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Biophysical Chemistry 100 (2003) 593–605

Biophysical Chemistry

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Reciprocal interactions between cells and extracellular matrix during remodeling of tissue constructs

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Received 2 May 2002; accepted 18 June 2002

Abstract

Cells remodel extracellular matrix during tissue development and wound healing. Similar processes occur when cells compress and stiffen collagen gels. An important task for cell biologists, biophysicists, and tissue engineers is to guide these remodeling processes to produce tissue constructs that mimic the structure and mechanical properties of natural tissues. This requires an understanding of the mechanisms by which this remodeling occurs. Quantitative measurements of the contractile force developed by cells and the extent of compression and stiffening of the matrix describe the results of the remodeling processes. Not only do forces exerted by cells influence the structure of the matrix but also external forces exerted on the matrix can modulate the structure and orientation of the cells. The mechanisms of these processes remain largely unknown, but recent studies of the regulation of myosin-dependent contractile force and of cell protrusion driven by actin polymerization provide clues about the regulation of cellular functions during remodeling.

Keywords: Tissue engineering; Tissue remodeling; Myosin activation; Cell spreading; Mechanotransduction

1. Introduction

It seems appropriate in this issue honoring the life and work of Dr John T. Edsall to discuss some recent results that bear on the contemporary field of Tissue Engineering. The work carried out by Dr Edsall and other members of the research group of E.J. Cohn during the 1930s and 1940s on blood fractionation and the development of clinically applicable products derived from blood can truly be regarded as a precursor to contemporary tissue engineering. The aim then was to use fractionated blood products for specific clinical and surgical purposes. For example, a fibrin foam with thrombin developed by Dr Edsall was used to stop bleeding during surgery, especially brain surgery. A fibrin film was used as an effective replacement for portions, removed during surgery, of the dural membrane lining the brain [1].

Similarly, one of the tasks of contemporary tissue engineering is to generate tissue equivalents assembled from cells of specified types embedded in a matrix composed either of natural biological

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0301-4622/03/$ - see front matter
PII: S0301-4622(02)00308-3

extracellular matrix (ECM) material, e.g. collagen or chemically synthesized polymeric substitutes [2–5]. The practical purpose of these tissue equivalents is to replace damaged or diseased human tissues. Tissue equivalents also can supply test systems for the analysis of cell–matrix interactions, regulation of contractile force, functions of cytoskeletal components and a variety of other basic cell biological subjects. A major problem is to determine how to induce the cells to align, orient and interact among themselves and to remodel the matrix in ways that yield a tissue equivalent that mimics the mechanical properties of the endogenous tissue targeted for replacement.

It is remarkable that cells such as fibroblasts, smooth muscle cells or cardiomyocytes placed within a collagen matrix remodel the matrix by compressing and stiffening it and establish connections among themselves that seem directed toward forming a biological tissue (or toward healing a wound). Nevertheless, these reconstituted model tissues are still far from equivalent to their analogous natural biological connective, smooth muscle or heart tissues. To guide the remodeling process it is essential to understand how cells respond to the matrix, to each other, to imposed mechanical forces as well as to secreted factors as they reorganize themselves and the matrix.

An essential requirement for tissue remodeling is that cells exert contractile forces. That nonmuscle cells such as fibroblasts can exert substantial contractile forces was demonstrated by their ability to wrinkle deformable substrata to which they adhere [6]. This approach has been extended and others have been developed to provide quantitative information about these forces and their spatial distribution [7–9]. Because these forces far exceed what is required to propel cell locomotion, it has been proposed that their main function is morphogenetic rather than to drive cell migration. Harris and colleagues have demonstrated how these forces might operate in the formation of ligaments, tendons and muscles [10]. Furthermore, the compression of collagen matrices by embedded fibroblasts into a tissue-like structure has also been proposed as a model for wound healing [11]. Extensive studies have compared the contraction by fibroblasts of floating collagen gels and of collagen gels anchored to a substratum [12]. Floating and anchored gels respond to remodeling very differently. The floating gels are compressed into a skin-like tissue in which the cells have a stellate morphology with long processes and a diffuse cytoskeletal meshwork. After contraction of the matrix there is a marked decline in DNA synthesis with the cells apparently arrested in G_0 in part through down regulation of the extracellular signal regulated kinase (ERK) [13]. Some cells become apoptotic. Platelet derived growth factor (PDGF) receptors in fibroblasts in floating matrices lose their ability to autophosphorylate in response to PDGF. These cells decrease collagen and increase collagenase biosynthesis compared to cells in anchored matrices. Anchored matrices, although also compressed, remain under tension generated by the cells, which have a bipolar shape oriented along the lines of tension in the matrix with prominent stress fibers. These cells continue to synthesize DNA and to multiply [12].

By what mechanisms, then, do cells within a collagen matrix align, orient, interact among themselves, and remodel the matrix to form a tissue? According to a model for the early stages of gel compression cells align and exert traction force along the direction of the surrounding collagen fibrils [14]. The force exerted by the cells deforms the gel and further aligns the collagen to generate an anisotropic fibril organization [15]. Once the collagen matrix gels, the cells must adhere to it and begin to exert force in order to compress and stiffen the matrix. Several questions arise:

A. What is the time course of the compression and stiffening process?
B. What is the role or myosin-dependent contractile force?
C. How do cells adhere to the matrix?
D. What is the relationship between the process of cell adherence to the matrix, spreading, and contraction?
E. How is the remodeling process regulated?

We have begun to address these questions in studies of chick embryo fibroblasts either in collagen matrices or in monolayer culture. Formation of a reconstituted model tissue is initiated by suspending cells in monomeric collagen [16,17].

When the cell suspension is warmed to 37 °C, the collagen gels, and the cells, up to now spherical, spread into the matrix and adhere to it. Then the cells begin to exert contractile force on the collagen fibers to compress and stiffen the matrix. The resulting model tissues can be formed to be suitable for various applications, e.g. studies of the regulation of contractile force [16,18,19] or of matrix properties and cellular contributions to the mechanical properties of the tissues [17,20].

2. Materials and methods

2.1. Formation of fibroblast populated matrices (FPMs)

These methods, briefly summarized here, have been provided in greater detail [17]. Chicken embryo fibroblasts (CEFs), isolated from 11-day chicken embryos were maintained on cell culture dishes in standard monolayer culture conditions [Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum and penicillin (50 U/ml) and streptomycin (50 mg/ml)]. Monomeric collagen in 0.02 M acetic acid was neutralized at 4 °C and mixed with concentrated DMEM stock to yield a final normal DMEM concentration. The fibroblasts, suspended by trypsin, were mixed with the collagen solution and brought to 37 °C in a CO_2 incubator. The collagen gels within 30 min, entrapping the fibroblasts within the matrix. For mechanical measurements the cell suspension was poured into Teflon casting wells consisting of an outer cylinder and an inner core or mandrel. In these wells the collagen gel forms an annulus 3 mm thick and 3 cm in diameter, which could be removed intact from the well after gelation. Shortly after gelation the gel can be mounted on an isometric force transducer, as described below, and placed in an organ bath under conditions that preserve viability of the cells [17]. Then, as the cells compress and stiffen the matrix, the contractile force that they exert on the matrix can be recorded over time [21]. Alternatively, the gel may be retained within the casting molds in the incubator for a day or more. After this time the gel is compressed and stiffened and its thickness is reduced ~10-fold. This remodeled matrix can then be mounted on the force transducer to measure contractile force and the mechanical properties of the model tissue [17].

2.2. Mechanical measurements

The FPM ring is looped over a hook linked to an isometric force transducer and over a horizontal bar connected to a stepper motor controlled by computer as previously described [17]. The ring can then be stretched to prescribed extents and at prescribed rates while measuring the force required for stretching. The greater the stiffness of the matrix, the higher the force value reached by a defined degree of stretching.

2.3. Fluorescence microscopy

The cells in the FPMs were stained with the cytoplasmic fluorescent dye Cell Tracker (Molecular Probes), washed to remove excess dye and fixed with 3.7% formaldehyde as described [17]. Images were acquired using a laser scanning confocal microscope (Biorad).

2.4. Blocking integrin binding with antibodies

Monocolonal anti-β_1 integrin (Sigma) specific to chicken was used to block integrin binding to the collagen matrix. Antibodies (1:200 dilution) were incubated with cells before mixing with collagen (50 min) and after formation of FPM (60 min). The control sample was incubated similarly with bovine serum albumin replacing the anti-β_1 integrin antibody (0.2% w/v).

2.5. Myosin light chain phosphorylation

At selected times during cell spreading in the collagen gel, the gel samples were cut into small pieces by scissors in a solution containing 10% trichloracetic acid and 2 mM DTT and sonicated before and after each of two washes with 1 ml acetone containing 10 mM DTT. After a third acetone wash the precipitated protein was concentrated by centrifugation and then solubilzed in a loading buffer containing 9 M urea, 2 mM DTT, 20 mM Tris, 22 mM glycine and 250 mM sucrose.

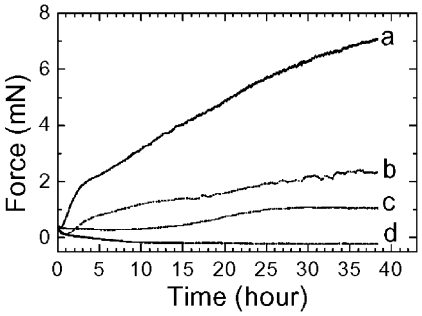

Fig. 1. Isometric force generation and actin cytoskeleton. The FPMs were attached to isometric force transducers within 30 min of gelation of the collagen, as described in Section 2. While the fibroblasts embedded in the collagen gel were spreading, they also began to exert an isometric force by pulling the collagen fibrils centripetally toward themselves. The increase in the isometric force continued for more than 35 h (a). The generation of the isometric force required an intact actin filament cytoskeleton. Relatively low concentrations of CD (0.05 μM) eliminated almost 60% of the isometric force generated by the control (b). Higher concentrations of CD (0.5 μM) further reduced the isometric force (c) and 2 μM CD abolished it completely (d).

The phosphrylated myosin regulatory light chains were separated in 1-mm-thick mini-gels containing 40% glycerol, 10% acrylamide, 20 mM Tris, 22 mM glycine at pH as previously described [22]. Western blots were incubated using a rabbit anti-myosin II regulatory light chain antibody [23]. RLC bands were visualized by ECL detection reagents (Amersham Pharmacia Biotech).

3. Results

3.1. Cell spreading and development of contractile force during remodeling

Fibroblasts suspended in a solution of monomeric collagen are approximately spherical. After gelation of the collagen the cells spread into and adhere to the matrix and adopt asymmetric, typically, elongated shapes [24]. Over the same time period the cells begin to exert a contractile force on the matrix as they compress and stiffen it. When collagen matrices containing cells are mounted on force transducers shortly after polymerization and gelation of the collagen, it is possible to observe the development of contractile force as the cells remodel the collagen matrix (Fig. 1). The change of cell shape can be separated from the development of contractile force. In the presence of serum cells both change shape and develop force, but in its absence the cells do not develop force although they adopt elongated shapes as before (Fig. 2a,b). Cytochalasin D (CD), which disrupts the actin cytoskeleton, inhibits the force increase during remodeling (Fig. 1). Diminished, but nonetheless significant, force develops even with CD concentrations as high as 0.5 μM. Although low concentrations of CD can slightly weaken the contractile force exerted by cells in collagen matrices, high CD concentrations (in the range of 2 μM) are required to cause major changes both to the organization of the actin

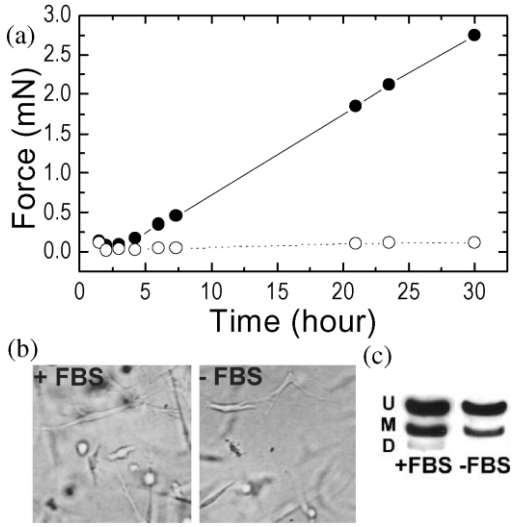

Fig. 2. Force generation and cell spreading in a collagen matrix. Generation of force by FPMs in serum-containing medium (10% FBS) was monitored for 30 h continuously. (a) The isometric force was generated within a few hours after formation of the collagen matrix and increased continuously (●). (b) Without serum in the culture medium cells in the collagen matrix still spread as well as in the presence of serum. There was, however, almost no increase in the baseline force (○in a). (c) After 30 h the total phosphorylated RLC increased in the presence of serum and decreased in its absence. The phosphorylated states of myosin II were analyzed on glycerol/urea gels, blotted to nitrocellulose, and probed with anti-myosin RLC antibody. U, M and D indicate, respectively, the unphosphorylated, monophosphorylated, and diphosphorylated states of RLC.

filaments and the mechanical properties of the cells and to the FPMs containing them [20]. Hence, a major disruption of the actin cytoskeleton is required to inhibit completely the development of force during the remodeling process.

The development of force is correlated with myosin activation measured by the phosphorylated levels of myosin regulatory light chain (RLC) (Fig. 2c). The total level of RLC phosphorylation was determined by normalizing the sum of intensities corresponding to mono- (M) and 2X di- (D) phosporylated bands by the total intensity of all bands including unphosphorylated (U) RLC. In the standard procedure for assembling FPMs this activation is accomplished by addition of fetal bovine serum (FBS). The myosin II activity of the sample incubated with FBS increased significantly compared to that without FBS. The components in FBS that are responsible for activation are unknown although it has been demonstrated that lysophosphatidic acid (LPA) a component of serum that has been shown to activate myosin via the small GTPase RhoA [25,26], can elicit a myosin-dependent increase of contractile force in 'mature' FPMs. The partial inhibition of force development by herbimycin (Fig. 3) implicates tyrosine kinases as participants in the signal transduction pathways that lead to myosin activation, particularly in its later stages.

In addition to the activation of myosin it is also necessary for matrix contraction that the cells adhere to the collagen fibrils. Cells adhere to collagen fibrils via the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins [27]. In earlier work we have demonstrated that cells lacking the α_1 and α_2 integrin subunits are unable to contract a collagen matrix [21]. Fig. 3 further demonstrates that a function-blocking antibody specific for β_1 subunits also inhibits the development of contractile force. That the inhibition is only partial may be due to residual interactions of cells by receptors other than the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins. The nearly complete absence of force development in FPMs assembled with cells lacking α_1 and α_2 integrin subunits [21], however, suggests that the incomplete inhibition of force development is due to incomplete blockage of these receptors by the antibody.

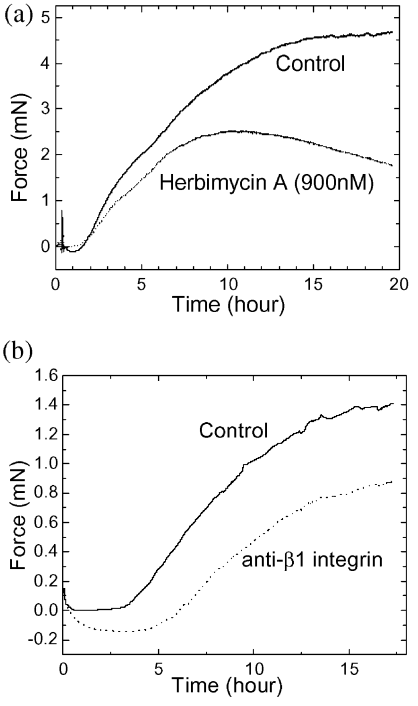

Fig. 3. Force generation and integrins. The generation of contractile force by the FPMs depends on the strength of the connection of the cells to the ECM through transmembrane proteins (integrins) that interact intracellularly with the actin cytoskeleton. Isometric force generated by the FPMs was significantly reduced, especially at later stages, by herbimycin A (900 nM), a general inhibitor of tyrosine phosphorylation (a). The inhibitor may have affected intracellular proteins, such as FAK and Paxillin. FAK is autophosphorylated and activated by interaction with integrins and integrin cluster formation. Paxillin is phosphorylated by activated FAK. More direct inhibition of the integrin connection to the ECM by anti-β_1 integrin antibodies (detail description in Section 2) during collagen gel formation also partially inhibited the generation of isometric contractile force (b). This inhibition occurred at a relatively early stage of force generation. The curves shown in (b) are the averages obtained from two duplicate samples. Control samples were incubated with bovine serum albumin as described in Section 2.

3.2. Stiffening of the matrix by the cells

As the cells remodel the collagen matrix, they compress and stiffen it. The extent both of compression and stiffening varies with the concentration of cells within the matrix [17]. When the FPM is stretched, the external force applied to it

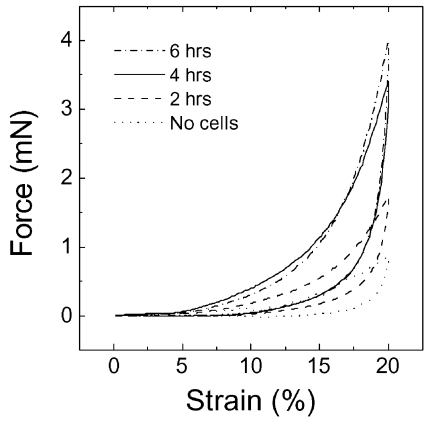

Fig. 4. ECM stiffening by the cells. During the generation of force by the FPMs, the cells remodel the ECM. After different periods in culture (2, 4 and 6 h) from the time of gelation of the collagen matrix, FPMs were treated with 2 μM CD to abolish the cellular contribution to the mechanical properties of the FPMs. The FPMs were stretched linearly over 30 min up to 120% of their original length and then returned at the same rate to 0% strain. The slope of the force response to the stretch indicates the stiffness of the FPMs. Compared to FPMs without cells, there was already significant stiffening of the ECM after only 2 h of incubation. The degree of stiffening increased with the incubation time. The stiffening of the ECM provides a good indicator of the remodeling activity of the cells.

is balanced by viscoelastic resistance both from the cells and the matrix. The attachment of the cells to the collagen matrix is mediated by integrins that bind to extracellular collagen and also link to the actin cytoskeleton inside the cells [27,28]. Treatment of a FPM with CD diminishes the contribution of the cells to the mechanical stiffness of the FPM both by decreasing the stiffness of the cells [29] and by disrupting the linkage of the matrix to the actin cytoskeleton via integrins. To a rough approximation we may suppose that the mechanical properties of a FPM are determined by the summation of the contributions from the cells and the matrix. Using CD to eliminate the cellular contribution reveals the matrix contribution [17]. The effect of remodeling on the matrix may be shown by treating FPMs with CD to eliminate the cellular contribution. Then the force resisting stretching is measured as the strain of the FPM is increased up to 20%. Fig. 4 illustrates the increase in matrix stiffness over the period of remodeling by comparing the force imposed on a collagen matrix containing no cells with FPMs that have been remodeled by cells for varying lengths of time and then treated by CD to eliminate the cellular contribution. Already within 2 h after gelation there is a significant increase in matrix stiffness that continues over the next several hours.

3.3. Stretching the collagen matrix induces polarization of the cells

Mechanical stimuli influence the shape and the orientation of cells subjected to stress or strain. One of the first observations of this was the fluid shear stress-induced alignment of endothelial cells parallel with the flow direction [30]. Later a cyclic stretch applied to the cells grown on an elastic substratum was found to induce realignment of cells perpendicular to the direction of strain [31]. The detailed mechanism of this process is still not well understood. When myotubes were subjected to a very slow ramp stretch (0.35 mm/min), however, instead of cyclic stretch, the cells elongated parallel with the direction of strain [32]. Similar cyclic stretches have different effects on cells grown in 2D and in 3D environments. Smooth muscle cells grown in a collagen gel align parallel with the direction of stretch [33]. When a fibroblast-populated matrix is held at both ends, we have observed the alignment of cells parallel with the edges [34]. This can be interpreted in terms of ‘contact guidance’ [14]. The force produced by the cells that builds up within the FPM, (cf. Fig. 1) could be responsible for inducing the alignment. Not only do forces generated by the cells remodel the collagen matrix, but also forces exerted by the matrix on the cells can influence their shape. A static stretch to 32% strain applied to the FPM immediately after the collagen gelation accelerated the rate of spreading. Both alignment of the collagen fibrils and stiffening of the matrix could contribute to this effect (cf. [35]). As shown in Figs. 5 and 6, a uniaxial stretch causes elongation of the cells along the stretch axis. The changes induced in shapes and alignment of cells are most likely regulated by signal transduction pathways activated by the mechanical stimuli [36].

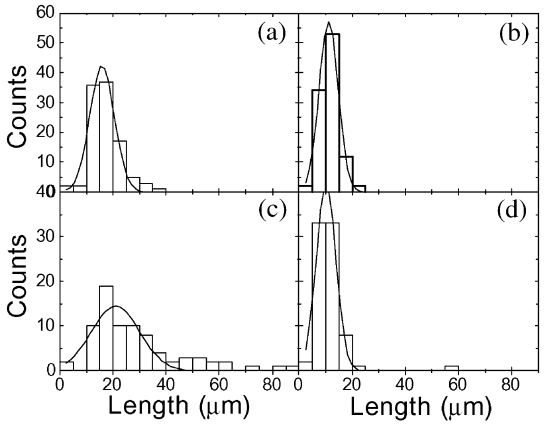

Fig. 5. Accelerated cell spreading induced by static stretch. The FPMs were cultivated in DMEM supplemented 10% FBS for 3 h with and without a static stretch (32% strain). During the last 20 min of culture, the FPMs were incubated with a cytoplasmic fluorescent dye (Cell Tracker). Then they were fixed with 3.7% (v/v) formaldehyde. The shapes of the cells within the samples were visualized by confocal microscopy. The shapes and sizes of the spreading cells were analyzed using the program NIH image to estimate individually their major and minor axes. Without stretch the distribution of the major axes indicated that the cells were just beginning to spread. Under the influence of stretch the distribution of major axes became broader indicating that a larger number of cells were spreading. The medians of the distribution were estimated by fitting the histogram to a normal distribution. Major axis (a) and minor axis (b) without stretch were 15.9 ± 4.6 μm and 11.2 ± 3.6 μm, respectively. Major axis (c) and minor axis (d) with stretch were 21.0 ± 9.1 μm and 10.2 ± 3.7 μm, respectively.

4. Discussion

The remodeling of collagen gels by cells provides a useful model for tissue development, wound healing, and response of tissues to external influences such as force loading and activation by growth factors or hormones. To understand the mechanisms responsible for this remodeling it is essential to study how the cells interact with the collagen matrix, change the properties of the matrix and are, in turn, affected by the changes of matrix properties. Initially, the cells embedded in a recently-gelled collagen matrix must spread into and adhere to the collagen fibrils. Then the cells exert a contractile force on the collagen that compresses and stiffens the gel. The data presented above illustrate the effects of disrupting the actin cytoskeleton on the development of contractile force in the matrix, the distinct roles played by myosin in cell spreading and the development of force, the effects of the remodeling process on gel stiffness, and the effects of forces exerted by the matrix on the polarization and orientation of the cells within the matrix.

4.1. Spreading of cells into the matrix and exertion of contractile force are separable

Whether a cell contracts into a compact shape as is typically found in suspension or spreads to maximize its contact with a substratum or a matrix depends on the balance of forces that drive extension or contraction. As demonstrated in Fig. 2, fibroblasts can spread into a matrix in the absence of myosin activation. Development of contractile force within the FPM, however, requires myosin activation. How, then, does myosin activation affect cell spreading? In previous studies on two-dimensional substrata we have shown that cell spreading is accelerated by inhibiting myosin acti-

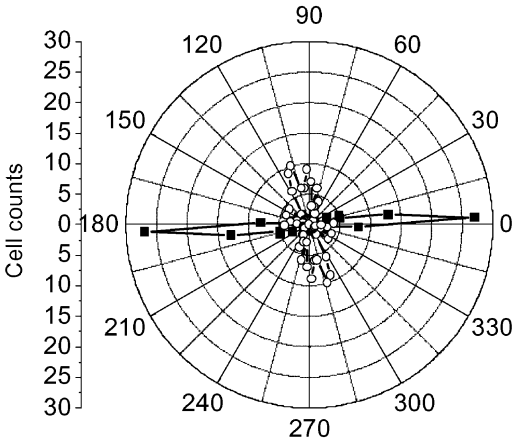

Fig. 6. Cells aligned parallel to the static stretch. The data shown in Fig. 5 were replotted to show the distribution of cell orientations relative to the direction of the fixed ends of the tissue and to the direction of stretch. The fixed ends were located at 0° and 180° and the stretch was oriented in the same direction. Without a static stretch cells oriented almost randomly with a slight preference for alignment perpendicular to the fixed ends. A static stretch clearly induced alignment of cells parallel with the direction of the stretch.

vation. Hence, myosin not only is unnecessary for but even inhibits cell spreading (Wakatsuki et al., submitted for publication). This might be expected from the presumption that a contractile force exerted by myosin should resist cell extension and suggests that forces responsible for cellular contraction and extension are differentially regulated.

We have also observed that cell spreading is inhibited by CD at nM concentrations as expected if the CD is binding to free barbed ends of actin filaments involved in polymerization. This is in contrast to requirement for much higher CD concentrations to achieve a major disruption of the actin cytoskeleton presumably by displacement of actin filaments from high affinity barbed end capping proteins [20]. In contrast to the inhibition of cell spreading by nM concentrations, μM concentrations of CD are required to inhibit the development of contractile force (Fig. 1). Hence, rather than simply inhibition of actin polymerization the latter process requires disruption of the actin cytoskeleton to inhibit the actin-myosin interactions that provide contractile force.

Both contraction and extension forces appear to be regulated by members of the Rho family of GTPases. Myosin-dependent contractile forces are activated by RhoA, which promotes the phosphorylation of the regulatory light chain of myosin II in non-muscle cells [25,26]. It is now thought that actin polymerization drives extension of cells as they spread and migrate [37,38]. Actin polymerization is regulated by the Rho-family proteins, Cdc42 and Rac1 [39]. Indeed it has been observed that during the initial phase of cell spreading, binding of integrins to ECM ligands diminishes RhoA activity (and therefore, myosin activation) [40–42]. Hence, RhoA is deactivated at a time when the resulting diminution of myosin contractile force would enhance cell spreading. Shortly thereafter RhoA is reactivated so that the resulting increase in myosin activity and contractile force can contribute to the formation of the focal adhesions and stress fibers seen in anchored fibroblasts [40].

4.2. Stiffening of the collagen matrix

During wound healing and tissue development cells exert contractile forces to compress and stiffen the extracellular matrix. The compression of the collagen matrix by fibroblasts during the formation of a FPM provides a model for these naturally-occurring processes. The mechanism of matrix remodeling remains a mystery. As demonstrated in Fig. 4, the matrix stiffens as it is compressed. This initial remodeling process is partially reversible. It has been shown that removal by detergent or by trypsin/EDTA of fibroblasts from contracted tissue constructs allowed a partial re-expansion of the gel. This partial re-expansion suggests that the collagen fibrils are initially condensed and held in place by the cells and then are stabilized by non-covalent interactions not requiring cells [43]. Over weeks covalent interactions either catalyzed by lysyl oxidase or by non-enzymatic glycation further stiffen and stabilize the collagen gel [44–46].

Stretching forces can disrupt some of the collagen fibril interactions that are responsible for the stiffening of 2-day-old gels prior to the formation of covalent cross-links. When FPMs are stretched several times at a constant rate to 20% over 30 min and then unloaded at the same rate, the peak force of the first stretch is substantially greater than that of the second stretch [17]. Smaller decreases in peak force occur with subsequent stretches. The increase of contractile force in response to serum activation was the same in FPMs that had not been stretched and that had been stretched 80 times (Wagenseil et al., manuscript in preparation). Hence, the decreases in force due to stretch result from changes in the structure of the gel rather than from damage to the cells. To understand how cells compress and stiffen FPMs it is essential to characterize the nature of the collagen interactions that stabilize the compressed gel and to learn how the cells promote their formation.

4.3. A role of FAK in cell spreading and protrusion

Clustering of integrins from dispersed focal contacts to form a focal complex is driven by myosin-dependent contractile activity [47]. Focal adhesion kinase (FAK) associating with integrins becomes autophosphorylated after the integrin clustering. FAK (−/−) fibroblasts isolated from

FAK(−/−) mouse embryos still forms focal contacts, yet the integrins do not cluster. More recently the importance of Src-family kinase binding to auto-phosphorylation sites of FAK has been shown using a cell line whose FAK expression is controlled by tetracylcine [48]. Under conditions of tetracylcine-induced FAK deficiency, the migration and spreading of FAK(−/−) fibroblasts are significantly impaired [48]. The reduction of the motility of FAK(−/−) fibroblasts suggests a role for FAK in recycling old focal contacts into new ones as well as in clustering of integrins.

Some other findings have begun to reveal the mechanism by which FAK promotes turn-over of focal contacts. The myosin activity becomes down-regulated upon cell adhesion to its substratum by lowering the activity of the small GTPase Rho, which is known to be in the upstream signal transduction pathway activating myoin [49]. FAK (−/−) fibrobasts do not down-regulate Rho; therefore, the rate of cell spreading is reduced [42]. Recently p190RhoGAP, which deactivates Rho by hydrolyzing its GTP, has been shown to be responsible for the down regulation of myosin activity. Furthermore, c-Src, which is a well-known binding partner of FAK through its tyrosine 397, is also shown to be involved in this signal transduction pathway.

Inhibition of myosin activity by the specific myosin light chain inhibitor, KT5926, and the general kinase inhibitor, staurosphorine, greatly enhances the rate of spreading of chicken embryo fibroblasts on 2D substrata (submitted for publication). On the other hand, the activation of myosin by LPA decreases the rate of cell spreading (unpublished results). Cells expressing a constitutively active mutant of Rho (V14Rho) also slow down the rate of cell spreading ([42] and our observation). In wild type fibroblasts, the focal complexes visualized using GFP labeled α-actinin turn over at the protrusive cell edge during the rapid phase of cell spreading. The fibroblasts expressing V14Rho fail to disperse α-actinin complexes, thereby significantly reducing the rate of cell protrusion.

To extend the cell edge actin polymerization must be accompanied by the dispersion of focal complexes. Downstream from FAK activation, Rac becomes activated through the FAK/Src-Cas/Crk/DOCK180 pathway. Then actin polymerization is induced by activated Arp2/3 complexes via WASP/Scar activation through Rac- and Cdc42-dependent pathways [50]. Lastly, recent reports suggest an antagonistic relationship between pathways involving Rho and Cdc42/Rac, which regulate cell spreading [47,51–53]. Rac activity is up-regulated [54] while Rho activity is down-regulated upon adhesion [49]. Therefore, cell adhesion to the substratum promotes cell spreading by shifting the balance between actin polymerization and myosin activity toward actin polymerization via upstream regulators such as Rac, Cdc42, Rho and Ca^{2+}-dependent signal transduction pathways.

Another molecule that may contribute to extension of the cell edge during cell spreading and protrusion is the mammalian homologue of *Drosophila* protein diaphanous, mDia, a member of the formin-homology (FH) protein family. The mDia protein produces non-cross-linked actin filaments upon binding to Rho via its FH domain. The presence of the mDia protein is the necessary and sufficient condition for extension of focal contacts induced by centripetal force applied to the cell [55]. The mDia-dependent extension of the focal contact does not depend on myosin activity but does require the existence of actin filaments.

4.4. Working hypothesis: cell spreading, contractility and cell alignment

These observations led us to a working model that describes the mechanism of cell protrusion at its leading edge. Myosin-dependent force exerted on actin filaments attached to integrins cluster the integrins to form focal complexes, thereby inducing autophosphorylation of FAK bound to integrins. Then c-Src binds to FAK, becomes activated, and initiates down-stream signal transduction events including activation of p190RhoGAP [40]. Rho becomes inactive by hydrolysis of bound GTP by p190RhoGAP [56]. Lower activity of Rho reduces the myosin activity which lowers the level of tension applied to the focal complex. Reduced tension decreases the FAK phosphorylation and in turn disperses the focal complex. How could this

negative feedback loop contribute to the protrusion of a cell at its leading edge?

By analogy consider two arms pulling a rope fixed at its end (cf. [57]). One hand grasps and pulls the rope. Once the force on the rope has become very strong, the hand grasping the rope sends two signals. One signal tells the arm to lower the pulling force and the second tells the other arm to extend forward and grasp the rope. At about the same time the hand of the extending arm grasps the rope and the other hand quickly releases it. Continuing the cycle of grasping and releasing the rope alternatively with two arms lets one pull the rope towards you or pull you towards the fixed end of the rope. If one does this exercise blindfolded, one can detect whether a rope is fixed or not by sensing the resistance. Cells may use a similar principle to regulate their remodeling and migratory interactions with substrata.

A pulling force applied by a micro-needle to a deformable substratum in the direction of protrusion increases its rate. If a pushing force is applied in the direction opposite to protrusion thereby releasing the tension, the cell retracts and moves away from the push [58]. When the same forces, however, are applied to a FAK(−/−) cell, the cell cannot respond to the stimulus. A retarded cell spreading is also observed when cells are plated on a highly compliant substratum. The degree of tyrosine phosphorylation of FAK is greatly reduced in these cells compared to that of cells grown on a stiffer substratum [59]. These observations suggest the existence of a mechanism similar to our hypothetical model that senses the mechanical environment surrounding cells. Most likely FAK plays a significant role in sensing the mechanical stimulus. Pulling a substratum away from the protruding cell is analogous to pulling the rope from the fixed end, thereby accelerating motion toward the fixed end (cf. Fig. 7). Pushing the substratum towards the protruding cell is analogous to releasing the fixed end thereby releasing the traction force.

The model could also explain the preferential alignment of cell spreading in the direction of the stretch (Figs. 5 and 6). In the model one of the arms is always extending while the other is pulling at the same time, analogous to the simultaneous

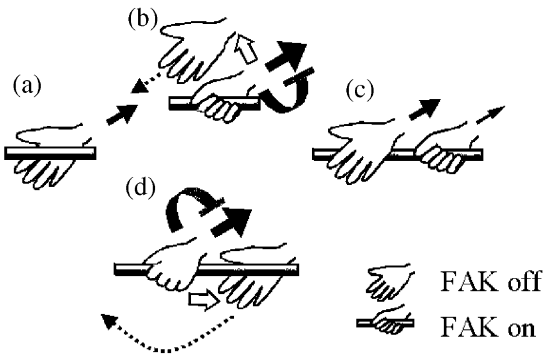

Fig. 7. Schematic model of the working hypothesis. A schematic working model describes the mechanism of cell protrusion. A hand represents a focal complex formed by integrins, FAK, and other proteins associated with the complex. The hand is connected to an arm that can extend and contract by polymerization of actin filaments and movement of myosin heads along the actin filaments, respectively. The first hand reaches a rope representing the ECM and grasps it. Increased pulling force by the arm, i.e. myosin activity, causes the hand to pull more strongly on rope. (The sizes of the black arrows indicate the magnitudes of the pulling forces.) This tension activates FAK to send two signals. One signal is sent to its own arm (gray symbol) to reduce the pulling force via p190RhoGAP, thereby reducing Rho activity, and therefore Rho-kinase activity, and then finally myosin activity. The other signal is sent to the second arm (white arrow) to extend it forward by activation of actin polymerization (dotted arrow). Then the second hand grasps the rope and starts to pull while the pulling force of the first arm is reduced. The reduction of pulling force releases the rope from the first hand by turning off FAK activity right after the second hand grasps the rope. The continuing cycle of grasping and pulling the rope by the two hands and arms drives protrusion of the cell edge along the ECM and compression of collagen within the vicinity of the cell (cf. [57]).

extension of a cell and its exertion of contractile force. It is still difficult to understand the mechanism by which the cells continuously compress the collagen matrix and produce contractile force after completion of cell spreading. An irreversible remodeling of the collagen matrix by actin-dependent cellular activity is detected already after 2 h of gelation (Fig. 4). Fibronecten (FN) secreted by the cells plays a significant role in this process. FN (−/−) cells cannot spread and remodel the collagen gel unless FN is mixed within the collagen gel [60]. Therefore, the mechanisms by which the cells polymerize and form fibrillar extracellular matrix structures along the fibrillar focal adhesion

may be responsible for the remodeling process. A recent report about the differences found between cells in natural tissues and those grown on 2D substrata, however, further complicates our understanding the mechanism of tissue remodeling. Adhesion strength, cell shape, and distribution of cytoskeletal proteins forming fibrillar focal adhesions in natural tissue are significantly different from those observed in the cells growing on a 2D substratum [61]. It has been suggested that the rigidity of the extracellular environment and the composition of extracellular proteins play an important role in causing the differences.

5. Conclusions

The production of tissue constructs that mimic the structure and mechanical properties of natural tissues is important both to provide useful models for basic research and to supply equivalents that can be used to replace tissues damaged by disease or trauma. Cells such as fibroblasts that contribute to wound healing and tissue development are naturally programmed to remodel the extracellular matrices in which they are embedded. These remodeling functions must, therefore, be guided to produce tissue constructs with desired properties. In collagen matrices fibroblasts spread and attach to collagen fibrils, develop contractile force, and compress and stiffen the matrix. As illustrated in this work, methods are available for monitoring all of these processes. The next task is to understand the mechanisms by which the cells remodel the collagen. We have proposed that differential regulation within individual cells of contraction and extension is central to their ability to compress the collagen, as indicated in the working hypothesis illustrated in Fig. 7. According to this model cells can alternately adhere to collagen fibrils and contract to concentrate the fibrils in the region of the cell and then release the fibril, extend, and then grasp another fibril. It is likely that contraction and extension are regulated by Rho and by Rac/Cdc42, respectively, possibly under the influence of tension-dependent feedback controls mediated through FAK. More work is required to test the details of this model and also to discover the mechanisms by which cells adhere to and detach from the matrix and how they stabilize the compressed matrix prior to formation of covalent cross-links.

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