Cell Communication & Adhesion

ISSN: 1541-9061 (Print) 1543-5180 (Online) Journal homepage: informahealthcare.com/journals/icac20

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To cite this article: Erik F. Young & Eugene E. Marcantonio (2007) A Novel Subcellular Collagen Organization Process Visualized by Total Internal Reflection Fluorescence Microscopy, Cell Communication & Adhesion, 14:5, 169-180, DOI: 10.1080/15419060701755552

To link to this article: https://doi.org/10.1080/15419060701755552

Published online: 11 Jul 2009.

Cell Communication and Adhesion, 14: 169–180, 2007

ISSN: 1541-9061 print / 1543-5180 online
DOI: 10.1080/15419060701755552

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A Novel Subcellular Collagen Organization Process Visualized by Total Internal Reflection Fluorescence Microscopy

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The $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins belong to a family of cell-surface molecules involved in structural contacts and signal-transduction events across the cell membrane. Employing two-dimensional substrates coated with fluorescently labeled type I collagen, we have discovered a novel subcellular matrix remodeling event that is particular to cells that express the fibrillar collagen receptor $\alpha 2\beta 1$. Cells expressing $\alpha 1\beta 1$ also perform this collagen organization process, but less proficiently. This work will provide a basis for subsequent studies of cell-mediated collagen fibril assembly.

Keywords collagen, integrin, matrix, remodeling

INTRODUCTION

Maintenance of tissue architecture in response to load or injury involves the destruction, synthesis, and reorganization of the extracellular matrix (ECM), seen especially in wound healing (Midwood et al. 2004). A principal ECM molecule involved in the structural integrity of various tissues is collagen. Collagens are a well characterized group of ECM proteins which comprise higher order load-bearing fibrils, sheet-forming branched fibrils, and other structures, many of which provide a scaffold in which living cells reside (van der Rest and Garrone 1991). The collagens which provide tensile strength in the skin, tendons, and other tissues are fibrillar collagens (Kucharz 1992).

Collagen is synthesized intracellularly and secreted in a pro-form with high solubility (Hulmes 2002). This protease-resistant collagen monomer, with its characteristic central triple helix, is then secreted into the extracellular space where metalloendoproteases specific for the N- and C-terminal pro-peptides cleave the ends of the monomer, drastically reducing its solubility (Gelman et al. 1979; Kadler et al. 1990; Miyahara et al. 1984; Prockop et al. 1998). *In vitro* self-assembly of collagen fibrils from monomeric subunits has been studied exhaustively for decades (Kadler et al. 1996), and the conditions under which this assembly occurs have

Received 29 September 2006; accepted 11 September 2007.
TIRF microscope setup was provided by NIH grant 1S10RR017911. We thank L. Hughes for technical assistance, and G. Gundersen and J. Schmoranzer for microscopy assistance and discussions.
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been known for some time (Wood 1960; Wood and Keech 1960).

In vivo, large-diameter, load-bearing collagen fibrils have been seen to be intimately associated with the fibroblasts in connective tissue of the tendon (Wasserman 1954). The diameter of these fibrils is regulated by collagen-associated ECM proteins. The proteins tenascin (Minamitani et al. 2004) and collagen V (Wenstrup et al. 2004) limit the diameter of collagen fibrils in skin while decorin (Danielson et al. 1997), lumican (Jepsen et al. 2002), and fibromodulin (Svensson et al. 1999) promote larger-diameter fibrils. Exactly how cells align collagen monomers for controlled polymerization and higher-order assembly is not fully understood, but this process undoubtedly involves receptors for ECM.

The cell-surface molecules that mediate the interaction between cells and the ECM are the integrins. Integrins are a superfamily of heterodimeric transmembrane glycoproteins responsible not only for structural connections to a wide variety of collagen and non-collagen ECM molecules, but are also competent signal-transducing molecules (Hynes 2002). The collagen-binding integrins $\alpha1\beta1$, $\alpha2\beta1$, and the more recently cloned $\alpha10\beta1$ and $\alpha11\beta1$ (White et al. 2004) demonstrate different affinities for fibrillar collagen. The integrin $\alpha2\beta1$ and its purified I-domain bind both fibrillar, type I collagen (Col I) and branched, type IV collagen (Col IV) but have a higher affinity for Col I, while $\alpha1\beta1$, also capable of binding both types of collagen, has a higher affinity for Col IV (Kapyla et al. 2000; Kern et al. 1993; Nykvist et al. 2000; Vandenberg et al. 1991). Integrins clearly play a role in arrangement of collagen monomers as $\alpha2\beta1$ not only facilitates cell adhesion to collagen but can also bind to the C-terminal pro-peptide (Weston et al. 1994), that is critical to modulating solubility of monomers.

Analysis of intact, isolated collagen fibrils from tissues has provided several advances in understanding of the architecture and *in vivo* assembly of collagen fibrils (Birk et al. 1996; Birk et al. 1995). While the requirements for the assembly of collagen have been revealed by *in vitro* studies, we wish to understand the mechanism by which individual cells initiate and direct the extracellular assembly of collagen fibrils. In characterizing this process, we seek to delineate the role of collagen-binding integrins $\alpha2\beta1$ and $\alpha1\beta1$ in several cell types.

MATERIALS AND METHODS

Preparation and Immunostaining of Fluorescent Collagen Substratum for Total Internal Reflection Fluorescence (TIRF) Microscopy and Epifluorescence Microscopy

Glass coverslips were coated with 500 μL each of a 100 μg/mL solution (160 ng/mm^2) of fluorescein isothiocyanate (FITC)–conjugated bovine type I collagen (Chondrex, Walkersville, MD) for one hour at 37°C. Media containing cells was added directly to the collagen-coated glass coverslips for the various times indicated. After incubation, cells were fixed with 4% (w/v) paraformaldehyde in PBS for 10 min. Primary antibody solution (monoclonal anti-vinculin from Sigma; anti–alpha 2 integrin-12F1 from BD Pharmingen) (10% normal goat serum in PBS with desired dilution of antibody; 70 μL/coverslip) was added to the parafilm and then the coverglass was laid down atop this staining solution and incubated thus in a humidified chamber for 30 min. The coverglass was washed twice in PBS. New 70 μL portions of secondary antibody solution were laid down on the parafilm and incubated as for the primary antibody. All secondary antibodies were from Molecular Probes.

TIRF Microscopy and Imaging

For TIRF microscopy, preparations were observed using a 60 X apo objective (NA = 1.45) on a Nikon TE2000-U microscope equipped with a TIRF illuminator and fiber optic–coupled laser illumination. Samples were excited at wavelengths of 488 nm and at 543 nm with Ar ion and HeNe lasers, respectively. This illumination was optimized for the fluorophores employed using filter cubes (Chroma Technology, Rockingham, VT). In

general, the penetration depth of illumination of the TIRF field was 100–150 nm. This depth was determined by aligning the angle of the laser such that we were just beyond the critical angle to get TIRF, using the Bertrand lens. Using the formulas on the web site http://www.microscopyu.com/ articles/fluorescence/tirf/tirfintro.html, we estimate the penetration depth to be approximately 100 nm, using the Nikon 60X objective with an aperture of 1.44 and considering the refractive index of aqueous media. Images were captured with a back-illuminated cooled CCD camera with a resolution of 6 μm/pixel and an image capture area comprised of 1344 pixels $\times$ 940 pixels (Princeton Instruments, Trenton, NJ). Images were captured and recorded using Metamorph^{TM} software (Universal Imaging Corp., West Chester, PA).

Cell Lines and Culture

HT1080 Cells (CCL-121) were acquired from American Type Culture Collection (ATCC; Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Primary human cardiac fibroblasts (HCF) were purchased from ScienCell Research Laboratories (San Diego, CA) or Cell Applications Inc. (San Diego, CA) and maintained in proprietary media provided by each company for the respective cell line. NIH3T3 fibroblast cell lines HA1 and HA2 cells were produced as described elsewhere (Briesewitz et al. 1993 and Kern and Marcantonio 1998). All NIH3T3 cell lines were cultured in DMEM with 10% Calf serum (CS).

RESULTS

Several factors were considered in examining the manipulation of extracellular collagen by cultured cells. Collagen-coated surfaces have been widely used to assay integrin binding affinity (Vandenberg et al. 1991) and integrin-mediated signal transduction (Wary et al. 1998). We initially chose the human fibrosarcoma line HT1080 because it has been used in experiments to characterize the collagen-binding function of the $\alpha 2\beta 1$ integrin, which is expressed robustly on the surface of these cells (Whittard et al. 2002). We employed a commercially available bovine FITC-conjugated Col I preparation (FITC Col I) to monitor manipulation of immobilized monomeric collagen on glass coverslips. Glass coverslips were coated with the material and examined after cells had interacted with the matrix.

Many different microscopic techniques are available for the examination of fibrillar collagens (Meek and Fullwood 2001). Of these different methods, total internal reflection fluorescence (TIRF) microscopy proved to be the most useful. With TIRF microscopy, fluorescent excitation is provided by an evanescent wavefront that propagates normal to the plane of incidence of illuminating laser light. The intensity of this evanescent wave decays exponentially as distance from the substrate increases, thereby reducing noise and preferentially illuminating substrate proximal fluorophores (Axelrod 2001; Toomre and Manstein 2001). TIRF microscopy has already been utilized to characterize the adhesion apparatus of cells and its interaction with the cytoskeleton (Axelrod 1981). We chose to use TIRF microscopy to examine the interface between cells and the collagen-coated glass substrates.

To ensure that collagen fibril assembly did not occur autonomously, cell-free zones of the substrate (identified by the lack of vinculin staining) were checked after a three-hour incubation and no fibrils were detected (Fig. 1 (a)). HT1080 cells plated on FITC Col I organized this material into fibrils, which were seen in both fixed and live-imaged cells (Fig. 1 (b)). To determine whether the organized collagen fibrils were extracellular or intracellular, cells were plated down on FITC Col I and allowed to spread and organize the matrix for three hours and then removed by treatment with 0.4 g/L EDTA for 15 min, causing integrins to disengage from the substrate. Collagen fibrils that were organized by cells remained associated with the glass substrate (Fig. 2).

Mixed matrix systems are a complicated environment and do not permit study of collagen manipulation via individual integrin subunits. Importantly, HT1080 cells express the $\alpha 5\beta 1$ integrin with which

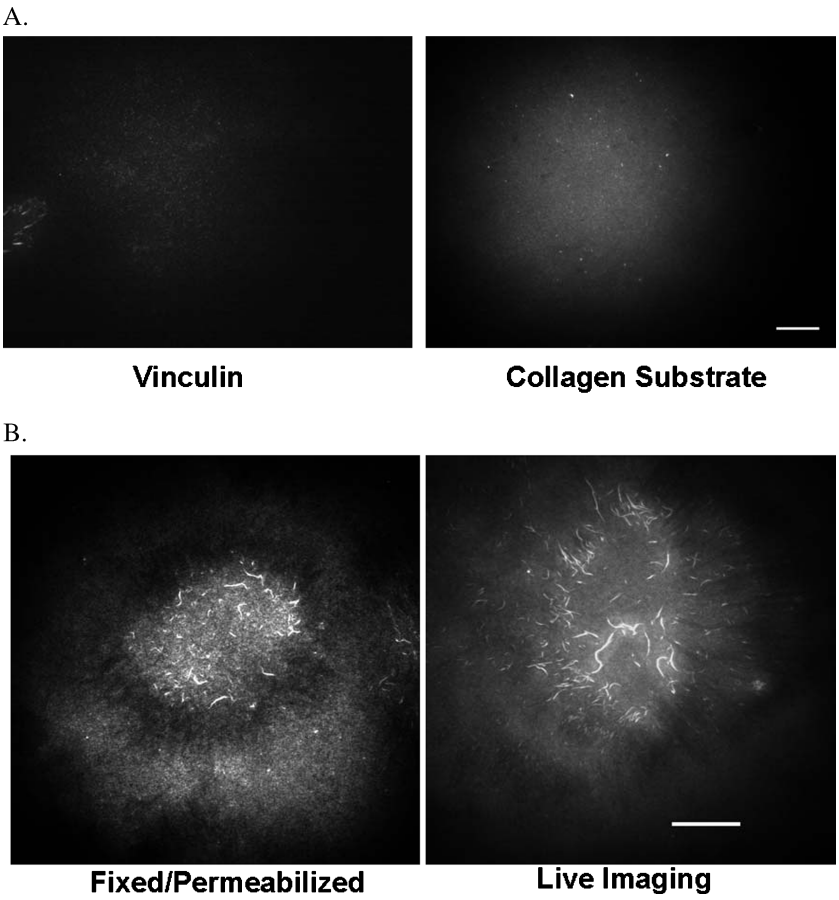

Figure 1. TIRF microscopy of HT1080 cells on FITC Col I. Glass coverslips were coated with FITC Col I as described in Methods. When incubated in culture conditions with cells for three hours, those areas that are free of cells (identified by the lack of staining using antibodies raised against the focal adhesion protein vinculin) do not exhibit collagen fibril assembly as shown in (a). HT1080 cells were plated on glass coverslips coated with FITC Col I and allowed to spread for two hours in culture before examination by live TIRF microscopy shown in (b). As indicated, other cells were fixed with 2% paraformaldehyde and permeabilized with 0.2% Triton-X 100 before examination by TIRF microscopy. FITC Col I is organized in both fixed and living cells and this organization does not occur in cell-free zones of the substratum. Bar = 15 μm.

these cells organize fibronectin matrices that they secrete themselves (Brenner et al. 2000). Attributing specific integrins to matrix reorganization events is therefore complicated given that fibronectin and collagen may bind to each another. Fibronectin and collagen are known to be intimately associated with each other *in vivo* (Little and Chen 1982), and the association of fibronectin with collagen has been found to modulate *in vitro* collagen assembly (Wood 1960a; Kleinman et al. 1981; Speranza et al. 1987). Cell-mediated collagen fibril assembly using fluorophore conjugated collagen has already been studied in primary vascular smooth muscle cells using a mixed matrix system (Li et al. 2003). In this work, cells were plated on a fibronectin substrate to which collagen was added. This added collagen became incorporated, organized and concentrated in the resultant matrix fashioned by the cells.

In order to focus on collagen interactions with integrin receptors, we needed to ensure that our system did not rely on a fibronectin matrix. To rule out matrix molecule production by cells in fibril assembly, the protein synthesis inhibitor cycloheximide was used. HT1080 cells continued to perform collagen

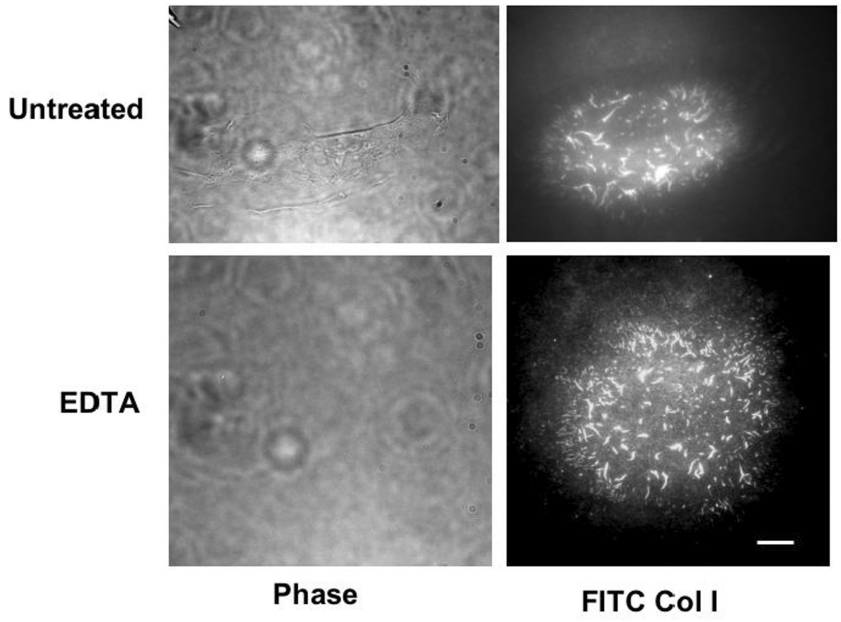

Figure 2. TIRF microscopy of HT1080 cells and their adhesion sites. HT1080 cells were plated on FITC Col I coated coverslips and allowed to spread for three hours in culture. Cells were subsequently left untreated or were treated with 0.4g/L EDTA for 15 minutes as indicated to permit cell detachment. The resultant cell-bearing, or cell-depleted coverslips were fixed in paraformaldehyde in parallel and mounted for analysis by TIRF microscopy. Bright-field and FITC fluorescence images were captured at adhesion sites on coverslips with and without cells. Organized collagen fibrils remain associated with the substrate when cells are detached from the substratum. Bar = 15 μm.

fibril organization with cycloheximide treatment relative to their untreated counterparts, demonstrating that protein synthesis is not required for this organization (Fig. 3). In this particular experiment, there was less fibril formed than in some other experiments shown. With the exception of this experiment, all other work shown is performed with a single preparation of FITC Col I. While we observe variation in

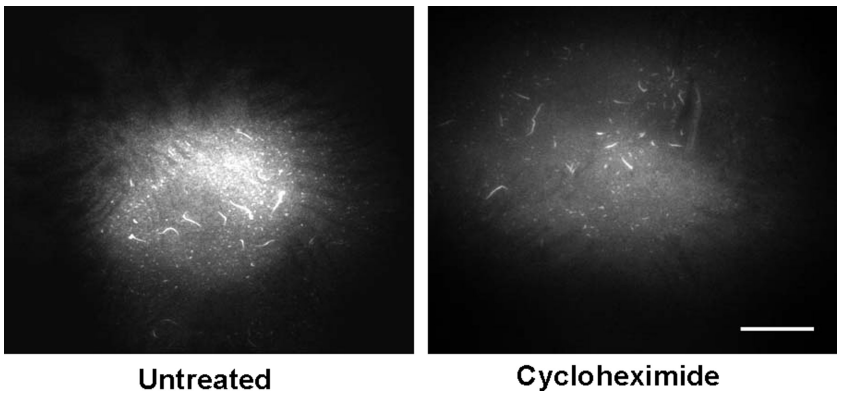

Figure 3. TIRF microscopy of Cycloheximide treated HT1080 cells. HT1080 cells in culture were treated with 40-μg/mL cycloheximide or vehicle alone for two hours and then trypsinized and then plated on FITC Col I–coated coverslips and allowed to spread for two hours in the continued presence of 20-μg/mL cycloheximide or vehicle alone. Cells were subsequently fixed in paraformaldehyde and mounted for analysis by TIRF microscopy. *De novo* synthesis of proteins is not required for subcellular collagen organization. Bar = 15 μm.

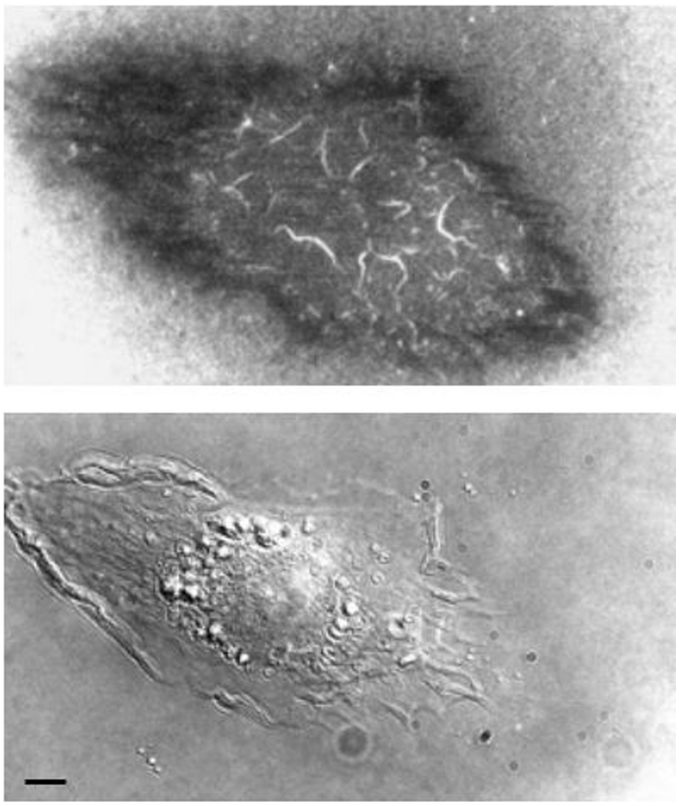

Figure 4. HT1080 morphology on FITC Col I. HT1080 cells were allowed to spread for two hours on FITC Col I and were subsequently fixed and visualized using TIRF and DIC. The top panel shows the FITC collagen image, with darkened areas in the periphery and fibrils under the center of the cell. The bottom panel shows the outline and edges of the cell membranes via DIC. Bar = 15 μm.

the degree of fibril formation depending on the lot of FITC Col I employed (not shown), cycloheximide did not impair the degree of fibril formation of any particular preparation.

In the process of characterizing the fibrils organized by cells, several observations were made. Concurrently with fibril formation under cells, darkened zones of excluded material are generated adjacent to visible fibrils formed, which are primarily concentrated under the center of the cell (Fig. 4). The adhesion apparatus of the cell was visualized with antibody staining for the focal adhesion protein vinculin and for the collagen binding alpha 2 integrin (Fig. 5). We find that focal adhesions overlap with the areas of collagen depletion and are probably the sites of incipient fibril formation, because fibrils that are very close to the radial focal adhesions are aligned with those focal adhesions (Fig. 5). Closer to the cell centroid, however, fibrils are not oriented in an orderly fashion when compared to fibrils closer to the collagen depleted sites.

To further characterize fibril formation, the kinetics of fibril lengthening were examined in primary human cardiac fibroblasts that express $\alpha 2\beta 1$ and $\alpha 1\beta 1$ integrins. These collagen binding integrins are important in human cardiac fibroblast (HCF) biology. IGF-1 treatment of neonatal rat cardiac fibroblasts, for example, resulted in increased migration and gel contraction, and this was blocked with antibodies to both $\alpha 1$ and $\beta 1$ (Kanekar et al. 2000). The $\alpha 1\beta 1$ integrin is also expressed in adult rat cardiac fibroblasts, and is critical for migration on collagen substrates and contraction of 3-D collagen gels. Furthermore, the surface expression of $\alpha 2\beta 1$ in these

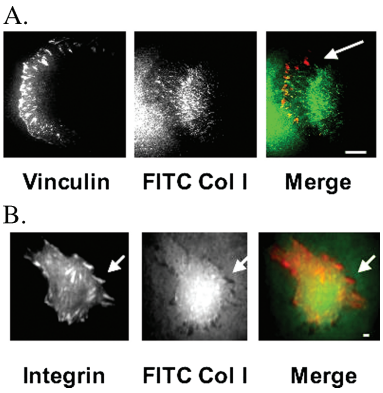

Figure 5. TIRF microscopy of focal adhesions in HT1080 cells plated on FITC Col I. HT1080 cells were allowed to spread for two hours on FITC Col I and were subsequently fixed, permeabilized and stained as described in Methods, with (a) antibodies raised against the focal adhesion protein vinculin, or (b) the collagen-binding integrin $\alpha 2\beta 1$. Merged images show antibody staining in red and FITC Col I in green. Darkened zones of collagen depletion (arrows) are created as cells reorganize the collagen substrate and these zones co-localize with focal adhesions, as shown by both (a) vinculin and (b) alpha 2 integrin. Bar = 15 μm.

cells is robust (Carver et al. 1995). We found that the number of fibrils formed in the cardiac fibroblasts was lower than that of HT1080 cells, making quantitation of fibrils easier in HCFs. When observed over a three-hour time course, the number and length of fibrils increased in a time-dependent fashion (Fig. 6).

To examine the contribution of individual I-domain–containing collagen receptors in this collagen organization process, NIH 3T3 mouse embryo fibroblast lines were used. These cell lines have been described by our laboratory previously and have no endogenous collagen receptor expression. These cells were stably transfected with cDNAs encoding the human $\alpha 1$ integrin (HA1 cells) or the human $\alpha 2$ integrin subunit (HA2 cells). These transfected subunits dimerized with the endogenous $\beta 1$ subunit and form functional collagen receptors and are expressed on the cell surface at comparable levels as assayed by flow cytometry (Briesewitz et al. 1993; Kern and Marcantonio 1998). We found that with the parental 3T3 cell line, virtually no spreading or adhesion took place as expected based on our previous work (data not shown). HA1 cells and HA2 cells were able to spread on the FITC Col I matrix and perform the collagen organization process as with HT1080 cells. However, in agreement with the higher affinity for $\alpha 2\beta 1$ integrin with Col I, HA2 cells were much more proficient at collagen fibril assembly than HA1 cells (Fig. 7).

In order to determine what role the $\alpha 2\beta 1$ integrin played in facilitating this collagen organization process, both HA1 and HA2 cells were stained with monoclonal antibodies to determine the location of the focal adhesion apparatus containing the recombinant collagen binding integrins exogenously expressed in these cells. HA1 cells and HA2 cells both form focal adhesions on the collagen substrate provided and both demonstrate alignment of the focal adhesions with darkened areas of collagen depletion from the substrate. HA2 cells, expressing the $\alpha 2\beta 1$ integrin exhibit more robust fibril formation (Fig. 7). As shown above in Figure 5, focal adhesions formed by the integrin alpha 2 on FITC–collagen appear in depleted zones of collagen fluorescence.

DISCUSSION

This work describes the cell-mediated assembly of higher order collagen fibril structures in culture. This novel collagen matrix organization process takes place in two-dimensional (2-D) culture where cells are plated on a collagen-coated glass substrate. This collagen fibril organization occurs in multiple cell types which endogenously express the $\alpha 2\beta 1$ integrin: the N-*ras*–transformed fibrosarcoma line HT1080 (Rasheed et al. 1974), and primary human cardiac fibroblasts. We also found that NIH3T3 mouse embryo fibroblasts, devoid of a functional collagen adhesion apparatus, executed this process when transfected with either $\alpha 2$ or $\alpha 1$ integrin subunits.

As all collagen fibril organization in this work is commensurate with collagen-binding integrin expression, the integrins $\alpha 2\beta 1$ and $\alpha 1\beta 1$ appear to be necessary for fibril formation. As this process of depleting collagen from the substrate and concentrating it in higher order collagen structures is only somewhat understood, a claim as to whether

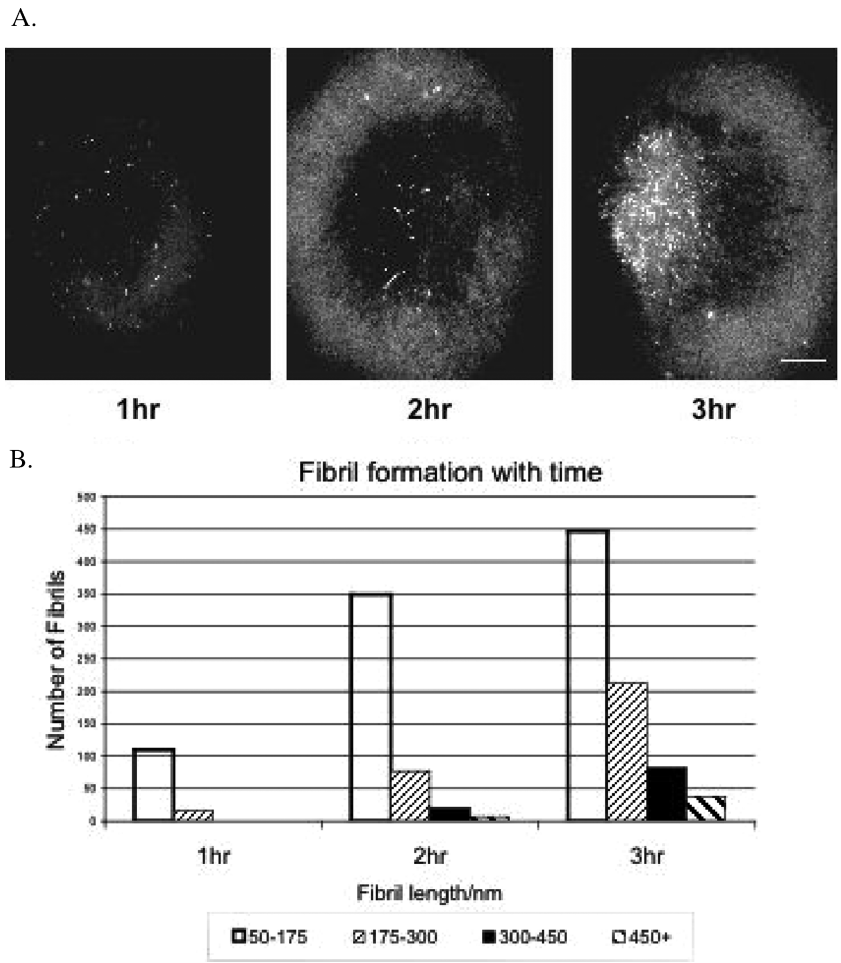

Figure 6. Quantitation of fibril size with time in primary human cardiac fibroblasts. Cells were allowed to spread for one, two or three hours on FITC Col I and were subsequently fixed, permeabilized and stained per Methods with monoclonal antibodies raised against the focal adhesion protein vinculin. Twenty rounded cells were imaged from each coverslip and of these twenty images, ten images were selected at random for quantitation. Quantitation was performed using the METAMORPH^{TM} software, which recorded the number and length of fibrils traced with a mouse pointing device. These data sets were exported to Excel (Microsoft; Redmond, Washington) spreadsheets and histograms were generated using the Data Analysis suite within Excel. Representative cells from each time point were imaged by TIRF microscopy and are shown in (a). Binned fibril length and number data from each experiment were plotted using Excel as shown in (b). The number and length of fibrils organized by primary human cardiac fibroblasts increases with time.

integrins are sufficient for this process is difficult. While these integrins mediate adhesion and spreading on a collagen substrate, these functions are unrelated to changes in the physical chemistry of the subcellular compartment that alone could support collagen fibril organization.

There were several advantages to the system outlined in this work. The integrin repertoire of HT1080 cells was known and advances in integrin-mediated signal transduction have been made using this same cell line. While the number of integrin receptor types was limited in this system, it was important to consider that even among the two well-characterized I-domain–containing collagen binding integrins, different signaling occurs. The $\alpha 1\beta 1$ integrin was Shc-associated as compared to $\alpha 2\beta 1$ (Wary et al. 1996; Wary et al. 1998) and signals through Erk to proliferative pathways. The

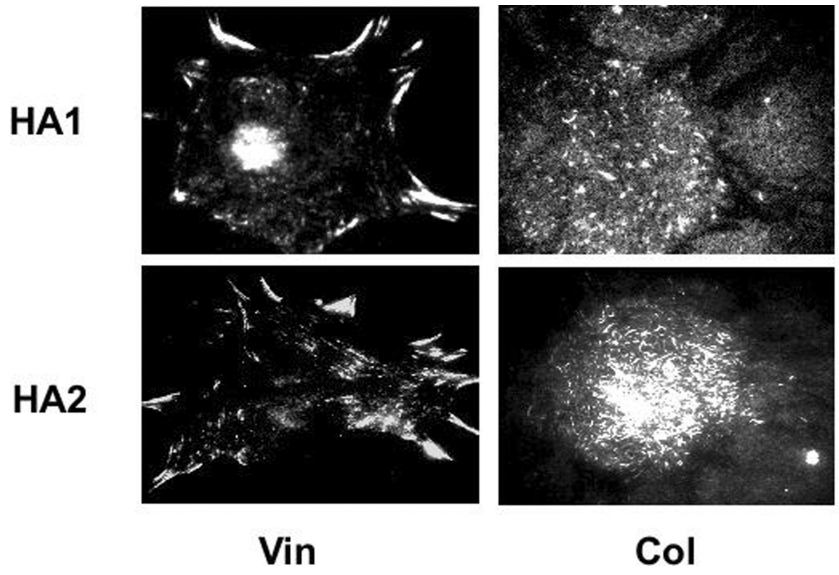

Figure 7. TIRF microscopy of HA1 and HA2 cells on FITC Col I. HA1 cells and HA2 cells were allowed to spread for three hours and one hour, respectively, on FITC Col I and were subsequently fixed, permeabilized and stained with monoclonal antibodies raised against the focal adhesion protein vinculin per Methods. Cells expressing the $\alpha 2\beta 1$ integrin are more proficient at collagen organization when compared to cells expressing the $\alpha 1\beta 1$ integrin. Bar = 15 μm.

$\alpha 2\beta 1$ integrin preferentially signals via p38 and resulted in both MMP and collagen expression (Ivaska et al. 1999; White et al. 2004). Differential signaling from $\alpha 1$ and $\alpha 2$ could also stem from the differences in collagen cross linking. The $\alpha 2\beta 1$ integrin bound Col I with or without hydroxyproline modification. For high-affinity binding, however, $\alpha 1\beta 1$ required hydroxyproline linkages in collagen (Perret et al. 2003). While differential signaling can complicate interpretation, absence of post-translational crosslinking in this system could also complicate interpretation of these findings.

Our experience in this work has shown that there is distinct variation in the nature of the commercially prepared FITC Col I. Occasionally preparations have significant numbers of non-cell-associated fibrils, which form independently of the cells when coated on glass coverslips. These preparations were not very useful, although the cells could still increase the fibril formation when plated on this substrate. In other preparations, a general decrease in the number of fibrils formed on plating was observed. Clearly, some unknown factor introduces variation from one FITC Col I preparation to another that can alter the overall

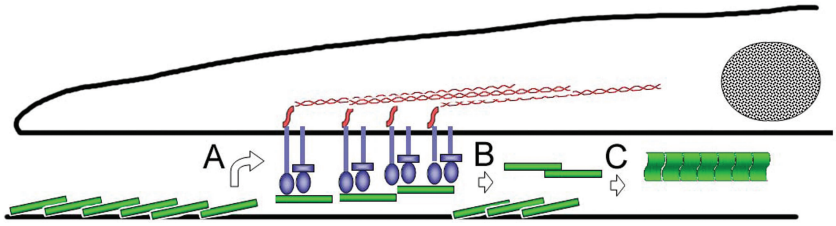

Figure 8. Model for integrin involvement in collagen fibril formation. (A) Monomeric collagen (green bars) coated onto the glass substratum is bound by integrins (blue) at sites of focal adhesions. (B) Bound collagen is concentrated into higher order collagen structures which are not integrin-associated and become less aligned with focal adhesions and (C) accumulate beneath the cell centroid.

number of fibrils. This variability seems to have a generalized effect and uniformly affects fibril formation among cell types tested.

One of the main limitations of this study was its constraint to 2-D analysis. The growth of HT1080 cells in 3-D requires functional MMP-14, but the presence or absence of MMP-14 in 2-D culture had no bearing on growth (Hotary et al. 2003). FAK phosphorylation, a conserved and early signal-transduction event common to integrin signaling, does take place on 2-D substrates, but appears reduced in 3-D gels (Ivaska et al. 1999). Also, $\alpha 1\beta 1$ and $\alpha 2\beta 1$ signal differently in 3-D culture as compared with culture on a 2-D substrate. When cultured in 3-D collagen gels, cells exert force on the matrix and contract the gel, reducing its area. Depending on the cell type utilized, this gel contraction can be mediated by either $\alpha 1$ or $\alpha 2$ and was dependent on tyrosine phosphorylation (Broberg and Heino 1996; Carver et al. 1995; Gotwals et al. 1996). Depending on whether cells were cultured in 2-D or 3-D systems, the expression of collagen and MMPs differed depending on whether $\alpha 1$ or $\alpha 2$ was ligated (Langholz et al. 1995).

We observe that integrins are not associated with higher-order collagen fibrils formed under cells. Instead, integrins remain in focal adhesions that appear to colocalize with collagen depletion sites. We propose a model in which higher-order collagen fibrils are nucleated at sites of integrin binding (focal adhesions). As focal adhesion turnover progresses and integrins detach from already nucleated fibrils, the fibrils can then increase in diameter autonomously as prior studies on self-assembly have shown.

Our observations reveal that the more ordered fibrils that are proximal to focal adhesions become progressively less ordered and more randomly coiled as their proximity to the cell center increases. We propose that integrins bind to collagen at these sites of depletion and that nascent, incipient fibrils are formed at these sites before being drawn underneath the cell by the isometric contraction of the cytoskeleton (Fig. 8). Future studies employing dynamic analysis of this fibril formation process and electron microscopy could reinforce this model.

Future work to be conducted using this system will focus on refining the understanding of cell-mediated collagen fibril assembly. Additional factors known to affect fibril formation or diameter such as fibronectin, decorin, lumican or fibromodulin can also be included in the substratum to determine their effects, if any, in this 2-D system of study. In addition the role of MMPs in collagen fibril organization in this system remains unclear.

Future studies will also include analysis of the dynamics of fibrillogenesis. Experiments using living cells in real time can be conducted to explore the factors required for initiation and the overall kinetics of cell mediated fibril assembly. The role of different collagen binding integrins and their contributions to fibril organization can be explored by examining stably transfected cells expressing $\alpha 1$, $\alpha 2$, $\alpha 10$ or $\alpha 11$ as they organize fibrils in real-time recordings. Results from this work and future studies will hopefully build upon the decades of biochemical studies on *in vitro* collagen fibril self assembly and expand our understanding of how cells organize secreted collagens into higher-order structures.

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