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Cell–Matrix Entanglement and Mechanical Anchorage of Fibroblasts in Three-dimensional Collagen Matrices

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Fibroblast-3D collagen matrix culture provides a physiologically relevant model to study cell–matrix interactions. In tissues, fibroblasts are phagocytic cells, and in culture, they have been shown to ingest both fibronectin and collagen-coated latex particles. Compared with cells on collagen-coated coverslips, phagocytosis of fibronectin-coated beads by fibroblasts in collagen matrices was found to be reduced. This decrease could not be explained by integrin reorganization, tight binding of fibronectin beads to the collagen matrix, or differences in overall bead binding to the cells. Rather, entanglement of cellular dendritic extensions with collagen fibrils seemed to interfere with the ability of the extensions to interact with the beads. Moreover, once these extensions became entangled in the matrix, cells developed an integrin-independent component of adhesion. We suggest that cell–matrix entanglement represents a novel mechanism of cell anchorage that uniquely depends on the three-dimensional character of the matrix.

INTRODUCTION

Compared with collagen-coated material surfaces such as plastic or glass, fibroblasts exhibit unique features when they interact with three-dimensional collagen matrices (Cukierman *et al*., 2002; Grinnell, 2003). The stiffness of fibroblasts is similar to that of three-dimensional (3D) collagen fibrils in the matrix (Wakatsuki *et al*., 2000). As a result, the interaction between cells and the matrix can lead not only to changes in cell shape but also to matrix remodeling and contraction (Grinnell, 1994; Brown *et al*., 1998; Tranquillo, 1999; Petroll and Ma, 2003; Vanni *et al*., 2003; Wakatsuki and Elson, 2003). By contrast, the difference in stiffness between cells and rigid material surfaces such as glass and plastic is so great (Wakatsuki *et al*., 2000; Callister, 2001) that cell shape change predominates even if the material surface has been modified to make it somewhat flexible (Balaban *et al*., 2001; Beningo and Wang, 2002).

Fibroblasts interact with and attach to collagen matrices primarily through integrin $\alpha 2\beta 1$ but also with other integrins, including $\alpha 1\beta 1$, $\alpha 11\beta 1$, and $\alpha v\beta 3$ (Klein *et al*., 1991; Schiro *et al*., 1991; Carver *et al*., 1995; Cooke *et al*., 2000; Tiger *et al*., 2001; Jokinen *et al*., 2004). Attached cells, rather than forming flattened, lamellar extensions as occurs on impenetrable glass or plastic surfaces, range in shape from dendritic to stellate to bipolar, depending on matrix stiffness and tension (Grinnell, 2003). Similar morphological features have been described for cells in tissues (Breathnach, 1978; Doljanski, 2004; Goldsmith *et al*., 2004; Langevin *et al*., 2005). In part, differences in morphology between cells in two-dimensional (2D) versus 3D culture may result from the symmetric adhesive interactions in 3D matrices versus the forced asymmetry of 2D surfaces (Beningo *et al*., 2004).

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In tissues, fibroblasts are phagocytic cells (McGaw and Ten Cate, 1983; Everts *et al*., 1996, 2003), and in culture they have been shown to ingest both fibronectin (FN) and collagen-coated latex particles (Grinnell and Geiger, 1986; McCulloch and Knowles, 1993). Cell adhesion and spreading on 2D surfaces was reported to constrain phagocytic activity (Arora *et al*., 2003). Because fibroblasts in 3D matrices initially spread without forming stress fibers and have few focal adhesions (Vaughan *et al*., 2000; Tamariz and Grinnell, 2002), we anticipated that they might have increased phagocytic activity. When we tested this possibility, however, we observed just the opposite. That is, compared with cells on collagen-coated coverslips, phagocytosis of FN beads by fibroblasts on collagen matrices was found to be reduced. This decrease could not be explained by integrin reorganization, tight binding of the beads to the collagen matrix, or by differences in overall bead binding to the cells. Rather, entanglement of cellular dendritic extensions with collagen fibrils seemed to interfere with the ability of the extensions to interact with the beads. Moreover, once these extensions became entangled in the matrix, cells developed an integrin-independent component of adhesion. We suggest that cell–matrix entanglement represents in a novel mechanism of cell anchorage that uniquely depends on the three-dimensional character of the matrix.

MATERIALS AND METHODS

Materials

Type I collagen (Vitrogen) was purchased from Cohesion (Palo Alto, CA). DMEM, 0.25% trypsin/1 mM EDTA solution, and 0.25% trypsin solution were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Gemini (Woodland, CA). Human plasma FN was obtained from the New York Blood Center (New York, NY). Rabbit anti-fibronectin was prepared in our laboratory. Two-micrometer polystyrene latex beads were obtained from Polysciences (Warrington, PA). Bafilomycin A1 was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Platelet-derived growth factor (PDGF) BB isotype was obtained from Upstate Biotechnology (Lake Placid, NY). Fibronectin peptide GRGDSP and control peptide GRGESP were obtained from AnaSpec (San Jose, CA). Fatty acid-free bovine serum albumin (BSA) and lysophosphatidic acid were obtained from Sigma-Aldrich (St. Louis, MO). Tetramethylrhodamine B isothio-

cyanate (TRITC)-conjugated phalloidin, FITC-conjugated goat anti-rat IgG (H+L) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (H+L) were obtained from Molecular Probes (Eugene, OR). Mouse anti-$\alpha 2\beta 1$ was obtained from Chemicon International (Temecula, CA), and rat anti-$\beta 1$ integrin (clone 9EG7) was purchased from BD Biosciences PharMingen (San Diego, CA). Fluoromount G was obtained from Southern Biotechnology Associates (Birmingham, AL).

Monolayer and Collagen Matrix Culture

Fibroblasts were from human foreskin specimens and cultured up to 10 passages in Falcon 75-cm^2 tissue culture flasks in DMEM supplemented with 10% FBS at 37°C in a 5% CO_2-humidified incubator. The culture medium was changed every 3–4 d. Cells were harvested by trypsin/EDTA for 4 min at 37°C followed by DMEM/10% FBS. For monolayer culture experiments, harvested cells (10^5 in 2 ml of DMEM containing 5 mg/ml BSA and 50 ng/ml PDGF) were incubated on 22-mm^2 glass coverslips (Fisher Scientific, Chicago, IL) that previously had been coated with collagen (50 μg/ml in DMEM for 15 min). Collagen matrix cultures were prepared using Vitrogen 100 collagen. Neutralized collagen solution (1.5 mg/ml) in DMEM was prewarmed to 37°C for 4 min, and then aliquots (200 μl) were placed on an area outlined by a 12-mm-diameter circular score within a well of 24-well culture plates (Greiner Bio-one, Frickenhausen, Germany) and allowed to polymerize for 60 min at 37°C in 5% CO_2-humidified incubator. Harvested cells (10^4 in 1 ml of DMEM containing 5 mg/ml BSA and 50 ng/ml PDGF) were seeded on top of the matrices and incubated as described above.

Immunofluorescence Microscopy

Cells on matrices or on coverslips were fixed for 10 min with 3% paraformaldehyde in phosphate-buffered saline at room temperature, blocked with 1% glycine/2% BSA in DPBS (150 mM NaCl, 3 mM KCl, 1 mM KH_2PO_4, 6 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.2) for 30 min, and then permeabilized where indicated for 15 min with 0.5% Triton X-100 in DPBS. Subsequently, the samples were washed with DPBS and treated for 30 min with 1% BSA in DPBS. Primary antibody against fibronectin or $\beta 1$ integrin was diluted in 1% BSA in DPBS and added to cells for 1 h at 37°C. After washing with DPBS, samples were treated for 30 min with 1% BSA in DPBS, and then FITC-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-rabbit IgG diluted in 1% BSA in DPBS was added to cells for 30 min at 37°C. Dilutions were generally around 1:150 but adjusted from lot to lot according the staining intensity obtained. In some cases, samples also were treated with 2 U/ml TRITC-conjugated phalloidin in DPBS containing 1% BSA for an additional 30 min at 37°C. After washing, samples were mounted on glass slides with Fluoromount G. Observations were made and images collected with a Nikon Elipse 400 Fluorescent microscope equipped with a Photometrics SenSys camera and MetaView workstation.

Phagocytosis Assay

Two-micrometer polystyrene latex beads (6.25 μl) were incubated with 250 μl of DMEM containing FN (2 μg/ml) for 5 min at room temperature after which an additional 250 μl of DMEM containing BSA (5 mg/ml) was added to the incubations. Final bead concentration was $\sim 7.6 \times 10^7$/ml based on the manufacturer's product sheet. FN beads were dispersed by sonication for 20 s at 60 W (Branson Ultrasonics, Danbury, CT) immediately before addition to the cells. Cells on coverslips or matrices were incubated with beads in DMEM containing 5 mg/ml BSA and 50 ng/ml PDGF for the time periods indicated in the figure legends. At the end of the incubations, samples were fixed and processed for scanning electron microscopy (see below) or stained with anti-FN. If staining was carried out without permeabilization of the cells, then only extracellular FN beads were detected. Alternatively, if unfixed cells were first subjected to trypsin treatment for 3 min (coverslips) or 6 min (matrices) at room temperature to digest the FN and fixed and permeabilized before staining with anti-FN, then only ingested beads were detected. Bafilomycin A1 (250 nM) was added to block intracellular FN degradation. To quantify bead uptake, internalized beads per cell were counted for 50 cells in randomly selected microscope fields. Data presented are from representative experiments, and all experiments were carried out multiple times.

Scanning Electron Microscopy (SEM) Analysis

For SEM analysis, collagen matrices were prepared on 13-mm-diameter Thermanox plastic coverslips (Nalge Nunc International, Naperville, IL). At the end of the incubations, samples were fixed overnight at 22°C with 2% glutaraldehyde in 0.1 M Na cacodylate, pH 7.0. Cells were washed twice for 15 min at 22°C in cacodylate buffer and postfixed with 1% OsO_4 (Polysciences) in cacodylate buffer for 30 min at 22°C. Cells were washed for 15 min at 22°C in cacodylate buffer and 15 min in glass-distilled H_2O at 22°C. Samples were dehydrated through a graded series of ethanol at 22°C, twice for 5 min each in 50, 70, 95% and three times in 100%. The dehydrated samples were critical-pointed dried with liquid CO_2 in a SAM DRI-795 (Tousimis, Rockville, MD) for 60 min. Finally, the samples were placed in an argon atmosphere at 1.33×10^{-4} to 1.33×10^{-5} Pa (Denton DV502A vacuum evaporator) and coated with ~10 nm from a 60% gold/40% paladium target using a sputter coater (Denton DSM-5). The coated specimens were viewed and photographed on a JEOL 840A scanning electron microscope.

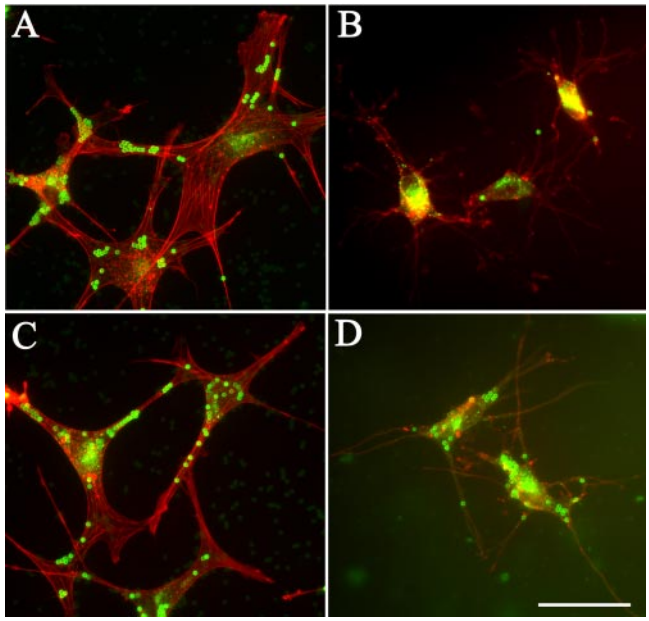

Figure 1. FN bead phagocytosis by fibroblasts on collagen-coated coverslips versus collagen matrices. Cells were attached for 30 min to collagen-coated coverslips (A) or matrices (B) and then incubated for 60 min with FN beads. Alternatively, FN beads were incubated for 60 min with collagen-coated coverslips (C) or matrices (D) and then for 60 min with cells. At the end of the incubations, samples were treated briefly with trypsin to destroy extracellular FN, fixed, permeabilized, and stained for FN (green) to identify FN-beads and actin (red) to show overall cell shape. Bar, 50 μm.

RESULTS

Phagocytosis of FN Beads by Human Fibroblasts on Collagen-coated Coverslips versus Collagen Matrices

Preliminary experiments established that BSA-coated latex beads did not bind to fibroblasts and were not phagocytosed unless the beads were precoated with FN before BSA (our unpublished data). The concentration of 2 μg/ml FN used to precoat latex beads before adding BSA was determined empirically to be sufficient to permit bead binding to the cells without causing extensive bead aggregation.

Figure 1A shows phagocytosis of FN beads by cells that were attached for 30 min to collagen-coated surfaces and then incubated for 60 min with FN beads. Overall cell morphology was visualized by fluorescent staining for actin (red). Collagen-coated coverslips are planar, smooth and rigid, and cells typically spread with flattened lamellae. Samples were treated briefly with trypsin before fixation to degrade extracellular FN and thereby make extracellular beads nonreactive with anti-FN antibodies. Trypsin treatment did not remove extracellular beads themselves, which still could be seen by phase contrast microscopy. After fixation and immunostaining of permeabilized cells, internalized FN beads (green) were identified based on their size, shape, and uniform fluorescence staining.

Figure 1B shows a parallel experiment in which fibroblasts were incubated on collagen matrices rather than coverslips before FN beads were added. Collagen matrices are fibrillar, porous, and compliant, and cells typically spread with den-

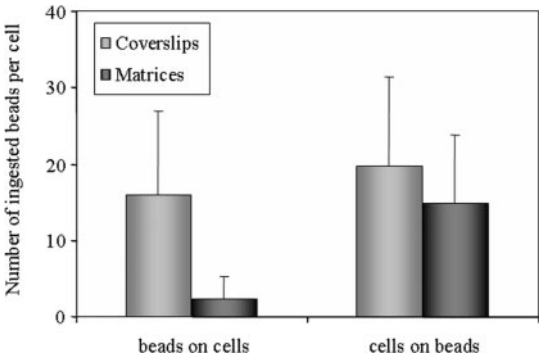

Figure 2. Decreased phagocytosis of FN beads by fibroblasts on collagen matrices. Quantification of the results of the experiment described in Figure 1. Internalized beads were counted for 50 cells in randomly selected microscope fields. Data show averages ± SD.

dritic extensions. In this case, perinuclear FN staining could be observed, but the cells contained few internalized FN beads. The results were quantified by counting the number of ingested beads/cell in randomly selected microscopic fields. Figure 2 shows that FN bead uptake by fibroblasts was much less by cells on collagen matrices compared with cells on collagen-coated coverslips.

One possible explanation for decreased phagocytosis was that FN beads bound to the collagen matrix so tightly that the beads could not be removed by the cells. To test this possibility, experiments also were carried out in which beads were placed on collagen-coated coverslips or matrices before the cells. Figure 1C compared with Figures 1A and 2 demonstrate that with collagen-coated coverslips, phagocytosis was increased only marginally if beads were added before cells. In marked contrast, Figure 1D compared with Figures 1B and 2 show that with 3D matrices, phagocytosis increased markedly if the beads were added first. Therefore, once fibroblasts interacted with collagen matrices, their ability to phagocytose fibronectin beads decreased. This decrease was observed even if the cells had attached to the matrices for only 15 min (see below).

Binding of FN Beads to Human Fibroblasts on Collagen Matrices

Bead ingestion by fibroblasts on collagen-coated coverslips or collagen matrices was integrin dependent because phagocytosis was inhibited ~80% by 5 mg/ml the fibronectin peptide GRGSDP but not by the control peptide GRGESP (our unpublished data). The inhibitory peptide had no effect on fibroblast adhesion or spreading.

In 2D culture, integrin reorganization and sequestration during cell adhesion and spreading was shown to reduce cell phagocytosis (Arora *et al.*, 2003). For example, bead uptake was greater if the beads were placed on the 2D surfaces before adding the cells compared with adding beads to cells that already had spread for 3 h. Integrin reorganization and sequestration seemed unlikely to explain why fibroblasts in collagen matrices became less phagocytic, however. Figure 3, C and D, show a typical example of a fibroblast interacting with matrices in which it can be seen that formation of actin stress fibers and integrin clustering did not occur under these incubation conditions. Previously, we reported that for fibroblasts in collagen matrices, integrin distribution on the cell surface was uniform and vinculin-containing focal adhesions were absent until after cells had remodeled and contracted the matrix (Tamariz and Grinnell, 2002). On the other hand, essentially every fibroblast attached 30 min to collagen-coated coverslips showed actin stress fibers and β1 integrins redistributed into focal adhesions (Figure 3, A and B), yet there was no decrease in phagocytosis by these cells (Figure 2).

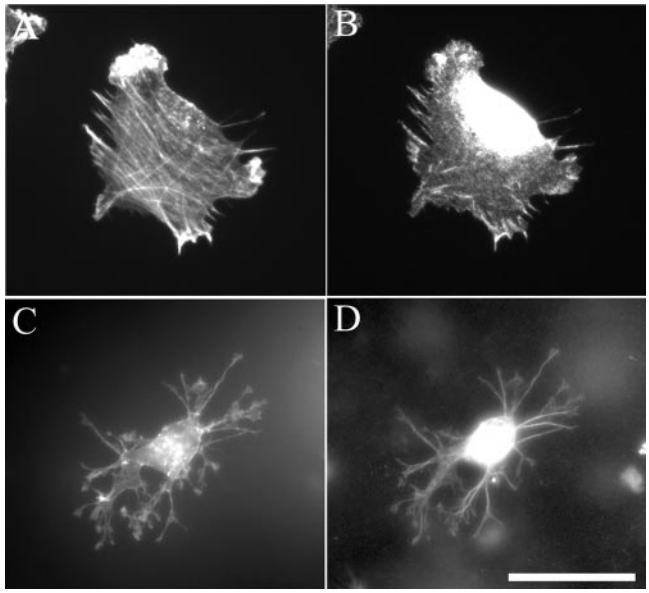

Figure 3. Distribution of β1 integrin and actin in fibroblasts on collagen-coated coverslips versus collagen matrices. Cells were attached for 30 min to collagen-coated coverslips (A and B) or matrices (C and D). At the end of the incubations, samples were fixed, permeabilized, and stained for actin (A and C) or β1 integrin (B and D). Bar, 30 μm.

Another possible explanation for decreased phagocytosis by cells in matrices was a decrease in bead binding. Figure 4, A (cells attached for 15 min followed by beads for 60 min) and C (beads attached for 60 min followed by cells for 60 min), shows the distribution of extracellular FN beads in relationship to the cells. In this case, extracellular beads were observed selectively because the samples were not permeabilized before adding anti-FN antibodies. If beads were added first, then some beads could they be seen directly beneath the cells (Figure 4C). Counts made on multiple (50) cells in randomly selected microscope fields showed that approximately the same number of beads overlapped with the cells under either condition, e.g., ~65 beads/cell in the experiment illustrated in Figure 4. Parallel samples were trypsin treated and permeabilized before immunostaining. Figure 4, B and D, show, as was observed in Figure 1, B and D, that little phagocytosis occurred if the cells had attached to the matrices before the beads were added. It seemed unlikely that the fivefold difference in bead ingestion between cells added first versus beads added first could be explained based on a difference in bead binding.

To gain a better understanding of the relationship between cells, FN beads, and matrices, additional observations were made by scanning electron microscopy. Figure 5 shows a typical example of a cell that had been incubated on the collagen matrix for 30 min after which FN beads were added for 60 min. Some ingested beads can be seen (arrow). Numerous extracellular beads seemed to be attached at cell margins and cellular dendritic extensions except where the extensions were within the collagen matrix.

Formation of dendritic extensions can be seen in Figure 6, which shows representative examples of cells interacting

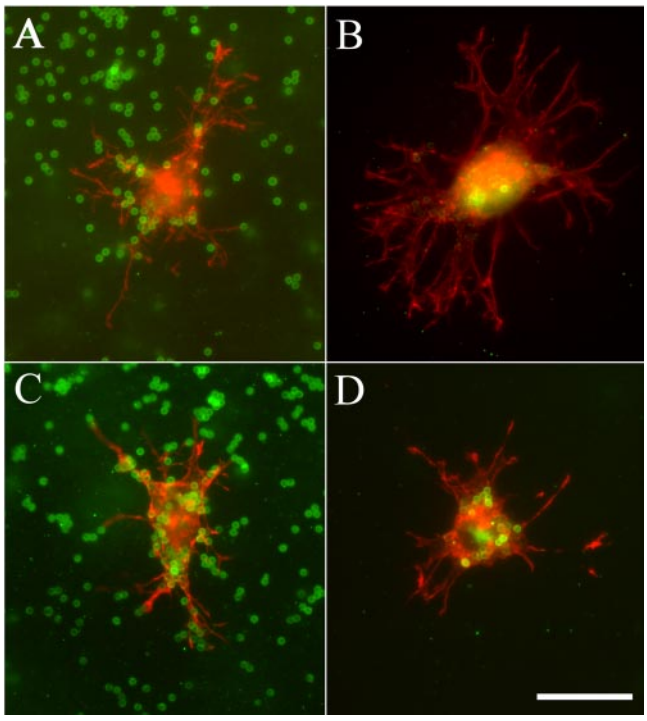

Figure 4. FN bead binding and phagocytosis by fibroblasts on collagen-coated matrices. (A and B) Cells were attached for 15 min to collagen matrices and then incubated for 60 min with FN beads. (C and D) FN beads were incubated for 60 min with matrices and then for 60 min with cells. At the end of the incubations, samples A and C were fixed and stained for FN (green) and actin (red). Samples B and D were treated briefly with trypsin to destroy extracellular FN and then fixed, permeabilized, and stained as described above. Bar, 30 μm.

with collagen matrices after various incubation times. Dendritic extensions began to protrude as early as cells could be observed to attach to the collagen matrix (5 min). Subsequently, the extensions increased in length and penetrated into the porous collagen network, eventually becoming difficult to distinguish by scanning electron microscopy from the collagen fibrils themselves. As will be suggested below, entanglement of the extensions with collagen fibrils may make them unavailable for interacting with FN beads.

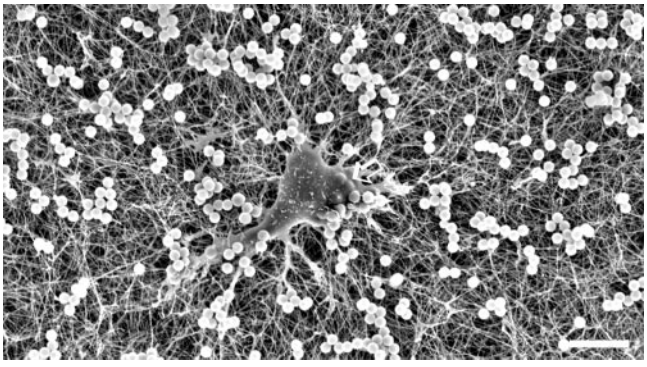

Figure 5. Distribution of FN beads on fibroblasts in 3D collagen matrices. Cells were attached for 30 min to collagen matrices and then incubated for 60 min with FN beads. Subsequently, the samples were fixed, processed, and examined by scanning electron microscopy. Arrow points to ingested FN bead. Bar, 10 μm.

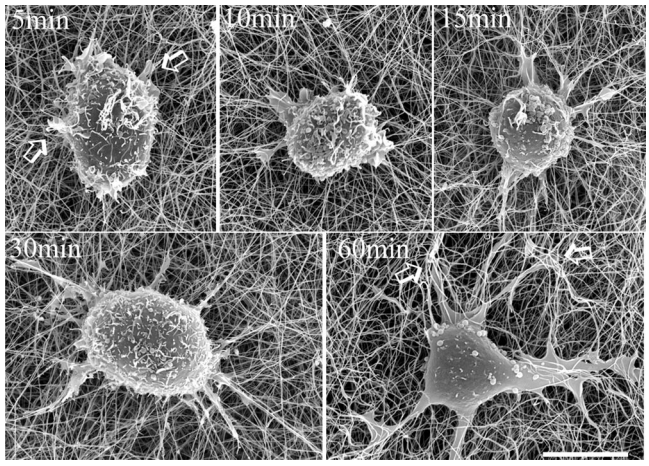

Figure 6. Protrusion of dendritic extensions into the collagen matrix. Cells were attached to collagen matrices for the time periods shown. Subsequently, the samples were fixed, processed, and examined by scanning electron microscopy. At 5 min, arrows show dendritic extensions beginning to form. At 60 min, arrows show ends of extensions becoming difficult to distinguish from the collagen fibrils. Bar, 10 μm.

Differential Trypsin Sensitivity of Fibroblasts Attached to Collagen Matrices versus Collagen-coated Coverslips

In developing the trypsin technique to distinguish ingested FN beads, we noticed a difference in sensitivity of the cells on coverslips versus matrices. That is, addition of 0.25% trypsin for 6 min to cells on collagen matrices was optimal to degrade FN, and this treatment had no detectable effect on cell shape. On collagen-coated coverslips, by contrast, the same treatment caused cells to start rounding up and detach. For this reason, we routinely used a shorter time (3 min) for treating cells on coverslips.

The difference in trypsin sensitivity suggested that there might be differences in mechanisms of adhesion for fibroblasts on coverslips versus matrices. Additional experiments confirmed this possibility. Figure 7 shows that incubation with 0.25% trypsin-1 mM EDTA (trypsin/EDTA) caused essentially 100% removal of cells that had previously attached for 30 min to collagen-coated coverslips but caused only rounding and no detachment of fibroblasts that were attached to collagen matrices.

Cells were examined by scanning electron microscopy before and after trypsinization. Figure 8A shows a typical fibroblast that had spread for 30 min with dendritic extensions projecting into the matrix, and at higher magnification entanglement of the tips of extensions with collagen fibrils (Figure 8B). Figure 8C shows a representative cell beginning to round up after treatment with trypsin/EDTA for 20 min. Disentanglement of the extensions and collagen fibrils did not occur, but breaks were observed between the cell bodies and extensions (Figure 8, C, arrow, and D). Before trypsin treatment, only 1 of 10 cells had such breaks in dendritic extensions; whereas after trypsin treatment, slight more than half (52%) of the cells had entangled extensions with breaks (our unpublished data). These findings suggested that entanglement of fibroblast extensions with collagen fibrils resulted in strong cell anchorage. Such an anchorage mechanism would be unique to 3D matrices and, given the trypsin results, potentially independent of integrins.

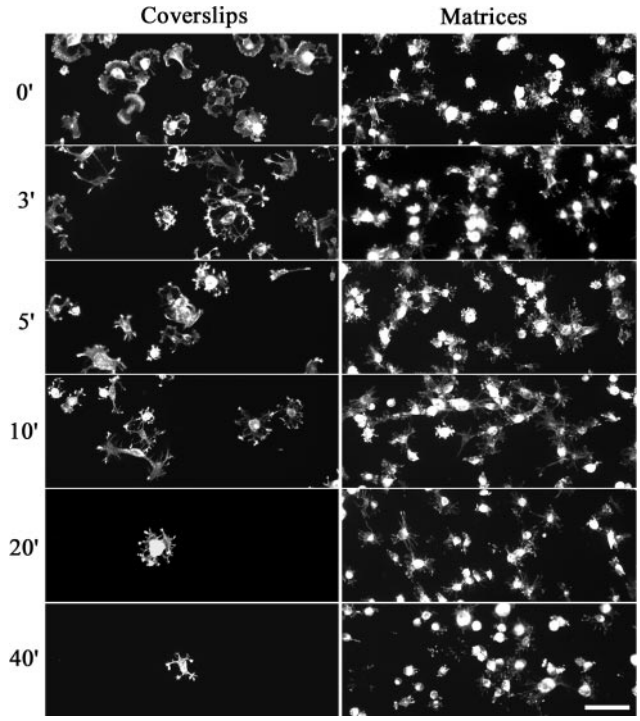

Figure 7. Differential detachment of fibroblasts on collagen-coated coverslips and collagen matrices in response to trypsin/EDTA. Cells were attached for 30 min to collagen-coated coverslips or matrices, washed, and then incubated with medium containing trypsin/EDTA. At the times indicated, the samples were fixed, permeabilized, and stained for actin. Bar, 100 μm.

Evidence That Anchorage of Fibroblasts to Collagen Matrices Develops an Integrin-independent Component

In collagen matrices, integrin $\alpha 2\beta 1$ has been implicated in fibroblast adhesion although other integrins may also be important, including $\alpha 1\beta 1$, $\alpha 11\beta 1$, and $\alpha v\beta 3$ (Klein *et al*., 1991; Schiro *et al*., 1991; Carver *et al*., 1995; Cooke *et al*., 2000; Tiger *et al*., 2001; Jokinen *et al*., 2004). Addition of blocking antibodies against $\alpha 2\beta 1$ (10 μg/ml) inhibited human fibroblast adhesion to collagen-coated but not FN-coated coverslips (our unpublished data). Figure 9 demonstrates that this concentration of blocking antibody also decreased cell attachment and spreading on collagen matrices compared with untreated cells.

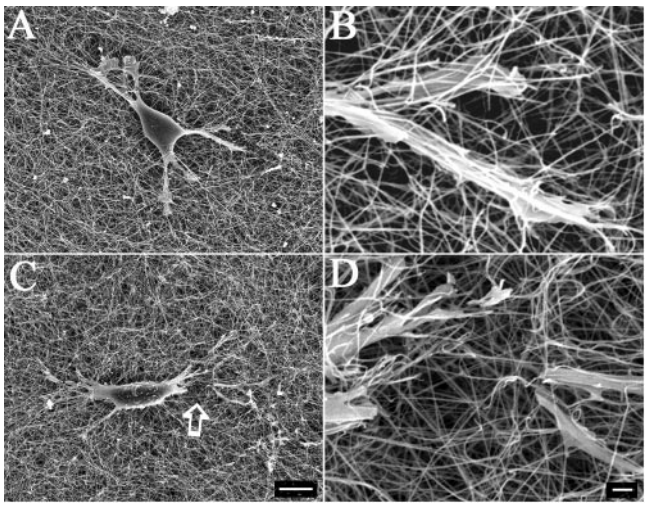

Figure 8. Entanglement of collagen fibrils with fibroblast dendritic extensions. Cells were attached for 30 min to collagen matrices and then fixed (A and B) or washed and treated for 20 min with trypsin/EDTA (C and D). Subsequently, the samples were fixed, processed, and examined by scanning electron microscopy. Arrow points to breaks in cellular dendritic extensions. Bar, 10 μm (A and C); 1 μm (B and D).

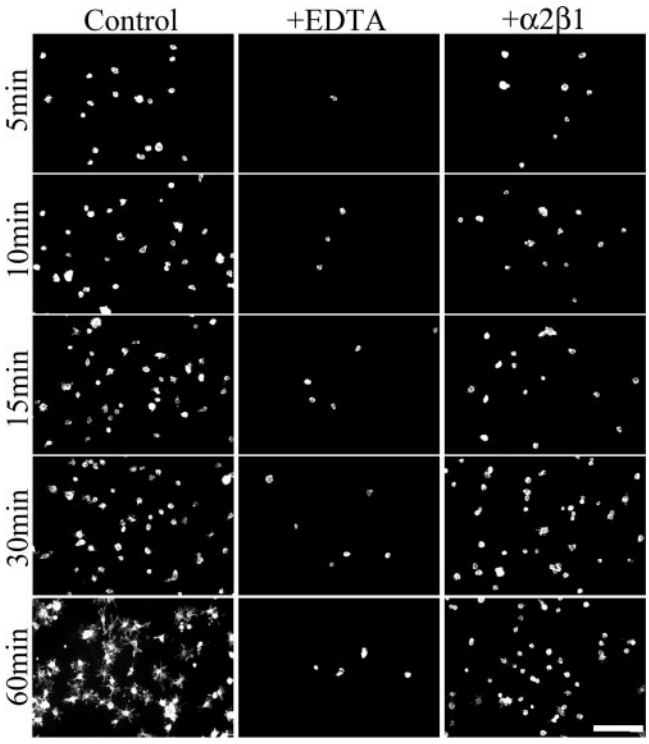

Figure 9. Effect of EDTA and integrin $\alpha 2\beta 1$ blocking antibody on cell adhesion to collagen matrices. Cells were attached to collagen matrices for the time periods shown in medium containing 10 μg/ml anti-integrin $\alpha 2\beta 1$ blocking antibody or 10 mM EDTA as indicated. At the end of the incubations, the samples were fixed, permeabilized, and stained for actin. Bar, 200 μm.

Integrin function also depends on divalent cations (Gailit and Ruoslahti, 1988; Leitinger *et al*., 2000). Figure 9 shows that fibroblast adhesion to collagen matrices was almost completely inhibited in the presence of 10 mM EDTA consistent with integrin dependence of adhesion. If, however, fibroblasts had attached to the matrix for 30 min, then as demonstrated by Figure 10, incubation with 10 mM EDTA was unable to release the cells from the matrix. Fibroblasts that had previously attached and spread on collagen-coated coverslips were detached completely. Together, the abovementioned findings demonstrated that fibroblast adhesion to the collagen matrix was initially integrin dependent, but then an integrin-independent component of anchorage developed, which did not occur on collagen-coated coverslips.

DISCUSSION

Fibroblasts in tissues are phagocytic cells (McGaw and Ten Cate, 1983; Everts *et al*., 1996, 2003), and in culture they have been shown to ingest both fibronectin and collagen-coated latex particles (Grinnell and Geiger, 1986; McCulloch and

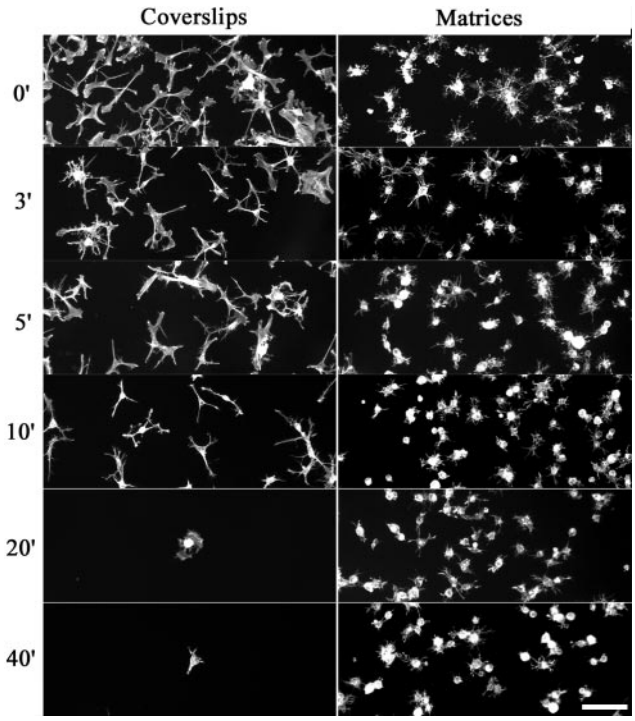

Figure 10. Differential detachment of fibroblasts on collagen coated-coverslips and collagen matrices in response to 10 mM EDTA. Cells were attached for 30 min to collagen-coated coverslips or matrices, washed, and then incubated with medium containing 10 mM EDTA. At the times indicated, the samples were fixed, permeabilized, and stained for actin. Bar, 100 μm.

Knowles, 1993). In the present study, we found that phagocytosis of fibronectin-coated beads by fibroblasts on collagen matrices was reduced compared with cells on collagen-coated coverslips. As will be discussed, this decrease seemed to occur because entanglement of cellular dendritic extensions with collagen fibrils interfered with the ability of the extensions to interact with the beads. Moreover, once these extensions became entangled in the matrix, cells developed an integrin-independent component of adhesion.

Decreased phagocytosis by fibroblasts on collagen matrices versus collagen-coated coverslips did not seem to result from receptor differences. In either case, phagocytosis was inhibited by the fibronectin peptide GRGDSP, and integrin clustering was less by cells on matrices compared with coverslips. Also, cells on matrices phagocytosed FN beads as well as cells on coverslips if the beads were allowed to bind to the matrices before the cells were added.

With cells on collagen matrices, comparing the conditions cells added first versus FN beads added first, overall bead binding seemed similar even though there was a fivefold difference in bead uptake. Examination of the samples by scanning electron microscopy showed that when the cells were added first, FN beads seemed to be attached at cell margins but not to dendritic extensions where the extensions penetrated into the collagen matrix. These extensions began to protrude as early as cells could be observed to interact with the collagen matrix and increased in length within the porous collagen network, eventually becoming difficult to distinguish by scanning electron microscopy from the collagen fibrils themselves. Because phagocytosis by fibroblasts uses cell membrane ruffles (Grinnell and Geiger, 1986; Arora *et al*., 2003), and ruffling activity of fibroblast dendritic extensions has been shown to occur primarily at the tips of the extensions (Grinnell *et al*., 2003), constraint of dendritic extensions by their entanglement with the collagen matrix likely accounts for decreased phagocytosis.

Entangled dendritic extensions seemed to be strongly anchored in the collagen matrix, and breaks between the cell bodies and extensions occurred when cell were treated with 0.25% trypsin-1 mM EDTA. Surprisingly, this treatment did not cause cells to detach from collagen matrices although essentially 100% of the cells were removed from collagen-coated coverslips. This difference in trypsin sensitivity suggested that the cells might be anchored to collagen matrices at least in part by an integrin-independent mechanism.

Fibroblasts interact with and attach to collagen matrices primarily through integrin $\alpha 2\beta 1$ but also with other integrins, including $\alpha 1\beta 1$, $\alpha 11\beta 1$, and $\alpha v\beta 3$ (Klein *et al*., 1991; Schiro *et al*., 1991; Carver *et al*., 1995; Cooke *et al*., 2000; Tiger *et al*., 2001; Jokinen *et al*., 2004), and blocking antibodies against integrin $\alpha 2\beta 1$ slowed down the time course of fibroblast adhesion and spreading on collagen matrices consistent with previous findings.

Divalent cations also have been implicated in integrin activity (Gailit and Ruoslahti, 1988; Leitinger *et al*., 2000), and in the presence of 10 mM EDTA, cell adhesion to collagen matrices was almost completely inhibited. Yet, once the cells were attached, treatment with 10 mM EDTA was unable to release cells from collagen matrices, even though cells on collagen-coated coverslips detached completely. Therefore, in contrast to cell adhesion on collagen-coated coverslips, cell adhesion to collagen matrices seemed to develop an integrin-independent component of anchorage.

Although biochemical adhesion mediated by integrins and other cell–matrix receptors have been the primary focus of research to understand how cells interact with their surrounding matrices, mechanical interactions independent of integrins have been implicated in the amoeboid propulsion of leukocytes and some tumor cells as they squeeze through the interstices of 3D matrices (Friedl *et al*., 1998; Wolf *et al*., 2003). Our findings indicate that mechanical interactions independent of integrins can be important not only in cell migration but also in cell anchorage.

In conclusion, we suggest that cell–matrix entanglement provides a novel mechanism of integrin-independent cell anchorage not previously recognized and reflects a mechanism of anchorage uniquely reliant on the three-dimensional character of the matrix. As advances in tissue engineering lead to development of new fabrics to be used as cell scaffolds (Li *et al*., 2002; Mo *et al*., 2004; Smith and Ma, 2004; Kwon *et al*., 2005), the potential to regulate cell anchorage to these scaffolds through mechanical entanglement should become an important design element.

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