 0.05 mM Tris-HCl, pH 7.4, 2 mM CaCl2) at 4 °C for 18 h. Western blot analysis of CaCl2 fractions, prepared as previously described (24), showed that fibrillin-1 was present in the CaCl2 density gradient fractions 9 and 10 (Fig. 1C). Microfibril purity of these fractions was, in all preparations, confirmed by electron microscopy (Fig. 1D) or by tapping mode atomic force microscopy in air using a Multimode AFM with a Nanoscope IIIa controller at a resonant frequency of 250–300 kHz.

**Cell Attachment and Spreading Assays**—Standard cell attachment assays and cell attachment inhibition assays were performed using microtitre plates (Costar Corp.) according to well defined methodologies (25). Purified microfibrils, recombinant fibrillin-1 protein fragments, human fibronectin, and the fibrillin-1 protein fragments were diluted in column buffer (0.1 M NaCl, 0.01 mM Tris-HCl, pH 7.4, 2 mM CaCl2) adsorbed to wells of 96 well microtitre plates, then incubated at room temperature for 1 h. The buffer and unbonded ligand were aspirated from wells, and nonspecific binding was blocked by the addition of 200 μl/well of 10 mg/ml heat-denatured BSA diluted in 130 μl NaCl, 3 μl Tris-HCl pH 7.4, at room temperature for 1 h. Cell suspensions were prepared by washing the cell layers with trypsin-EDTA and incubating with 1 ml of trypsin-EDTA/10-cm2 flask area at 37 °C for no more than 4 min. Resulting cell suspensions were neutralized with 5 volumes of DMEM containing 10% fetal calf serum, 2 mM glutamine, then centrifuged at 800 × g for 5 min. Cell pellets were resuspended in 5 ml of warm DMEM/HEPES gassed with a 5% CO2–95% air mixture (v/v), and cell numbers were determined using a haemocytometer. Cell suspensions were centrifuged at 800 × g for 3 min, and the cell pellets were then resuspended in an appropriate volume of warm DMEM/HEPES (contains Mg2+, Ca2+, and Mn2+) gassed with 10–5% (v/v) CO2 to give a final cell density of 5 × 105 cells/ml. The cell suspensions were then incubated for 20 min at 37 °C with the lid off in a 5% CO2 incubator. The BSA-blocking solution was aspirated from the ligand-coated wells, which were then washed with 200 μl of column buffer. To determine cell attachment to adhesion ligands, 100 μl of cells (mixed prior to use by gentle pipetting) was added to the appropriate wells. Alternatively, to examine the effects of an exogenous agent on attachment, 50 μl of 2% exogenous agent (antibody, peptide, or heparin) was added to mixed cell suspensions, which were allowed to attach when examined the effect(s) of exogenous agents, 50 μl of column buffer followed by 50 μl of 2% cells were added to control wells.

To estimate 100% attachment, cells were initially diluted to 5 × 104 cells/ml then further diluted to 20, 50, and 100% of the working cell suspension using warm DMEM/HEPES gassed with 5–10% (v/v) CO2, and 100 μl of cells was added to uncoated, unblocked wells. The microtitre plates were incubated for 20 min at 37 °C in a 5% CO2 incubator with the lid removed. Cells in the wells to be used for determining 100, 50, and 20% cell attachment were fixed by adding 10 μl of 50% (v/v) glutaraldehyde. Non-adherent and loosely attached cells were removed from the other wells by gentle tapping of the plate and aspirating the wells. Attached cells were fixed by adding 10 μl of 4% (v/v) glutaraldehyde per well. The microtitre plates were incubated at room temperature for 30 min then washed three times with 200 μl of DMEM. Cells were stained by adding 100 μl of 0.1% (w/v) crystal violet in 0.2 M MES, pH 5.0 to each well and incubating the microtitre plates at room temperature for 1 h, then washed once with 200 μl of DMEM/HEPES, three times with 400 μl of D2O. The dye was solubilized in 100 μl of 10% (v/v) acetic acid, and the absorbance at 570 nm measured on a Dionex MRX II microtitre plate reader. Background crystal violet staining readings were subtracted from all experimental and 100% attachment results. Data from 20, 50, and 100% cell number standards were plotted (A100 versus cell density) using Microsoft Excel. The slope of the linear portion of this line was determined and then fixed at an arbitrary value, which was used to express data as percent attachment. In all experiments, triplicate or quadruplicate wells were prepared. To examine the effects of cations on attachment, 50 μl 2x EDTA or PBS containing cations followed by 50 μl 2x cells resuspended in PBS minus cations (mixed prior to use by gentle pipetting) were added to ligand-coated and control wells after BSA-blocking.

To determine cell spreading, the wells of microtitre plates were ligand-coated and BSA-blocked as for cell attachment assays. The cells were trypsinized, quenched, and counted as before, then adjusted to 2 × 105 cells/ml with warm DMEM/HEPES gassed with 5–10% (v/v) CO2. Aliquots of cells were added to the appropriate wells. As negative controls, cells were added to wells coated with 50 μl of PBS. Each well was incubated for 40 min at 37 °C in a 5% CO2 incubator with the lid removed. The cells were immediately fixed with the addition of 10 μl of 37% formaldehyde directly to the well for 20 min. The formaldehyde was aspirated, and the wells were filled with PBS before layering a...
glass coverslip onto the plate. The level of cell spreading was determined by phase contrast microscopy.

**FACS Analysis**—Cell suspensions were prepared by washing confluent cultured cell layers with PBS and incubating with 1 ml of 5 mM EDTA in Hanks’ buffered saline solution/10-cm² flask area at 37 °C for no more than 30 min. The resulting cell suspensions were neutralized with 5 volumes of DMEM-5 (Dulbecco’s minimal essential medium, including 25 mM Hepes, 500 mg/liter glucose, 4 mg/liter pyridoxine) supplemented with 10% (v/v) fetal calf serum and 2 mM L-glutamine and centrifuged at 800 × g for 5 min. The cell pellets were resuspended in

![Image of domain organization of the recombinant fibrillin-1 protein fragments compared with full-length fibrillin-1. SDS-PAGE analysis of nickel affinity-purified recombinant fibrillin-1 protein fragments under non-reducing (i) or reducing (ii) conditions or after reduction and pretreatment with peptide-N-glycosidase F (iii) (peptide-N-glycosidase F is indicated with an asterisk). B, protein concentration profile of CsCl density-purified microfibrils. (The arrow indicates fractions containing microfibrils.) Diamonds, CsCl concentration; squares, protein concentration. C, dot blot detection of fibrillin-1 from CsCl density-purified protein fractions using the anti-fibrilllin-1 antibody 11C1. D, electron microscopy of CsCl-purified microfibrils taken from fraction 10.](image-url)
1 ml of supplemented DMEM/HEPES, and the cell density was calculated using a Neubauer hemocytometer. The cell suspension was centrifuged at 800 × g for 3 min, and the cell pellets were resuspended in an appropriate volume of supplemented DMEM/HEPES to give a final cell density of 1 × 10^6 cells/ml. Cells (50 μl) were added to FACS tubes followed by 50 μl of primary antibody (K20, JBS5, JA218, 12G10, LM609, or mouse IgG control) diluted to 10 μg/ml in PBS-2 containing 0.02% (w/v) sodium azide and incubated at 4 °C for 1 h. The cell-antibody mixtures were centrifuged at 800 × g for 4 min, and the cell pellets were washed three times in PBS-2 (Dulbecco’s phosphate-buffered saline (without Ca^{2+} and Mg^{2+})) containing 1% (v/v) fetal calf serum. The resulting cell pellets were resuspended in 50 μl of PBS-2 containing FITC-conjugated anti-mouse secondary antibody (1:200 dilution) and 10% (v/v) human serum and incubated at 4 °C for 45 min. The antibody-cell mixtures were centrifuged at 800 × g for 4 min, and the cell pellets were washed twice in PBS with cations containing 1% (v/v) fetal calf serum and once with PBS with cations. Cells were fixed by the addition of 100 μl of 2% (v/v) formaldehyde followed by 400 μl of PBS with cations and incubated at room temperature for 20 min. Cells from each sample (20,000 in total) were counted using a FACScan flow cytometer (BD Biosciences, Oxford, UK) at a flow rate of less than 200 events/s.

**Immunofluorescence**—Double antibody staining was performed on HDF cells plated on fibrillin or fibronectin ligands for up to 6 h. Coated wells were prepared and blocked as outlined under “Cell Attachment and Spreading Assays.” Primary antibodies were a rabbit polyclonal antibody (PF2) to a pepsin fragment of fibrillin-1 kindly supplied by Dr. R. Glanville (Portland, OR) (26) and a monoclonal antibody to human serum fibronectin (15T4) obtained from Sigma Chemical Co. (Dorset, UK). Secondary anti-rabbit IgG conjugated FITC (1:100 dilution) and
FIG. 3. A, cation dependence of HDF attachment onto 10 μg/ml microfibrils, PF9, PF10, and PF11. Squares, calcium; triangles, magnesium; circles, manganese; short dash, BSA control; long dash, DMEM control. Error bars indicate ± S.D. B, inhibition of HDF attachment to microfibrils (squares), PF9 (triangles), PF10 (crosses), or PF11 (circles) using antibodies raised against peptides corresponding to the RGD containing region of fibrillin-1 or fibrillin-2.
Fig. 4. A. anti-integrin antibody inhibition of HDF attachment onto 10 μg/ml microfibrils, PF9, PF10, or PF11. LM609 is an inhibitory anti-α5β1 antibody, mAb13 is an inhibitory anti-β1 antibody, K20 is a non-functional anti-β1 antibody, mAb16 and JBS-5 are both inhibitory anti-α5 antibodies, and JA218 is an inhibitory anti-α5 antibody. Antibodies were used at 10 μg/ml. B indicates background cell attachment to BSA-blocked wells. C indicates cell attachment in the absence of antibody. Error bars indicate ± S.D. B, dose-dependent inhibition of HDF cell attachment onto 10 μg/ml microfibrils, PF9, PF19, or PF11. Squares, LM609; triangles, mAb13; diamonds, mAb16; circles, JA218; dashed line, BSA background. Error bars indicate ± S.D. C, FACS detection of HDF cell surface integrins using the anti-β1 antibodies K20 (i) and 12G10 (iii), the anti-α5 antibody JA218 (ii), the anti-α5β1 antibody LM609 (iv), the anti-α5 antibody JBS-5 (v), and mouse Ig control antibody (vi). The geometric mean values of each graph are shown.
anti-mouse IgG-conjugated rhodamine (1:100 dilution) was used. Cell cultures were then visualized using a Leica DM RXA microscope fitted with a UV lamp and appropriate filters. Basic image acquisition and analysis were performed using IP Lab version 3.2. Advanced image analysis was performed using Adobe Photoshop version 6.0.

RESULTS

**HDF Attachment and Spreading on Microfibrils**—To determine whether assembled native fibrillin-rich microfibrils support HDF adhesion and spreading, microfibrils were immobilized onto 96-well microtiter plates and used as ligands in cell attachment and spreading assays (Fig. 2). Linear coating efficiencies within the concentration range 0–20 μg/ml were confirmed by enzyme-linked immunosorbent assay, and microscopy analysis confirmed that microfibrils readily adhered to the wells. Highest percentage of cell attachment was obtained using a ligand coating concentration of 2 μg/ml, which was then used in all subsequent assays. Fibronectin was used as a positive control and BSA as a negative control. HDF attached strongly to microfibrils (Fig. 2A) and spread well on microfibrils and on fibronectin (Fig. 2, B and C).

**HDF Attachment and Spreading on Recombinant Fibrillin-1 Protein Fragments**—To compare HDF attachment to microfibrils and to molecular fibrillin-1, cell adhesion to three overlapping fibrillin-1 recombinant protein fragments (Fig. 1A), two of which contained the RGD sequence, was examined (Fig. 2). The RGD-containing fibrillin-1 recombinant protein fragments PF9 and PF11 supported strong cell attachment, although slightly lower than microfibrils (Fig. 2A). The non-RGD-containing fragment PF10 also supported some cell adhesion, but this was much lower than PF9 and PF11. The RGD-containing protein fragment PF11 had the highest cell adhesion activity, followed by the RGD-containing protein fragment PF9 and then by peptide PF10. HDF spread rapidly on fibrillin-1 fragments PF9 and PF11 but very little on fragment PF10 (Fig. 2, B and C).

**HDF Attachment to Fibrillin-1 Ligands Is Divalent Cation-and RGD-dependent**—Divalent cations are known to regulate integrin-ligand interactions (25). To establish whether integrin receptors mediate cell adhesion to fibrillin-1 molecules and microfibrils, divalent cation dependence was assessed. For HDF attachment to microfibrils and RGD-containing protein fragments PF9 and PF11, calcium was unable to support attachment, whereas magnesium and especially manganese strongly enhanced binding (Fig. 3A). Addition of cations had no effect on HDF attachment to PF10 (data not shown).

RGD dependence of cell attachment was then examined directly using synthetic peptides to the fibrillin-1 and fibrillin-2 RGD sequences, and antibodies raised to these peptides. Immunoblotting demonstrated high immunoreactivity of each fibrillin isoform antibody for its own peptide but not for the other fibrillin peptide. HDF attachment to microfibrils was inhibited in a dose-dependent manner by both fibrillin-1 and fibrillin-2 RGD antibodies (Fig. 3B) and by synthetic peptides (not shown). HDF attachment to the RGD-containing recombinant fibrillin-1 protein fragments PF9 and PF11 was strongly inhibited by the fibrillin-1 RGD antibody and synthetic peptide but not the corresponding fibrillin-2 RGD reagents (Fig. 3B). The low level of HDF attachment to fibrillin-1 protein fragment PF10 was unaffected by addition of either peptides or antibodies. These studies confirmed that HDF attachment to fibrillin-1 fragments PF9 and PF11 is RGD-dependent and showed that ~45% of attachment to microfibrils is RGD-dependent with contributions from both fibrillin-1 and fibrillin-2.

**Integrin Receptors Mediating HDF Attachment to Fibrillin-1 Ligands**—The integrin receptors responsible for mediating RGD-dependent cell attachment to microfibrils were identified in cell attachment-inhibition studies (Fig. 4A). Microfibrils immobilized onto 96-well microtiter plates were used as ligands in inhibition assays of HDF cell attachment, with antibodies to integrin receptors at final concentrations of 20 μg/ml (α5β1, LM609, α5 (JBS5 and mAb16), α3 (JA218), and β3 (mAb13 and K20)). Antibodies JBS5 and mAb 16 (block α5 integrin subunit) both strongly inhibited cell attachment, suggesting that α5β3 integrin mediates HDF attachment to microfibrils. Addition of JBS5 resulted in 84% inhibition of attachment and of mAb16 led to 97% inhibition. mAb 13, which inhibits β3 integrins, inhibited cell attachment by 78%. No inhibition was observed using the K20 anti-β1 antibody, as expected, because this antibody does not block integrin function. A contribution to cell attachment was also shown for α5β3, because antibody LM609 inhibited cell attachment by 41%. Integrin α5β3 was not involved, because antibody JA218 did not inhibit cell attachment. Antibody dose-response curves confirmed the specific antibody effects and that α5β3 and α3β1 integrin receptors are responsible for the RGD-mediated HDF attachment to microfibrils (Fig. 4B).

Similar cell attachment-inhibition assays using anti-integrin antibodies were conducted with HDF plated on recombinant fibrillin-1 RGD fragments PF9, PF10, and PF11 (Fig. 4, A and B) to establish whether the same integrin receptors bind fibril-
lin-1 molecules and microfibrils. Neither JA218 nor K20 inhibited attachment to these protein fragments, as shown using microfibril ligands. mAb 16 caused 95% (PF9) and 86% (PF11) inhibition of HDF attachment, JBS5 caused 89% (PF9) and 96% (PF11) inhibition, and LM609 caused 67% (PF9) and 46% (PF11) inhibition. Thus, cell attachment to fibrillin-1 fragments PF9 and PF11 is mediated by \( \alpha_5\beta_1 \) and \( \alpha_5\beta_3 \). There was no inhibition of cell attachment to fragment PF10 with any of these antibodies.

FACS analysis confirmed the presence of \( \alpha_5\beta_1 \) and \( \alpha_5\beta_3 \) receptors on HDF cells (Fig. 4C). Abundant \( \beta_1 \) integrin was detected as well as \( \alpha_\_ \) and \( \alpha_\_ \) subunits and low levels of \( \alpha_\beta_3 \) integrin.
A cellular consequence of attachment to fibrillin ligands—When the morphology of HDF plated on microfibrils for up to 6 h was compared with that of HDF on fibronectin, pronounced shape differences were observed (Fig. 6). The cells on microfibrils were less extensively spread and had very prominent actin filaments and some ruffled edges. The cells on fibronectin were highly spread with well organized cytoskeletal structure. Immunocytochemical analysis revealed increased fibrillin-1 immunoreactivity when plated on microfibrils relative to cells on fibronectin (Fig. 6). On binding microfibrils, focal complexes containing FAK and paxillin were apparent as previously shown (11), and focal adhesion molecules paxillin and FAK were phosphorylated (not shown). These experiments confirmed that adhesion to microfibrils results in cell signaling, cytoskeletal organizational changes, and extracellular matrix expression.

Adhesion of Smooth Muscle Cells, HT1080 Fibrosarcoma Cells, and A375-SM Melanoma Cells to Microfibrils and Fibrillin-1 Protein Fragments—Adhesion of primary human coronary artery smooth muscle cells, HT1080 fibrosarcoma cells, and A375-SM melanoma cells to microfibrils and fibrillin-1 protein fragments was also examined (Fig. 7). HT1080 cells exhibited 20% cell attachment to PF11 (Fig 7A). This adhesion was strongly inhibited by anti-integrin α5 antibody mAb16 and anti-integrin β1 antibody mAb13, but anti-integrin αvβ3 antibody LM609 had little effect on cell adhesion. A375-SM melanoma cells exhibited 65% attachment to PF11 (Fig. 7B). This adhesion was strongly inhibited by anti-integrin αvβ3 antibody LM609, but anti-integrin α5 antibodies had little effect on cell adhesion. For smooth muscle cells, the percent cell attachment to fibrillin-1 protein fragments and microfibrils was lower than for HDF, but after pre-treatment with the β1 integrin activating antibody, TS2/16, activated SMC β1 integrins bound and spread on microfibrils and on fragments PF9 and PF11 (Fig. 7, C and D).

**DISCUSSION**

Fibrillins serve an essential role linking extracellular fibrillin-rich microfibril bundles and elastic fibers with cells of connective tissues such as skin and blood vessels. Loss of cell-microfibril interactions in Marfan syndrome, a severe heritable disease caused by mutations in fibrillin-1 (28), and in aging following microfibril degeneration may lead to altered cell-matrix interactions, which, in turn, could modify cell behavior. Studies have shown that recombinant fibrillin-1 fragments (12, 13) and fibrillin molecules purified using a denaturing protocol (11) can interact with cells, and αvβ3 integrin was identified as the major receptor. An aim of this study was to determine whether connective tissue cells also bind assembled microfibrils and the molecular basis of these interactions. We have shown that cells strongly adhere and spread on microfibrils that, in addition to αvβ3, α5β1 is an important receptor for microfibrils as well as for recombinant fibrillin-1 molecules, and that RGD-dependent adhesion to fibrillin ligands influences cell shape and ECM deposition.

Although cell adhesion studies generally utilize molecules or peptides as ligands, this study has examined cell adhesion to native assembled fibrillin-rich microfibrils which are very large polymers with a complex bead ultrastructure (1, 20, 26, 29). Using these physiological ligands, we have shown strong cation- and RGD-dependent adhesion of HDF to microfibrils, which is comparable to their adhesion to a 50-kDa fibronectin fragment that contains the central cell binding domain. Because interactions between cells and microfibrils are inhibited by fibrillin-1 and fibrillin-2 RGD peptides, the RGD sequence within the fourth fibrillin TB module must be accessible within assembled microfibrils. Such a location is consistent with pro-
posed models of fibrillin alignment in microfibrils, which predict that the RGD motif is within the interbead region (1, 20, 26, 30, 31). Strong inhibition of microfibril adhesion was seen with both fibrillin-1 and fibrillin-2 RGD peptide antibodies, which is consistent with fetal aortic microfibrils containing both molecules (3, 4). Thus for HDF, RGD-dependent adhesion to microfibrils, as well as to recombinant fibrillin-1 RGD protein fragments, is mediated mainly by $\alpha_5\beta_1$, although $\alpha_v\beta_3$, 

**Fig. 7.** A, anti-integrin antibody inhibition of HT1080 cell attachment onto 20 μg/ml PF10, PF11, or 50K fibronectin (type III repeats 6–10). Unshaded bars, no antibody; light gray, mAb16 the inhibitory anti-$\alpha_5$ antibody; mid gray, LM609 the inhibitory anti-$\alpha_5\beta_1$ antibody; dark gray, LM609 and mAb16 combined. Error bars indicate ± S.D. B, anti-integrin antibody inhibition A375-SM melanoma cell attachment onto BSA, PF11, or 50K fibronectin (type III repeats 6–10). mAb16 is an inhibitory anti-$\alpha_5$ antibody, and LM609 is an inhibitory anti-$\alpha_5\beta_1$ antibody. Unshaded bars, no antibody; light gray, mAb16 the inhibitory anti-$\alpha_5$ antibody; mid gray, LM609 the inhibitory anti-$\alpha_5\beta_1$ antibody; dark gray, LM609 and mAb16 combined. Error bars indicate ± S.D. C, human coronary artery smooth muscle cell adhesion to PF9, PF10, and PF11 in the presence (dark gray bars) or absence (unshaded bars) of the $\beta_1$ activating antibody TS2/16. The non-functional $\alpha_5$ antibody 10A4 (mid gray bars) was included as a control. Error bars indicate ± S.D. D, phase-contrast microscopy of human coronary artery smooth muscle cell spreading onto BSA (A and E), PF9 (B and F), RDG PF9 (C and G), and fibronectin (D and H) in the absence (A–D) or presence (E–H) of the $\beta_1$ activating antibody TS2/16.
receptors also bind these ligands. Adhesion to integrin αβ₁ is well known to be highly conformation-dependent (32). Smooth muscle cells adhered strongly to β₁ integrins only after antibody activation, indicating cultured cell-specific differences in αβ₁-activated status. A similar effect was noted for bovine auricular chondrocytes (11). Integrin-dependent HDF and smooth muscle cells spreading on fibrillin ligands showed that cell signaling events caused cytoskeletal changes. Prominent differences in cell shape when plated on microfibrils or on fibronectin indicated subtle differences in cellular responses to adhesion to these two RGD ligands even though adhesion was, in both cases, mediated largely by αβ₁ integrin. On microfibrils, HDF were partially migratory with some ruffled edges, whereas on fibronectin the cells were well spread. These differences have physiological implications for cell morphology and signaling when adherent to fibronectin or microfibrils in vivo.

Interactions between fibrillin ligands and αβ₁ integrin were confirmed using a fibrillin-1 RGD mutant which, as expected, showed ablated cell adhesion. These data allowed us to exclude other ECM molecules such as fibronectin as a potential αβ₁ ligand that might have been secreted during the 20-min cell adhesion assays. Moreover, mAb 333, which blocks fibronectin binding to integrins (33), resulted in no reduction of cell adhesion to fibrillin-1, although in fibronectin control experiments adhesion was ablated. Immunofluorescence studies also failed to detect fibronectin expression within the timeframe of the assays. RGD-dependent integrin αβ₁ binding to fibronectin is known to involve recognition of a PHSRN synergy site in the ninth fibronectin type III repeat (34). No similar synergy sequence is present within the flanking cbEGF domains in fibrillin-1, so any synergy site in this molecule must be a different sequence or a conformation-dependent epitope. A recent study of αβ₁-mediated binding to fibronectin has highlighted that the RGD domain serves to activate and align the αβ₁-fibronectin interaction, whereas the synergy site provides the mechanical strength to the interaction (32). Because fibrillin microfibrils serve an important mechanical role in connective tissues (35), it will be of interest to determine the molecular basis of their mechanical coupling to cells.

Because RGD-mediated cell adhesion accounted for ~50% of cell adhesion to assembled microfibrils, we examined whether microfibrils also interact with heparan sulfate, which, in turn, would suggest interactions with cell surface heparan sulfate proteoglycan receptors. Three heparan sulfate binding sequences have been localized to large regions of fibrillin-1 using recombinant fibrillin-1 peptides (27), but it is not clear whether these sequences are exposed in microfibrils or available for interactions with cell surface receptors. The N- and C-terminal heparan sulfate binding regions, in particular, may be located in the microfibril beads and/or involved in fibrillin-1 assembly (1, 20, 26, 36). Our data show that ~one-fifth of HDF attachment to microfibrils was inhibited by heparan sulfate. The RGD-independent adhesion to fibrillin-1 exons 41–52 that we have identified was not inhibited by heparan sulfate, so the molecular basis of this adhesion is unknown but could possibly be mediated by an integrin receptor that does not recognize the RGD motif. We have shown that cell adhesion to fetal aortic microfibrils involves interactions with fibrillin-1 and fibrillin-2, but interactions with other potential microfibril-associated molecules shown to interact with cells in vitro may also, if present, contribute. These include tropoelastin, which binds cells via the elastin-binding protein (37), and MAGP-2, which binds cells via αβ₂ (38).

HDF attachment to microfibrils and to fibrillin-1 protein fragments, in addition to promoting cell spreading and migration, morphology, also enhanced the extracellular deposition of fibrillin-1 relative to deposition when attached to fibronectin. This effect suggests a positive feedback mechanism for fibrillin-1 expression. The integrin αβ₁ plays a central role in pericellular fibrinogen assembly, mediating conformational changes that allow linear accretion (39). It will be of interest to determine whether αβ₁-mediated adhesion to newly synthesized molecules also influences fibrillin microfibril assembly. The physiological importance of fibrillin interactions with cells is clearly highlighted in blood vessel walls. Medial elastic fiber laminae have an outer mantle of microfibrils that is juxtaposed to smooth muscle cells at dense plaques. In a mouse model of Marfan syndrome, microfibril defects resulted in an unusually smooth surface of elastic laminae, loss of cell attachments normally mediated by fibrillin-1, and alterations in smooth muscle cell morphology and expression profiles (40). Subendothelial microfibrils deposited during aortic development interact with endothelial cells at dense plaques (5) and are critical in anchoring endothelial cells to the vessel wall. Juxtaposition of microfibrils and fibroblasts in skin and ligament further confirm the physiological importance of microfibril-cell interactions.

In summary, we have shown that assembled fibrillin-rich microfibrils are important adhesion ligands for connective tissue cells, αβ₁ is a major receptor for fibrillin-1 molecules and microfibrils, and RGD-dependent adhesion to fibrillin ligands influences cell phenotype. An implication of this study is that reduced or defective microfibrils in Marfan syndrome may directly alter cellular phenotype.

REFERENCES

1. Handford, P. A., Downing, A. K., Reinhardt, D. P., and Sakai, L. Y. (2000) Matrix Biol. 19, 457–470.
2. Kielty, C. M., Sherratt, M. J., and Shuttleworth, C. A. (2002) J. Cell. Sci. 115, 2871–2878.
3. Gambardello, F., Reinhardt, D. P., Charbonneau, N. L., Pophal, G., Sakai, L. Y., and Herken, R. (2002) Matrix Biol. 21, 637–646.
4. Charbonneau, N. L., Dzamba, B. J., Ono, R. N., Keene, D. R., Corson, G. M., Reinhardt, D. P., and Sakai, L. Y. (2003) J. Biol. Chem. 278, 2740–2749.
5. Davis, R. C. (1993) Cell Tissue Res. 272, 211–219.
6. Perera, L., D’Alessio, M., Ramirez, F., Lynch, J. R., Sykes, B., Pangilinan, T., and Bonadio, J. (1993) Hum. Mol. Genet. 2, 961–968.
7. Humphries, M. J. (2002) Arthritis Res. 4, 869–878.
8. Zhang, H., Arief, D., Hu, W., Davis, E. C., Sanguineti, C., Bonadio, J., Mechem, R. P., and Ramirez, F. (1994) J. Cell Biol. 124, 855–863.
9. Nagase, T., Nakayama, D., Nakajima, D., Kikuno, K., and Ohara, O. (2001) DNA Res. 8, 85–95.
10. Yuan, X., Downing, A. K., Knott, V., and Handford, P. A. (1997) EMBO J. 16, 6659–6666.
11. Sakamoto, H., Broekelmann, T., Cheres, D. A., Ramirez, F., and Bonadio, J. (1992) J. Biol. Chem. 267, 45192–45200.
12. Pfaff, M., Reinhardt, D. P., Sakai, L. Y., and Timpl, R. (1998) FEBS Lett. 384, 247–250.
13. D’Arrigo, C., Burl, S., Withers, A. P., Dobson, H., Black, C., and Boxer, M. (1998) Connect Tissue Res. 37, 29–51.
14. Kielty, C. M., Whittaker, S. P., Grant, M. E., and Shuttleworth, C. A. (1992) J. Cell. Sci. 103, 445–451.
15. Aquilina, A., Korda, M., Bergelson, J. M., Humphries, M. J., Farnsdale, R. W., and Tuckwell, D. (2002) Eur. J. Biochem. 269, 1139–1144.
16. Mould, A. P., Askari, J. A., Aota, S., Yamada, K. M., Irie, A., Takada, Y., Mardon, H. J., and Humphries, M. J. (1997) J. Biol. Chem. 272, 17283–17292.
17. Mould, A. P., Wheldon, L. A., Komorinya, A., Wayner, R. E., Yamada, K. M., and Humphries, M. J. (1999) J. Biol. Chem. 274, 40420–40424.
18. Tselipis, V. H., Green, L. J., and Humphries, M. J. (1997) J. Biol. Chem. 272, 21341–21348.
19. Ashworth, J. L., Murphy, G., Rock, M. J., Sherratt, M. J., Shapiro, S. D., Shuttleworth, C. A., and Kielty, C. M. (1999) Biochem. J. 340, 171–181.
20. Reinhardt, D. P., Keene, D. R., Corson, G. M., Poschl, E., Bachinger, H. P., Gambee, J. E., and Sakai, L. Y. (1996) J. Mol. Biol. 258, 104–116.
21. Reinhardt, D. P., Mechling, D. E., Boswell, B. A., Keene, D. R., Sakai, L. Y., and Bachinger, H. P. (1997) J. Biol. Chem. 272, 7368–7373.
22. Lin, G., Tiedemann, K., Vollbrandt, T., Peters, H., Batge, B., Brinckmann, J., and Reinhardt, D. P. (2002) J. Biol. Chem. 277, 50795–50804.
23. Kielty, C. M., Cummings, C. R., Whittaker, S. P., Shuttleworth, C. A., and Grant, M. E. (1991) J. Cell. Sci. 99, 797–807.
24. Kielty, C. M., Hanssen, E., and Shuttleworth, C. A. (1998) Anal. Biochem. 255, 168–172.
25. Mould, A. P., Garratt, A. N., Puxon-McLaughlin, W., Takada, Y., and Humphries, M. J. (1998) Biochem. J. 331, 821–828.
26. Baldock, C., Koster, A. J., Ziese, U., Rock, M. J., Sherratt, M. J., Kessler, K. E., ...
Integrin-mediated Cell Adhesion to Fibrillin

Shuttleworth, C. A., and Kielty, C. M. (2001) J. Cell Biol. 152, 1045–1056
27. Tiedemann, K., Batge, B., Muller, P. K., and Reinhartl, D. P. (2001) J. Biol. Chem. 276, 36035–36042
28. Robinson, P. N., and Booms, P. (2001) Cell. Mol. Life Sci. 58, 1698–1707
29. Cardy, C. M., and Handford, P. A. (1998) J. Mol. Biol. 276, 855–860
30. Downing, A. K., Knott, V., Werner, J. M., Cardy, C. M., Campbell, I. D., and Handford, P. A. (1996) Cell 85, 597–605
31. Qian, R. Q., and Glanville, R. W. (1997) Biochemistry 36, 15841–15847
32. Garcia, A. J., Schwartzbauer, J. E., and Boettiger, D. (2002) Biochemistry 41, 9063–9069
33. Nojima, Y., Humphries, M. J., Mould, A. P., Komoriya, A., Yamada, K. M., Schlossman, S. F., and Morimoto, C. (1990) J. Exp. Med. 172, 1185–1192
34. Aota, S., Nomizu, M., and Yamada, K. M. (1994) J. Biol. Chem. 269, 24756–24761
35. Kielty, C. M., Baldock, C., Lee, D., Rock, M. J., Ashworth, J. L., and Shuttleworth, C. A. (2002) Philos. Trans. R. Soc. Lond B Biol. Sci. 357, 207–217
36. Ashworth, J. L., Kelly, V., Rock, M. J., Shuttleworth, C. A., and Kielty, C. M. (1999) J. Cell Sci. 112, 1163–1171
37. Mochizuki, S., Brassart, B., and Hinek, A. (2002) J. Biol. Chem. 277, 44854–44863
38. Gibson, M. A., Levesley, D. I., and Ashman, L. K. (1999) J. Biol. Chem. 274, 13060–13065
39. Sechler, J. L., Rao, H., Cumiskey, A. M., Vega-Colon, I., Smith, M. S., Murata, T., and Schwachman, J. E. (2001) J. Cell Biol. 154, 1081–1088
40. Bunton, T. E., Biery, N. J., Myers, L., Gayraud, B., Ramirez, F., and Dietz, H. C. (2001) Circ. Res. 88, 37–43