Eur Biophys J (2006) 35:695–708
DOI 10.1007/s00249-006-0081-7

ARTICLE

Fibronectin organization under and near cells

Kathy L. De Jong · Heather C. MacLeod · Peter R. Norton · Nils O. Petersen

Received: 22 January 2005 / Accepted: 17 March 2006 / Published online: 31 August 2006

Abstract Polymerization of soluble fibronectin molecules results in fibres that are visible as networks using fluorescently labelled fibronectin protomers or by antibody labelling. Displacement of fibres composed of modified protomers in living cells provides information regarding matrix structure, organization, and movement. A static analysis of fibronectin structures and patterns of organization provide insight into their reorganization during adhesion and motility. Confocal microscopy and atomic force microscopy (AFM) reveal fibronectin-containing networks aligned in arrays perpendicular to the retracting cell edge and in apparently disordered networks of fibres under the cell. The change in patterns suggests a reorganization of fibronectin from disordered arrays used for adhesion into ordered arrays during movement of the cell. Comparison of confocal images with corresponding AFM images confirms that the fibres left on the surface as the cell moves away do contain fibronectin. The orientation of these fibres relative to the tail (uropod) and the receding edges of the cell leads us to propose that cells generate a force on the fibres that exceeds the adhesion force of the fibres to the surface causing them to pull fibronectin fibres into straight arrays. However, when the fibres are parallel to the direction of pull, the fibres remain attached to the surface. The data supports the hypothesis that disorganized, linear fibres are the product of Fn polymerization induced by the cell beneath it and serve to adhere the cell to the substrate as the cell spreads, whereas arrays of fibres found outside the cell are formed as existing fibrils and reorganize during cell motility.

Abbreviations

Fn	Fibronectin
ECM	Extracellular matrix
AFM	Atomic Force Microscopy

Introduction

The extracellular matrix (ECM) is an important factor in cell adhesion and motility and more generally, in the body's reaction to wounds and disease (Adams and Watt 1990; Chen et al. 1997; Hynes 1990). An understanding of ECM function affects tissue engineering and the development of more effective biomaterials (Anderson et al. 2004; Baugh and Vogel 2004; Renner et al. 2004). Fibronectin (Fn) is a major component of the ECM that mediates the interaction between the cell and other ECM proteins. Models of Fn structure and binding reflect its mechanical stability, the effect of stretching on Fn binding, and the role of sequential

Electronic supplementary material Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s00249-006-0081-7 and is accessible for authorized users.

K. L. De Jong · H. C. MacLeod · P. R. Norton · N. O. Petersen
Department of Chemistry, University of Western Ontario, London, ON, Canada N6A 5B7

Present Address:
K. L. De Jong
Xerox Research Centre Canada, Mississauga, ON, Canada L5K 2L1

N. O. Petersen (✉)
National Institute of Nanotechnology, 11421 Saskatchewan Drive, Edmonton, AB, Canada T6G 2M9
e-mail: nils.Petersen@nrc-cnrc.gc.ca

unfolding of Fn in polymerization (Baneyx et al. 2001, 2002; Baneyx and Vogel 1999; Craig et al. 2004; Zhong et al. 1998). It is known that Fn protomers form insoluble fibres as part of the ECM, however, the mechanism of Fn polymerization from soluble protomers to insoluble fibres in the ECM has yet to be elucidated (Gao et al. 2003). Once formed, receptors (integrins) on the cell can bind to the fibres, but it is not understood how the cell can subsequently move: do the receptors release or does the fibronectin network move?

The dynamics of the Fn matrix can be studied using fluorescence microscopy and fluorescently modified Fn protomers taken up by live cells (Pickering et al. 2000). Tracking the change in fibre positioning shows that the network reorganizes as a result of cell movement. While the cell uses the Fn matrix for traction, it is not a rigid network that the cell simply moves across (Dembo and Wang 1999; Sheetz et al. 1998; Hynes 1999). Some of the Fn fibres are remodelled as a result of forces exerted by the cell during motility. The ease of movement of Fn fibres is determined by the relative strength of attachment to the cell and to the substrate (Ohashi et al. 2002; Erickson 2002). Fn attachment is a result of specific molecular interactions between integrins and Fn monomers on the one hand and non-specific ionic interactions between Fn fibres and the surface to which the ECM is attached. The relative strength of interactions is key to determining Fn attachment to the cell and substrate.

Fibronectin monomers are composed of a number of repeating modules termed type I, II, and III (Potts and Campbell 1994). Short linkers join the modules like "beads on a string". The 10th type III module from the amino terminus is the only type III module that contains an RGD sequence forming an integrin binding loop (Potts and Campbell 1994; Krammer et al. 1999). Fn is known to be stretched in living cultures (Ohashi et al. 1999, 2002) and since the 10th type III module contains the integrin binding loop, stretching or unfolding of this module should have an impact on the strength of the Fn attachment to the cell (Baneyx et al. 2001, 2002; Baneyx and Vogel 1999; Geiger et al. 2001; Krammer et al. 2002; Zhong et al. 1998). Steered molecular dynamics simulations and modelling of the 10th type III module show that the RGD binding loop normally protrudes approximately 12 Å from the surface of the molecule and is fully accessible to cell surface integrins (Krammer et al. 1999). However, pulling on the beta strand closest to the integrin binding loop on the C-terminus side, causes the binding loop to be drawn closer to the surface of the molecule, from 12 to 8 Å, and to straighten. These changes are proposed to decrease the accessibility and selectivity of integrin binding to the 10th type III module. Further pulling results in full unfolding of the module (Krammer et al. 1999). Thus, the integrin-binding loop is proposed to act as a switch for integrin binding that can be controlled by stretching of the fibronectin molecule along the length of the fibre. If the connection between integrin and Fn remains intact, the cell can exert a force on the Fn matrix, influencing its structure and organization. Therefore, the structure and organization of Fn on the surface must reflect both how it is laid down to aid in adhesion and the mechanism by which cells move and cause strain on the matrix. A static analysis of Fn structures and their patterns will provide insight into how they are produced and how they are reorganized. In this work we use confocal microscopy to study the patterns of Fn networks to illustrate the remodelling which may occur during adhesion and motility. We propose that if cells generate the force required to pull Fn fibrils straight, Fn networks should reflect the direction of pulling and therefore the direction of motility. As a result, Fn patterns of stationary cells would differ from the patterns of motile cells and this difference should be observed in the fluorescence images. We propose further that since stretching Fn along its length reduces the affinity of the 10th type III binding loop for integrins, Fn fibres should be left attached to the substrate after the cell has moved. This pattern of reorganization due to motility should differ significantly from patterns due to polymerization for the purpose of adhesion only. We use atomic force microscope (AFM) to image Fn containing fibres located outside the cell boundary and to study their orientation relative to the tail (uropod) and receding edges of the cell.

Materials and methods

Cell culture

CV-1 cells are epithelial cells with fibroblast morphology originating from the kidney of an adult African green monkey. CV-1 cells were purchased from American Type Culture Collection (Rockville, MD, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Gaithersburg, MD, USA). The culture medium contained L-glutamine, 1% penicillin–streptomycin (Life Technologies, Gaithersburg, MD, USA) and 10% fetal bovine serum (FBS, Life Technologies). In experiments involving incorporation of modified Fn protomers, FBS was decreased to 5% to reduce the amount of Fn taken up by the cell from the FBS. Cells were plated on 12 or

22 mm glass cover slips for all experiments excluding live-cell experiments. Unless otherwise specified, the cells were grown for 24 h before fixation and membrane permeabilization. Prior to labelling, cells were rinsed with PBS (Phosphate buffered saline, Gibco Life Technologies, Burlington, ON, Canada) and fixed using a solution of 4.4% paraformaldehyde in PBS for 10 min. Cells were permeabilized with acetone at −20°C for 3 min before immunofluorescent labelling with the appropriate antibodies.

For Fn labelling, cells were incubated with 1.25 μg/ml rabbit anti-human fibronectin (Sigma, St. Louis, MO, USA) for 30 min followed by rinsing with PBS and PBS with 2% BSA (bovine serum albumin, Gibco Life Technologies). Cells were then incubated with 50 μg/ml goat anti-rabbit FITC conjugate (Sigma) or 20 μg/ml Alexa Fluor® 488 for 30 min. For non-permeabilization experiments, cells were fixed with paraformaldehyde for 10 min and then labelled with 1.25 μg/ml rabbit anti-Fn (Sigma) for 30 min followed by rinsing with PBS and PBS/BSA. Cells were then incubated with 50 μg/ml of goat anti-rabbit FITC (Sigma) conjugate for 30 min before rinsing, drying, and mounting on microscope slides. Without permeabilization of the membrane, Fn beneath the cell cannot be labelled as the antibodies are not able to access this area due to the intact membrane and the close adhesion of the cell to the surface. Thus, immunofluorescent labelling is not a technique easily adapted to in vivo imaging.

Fn-protomer modification

The Fn network is composed of Fn protomers produced and secreted by the cell in addition to Fn protomers available in the growth media. In fact, Fn protomers can be modified with a fluorescent molecule and incorporated into the ECM by the cell without significantly altering the biological activity (Baneyx and Vogel 1999; Baneyx et al. 2002; Pickering et al. 2000). This method provides a means of visualizing Fn networks in living systems. Fn protomers were modified with Alexa Fluor® 568 (AF568) by reaction with free lysines.

The Alexa Fluor® 568 succinimidyl ester was used to modify Fn protomers by reaction with amines. Five milligrams of plasma Fn (Sigma) was dissolved in 2 ml of 0.1 M sodium bicarbonate buffer, pH 9.0 and dialysed against 500 ml 0.1 M sodium bicarbonate buffer, pH 9.0 for 24 h at 4°C with one buffer change after 12 h. This dialysis removed traces of amine from the Fn storage buffer. Five milligrams of AF568 was dissolved in 500 μl DMSO and combined with the Fn. The mixture was stirred overnight at 4°C. The mixture was dialysed extensively against 500 ml of PBS, pH 7.2, at 4°C for 72 h with five to six changes to remove unreacted AF568 reagent. The reaction and dialysis were carried out at 4°C in order to inhibit Fn degradation. UV spectroscopy was used to determine the final concentration of the AF568 modified Fn protomers (Fn-AF568) by standard procedures. The degree of labelling for Fn-AF568 was determined to be 0.547 fluorescent probes per protomer.

To incorporate protomers into Fn networks, 50 μl Fn-AF568 protomers were added to 2 ml of cells in media containing 5% FBS. Cells in media containing Fn-AF568 protomers were plated on glass bottom Bioptechs Inc. ΔT dishes (Bioptechs, Butler, PA, USA). Cells were grown to confluency (24–48 h) before imaging.

Image collection

For live cell imaging, a Leica DMIRE2 inverted microscope (Leica Microsystems, Heidelberg, Germany) with Open Lab 3.1.3 Software by Improvision (Guelph, ON, Canada) was used. Prior to imaging, the cell media was replaced with Krebs buffer at pH 7.4. Mineral oil was applied to the top of the media to prevent evaporation. Live cells were imaged for a total of 140 min with images acquired every 10 min. Cells were maintained at 37°C for the entire experiment. Fluorescence images of Fn networks following immunofluorescent labelling and fixation were acquired using a Zeiss 410 confocal laser scanning microscope (Zeiss, Thornwood, NY, USA) or a Leica DMIRE2 TCS SP2 inverted confocal microscope (Leica Microsystems).

In some experiments, the same cells imaged by confocal microscopy were also imaged by AFM. Coverslips were fastened to a metal disc and mounted on the sample stage of a Digital Instruments NanoScope III Multimode scanning probe microscope (J scanner, Digital Instruments, Santa Barbara, CA, USA). Cells were located using the NanoScope optical viewing system. Images were taken at 0.4–2.0 Hz in contact mode using gold coated silicon nitride V shaped cantilevers of 200 μm length with a spring constant of 0.06 N/m (Digital Instruments). Special care was taken to ensure that images were collected at the same magnification by confocal and atomic force microscopy.

Results

Live cell imaging of modified Fn protomer networks shows movements of fibres

Incorporation of fluorescently modified Fn protomers into the Fn matrix by live cells provides a method of

visualizing Fn networks over time (Figs. 1, 2). The soluble Fn protomers produced by protein synthesis within the cell (cellular Fn) are secreted and assembled into fibres and networks by the cell. In addition, the cell will also use plasma Fn, or Fn protomers that are present in the surrounding culture media (Alberts et al. 2002) even if they are chemically modified to contain a fluorescent probe. Antibodies used in immunofluorescent labelling do not distinguish between these two sources of Fn and therefore the antibody will label all Fn in the assembled fibres. It would be expected therefore that images of Fn networks generated by cells supplied with the fluorescent Fn protomers will on average appear dimmer than Fn networks labelled using antibodies. Nevertheless, the fluorescence from the modified Fn protomers in Fn fibres is fairly equally distributed throughout the cell population in patterns of networks similar to those obtained with antibody labelling (data not shown) suggesting that they reflect the same fibres and their distribution.

Figures 1 and 2 show every fourth frame of a series of images taken from two movies (S1 and S2) of living cells plated with Fn-AF568 protomers (see Materials and methods) present in the medium and illustrate that the Fn matrix is a dynamic network even when the cells are not moving extensively because they are nearly confluent. In these images, fluorescent Fn is observed as bright spots (non-fibrillar Fn-AF568 protomers) and as fibres (Fn-AF568 protomers polymerized by the cell). The nuclear region of the cell is distinguished as a circular structure (dotted circle) often surrounded by bright spots (Figs. 1a, 2a, arrow A) or small fibres of Fn. These intracellular bright spots may represent Fn protomers in endocytotic vessels.

A close examination of the position and orientation of Fn fibres in Figs. 1 and 2 in the images shows movements that include changes in shape, length, density and orientation of the fibres over time. In Fig. 1, two fibres at time 0 identified by arrows B and C have changed shape during the 140 min. The segment of fibre identified with arrow D at time 0 has decreased in length by 19% and appears thicker in the final image (arrow D at 140 min). When the images are played in sequence (S1), it appears that the two ends of this segment are moving toward each other. It is impossible to determine whether the shortening is due to a compression of a normal fibre or a relaxation of a stretched fibre, but it does not appear that the increase in density arises from a lateral fusion of fibres into a larger bundle (Ohashi et al. 1999). Arrow E in images from 0 to 140 min tracks a fibre of variable brightness that stretches and then breaks at 140 min. The left portion

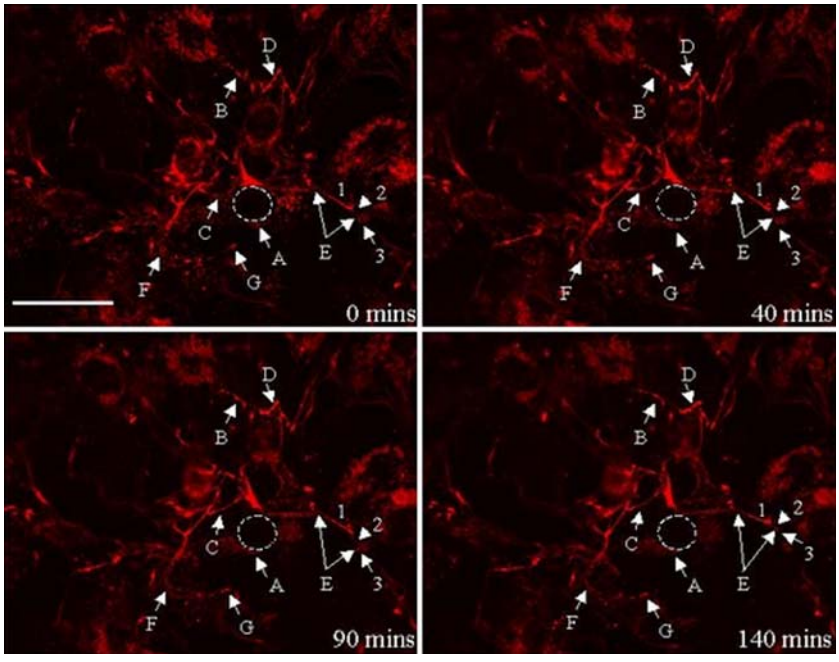

Fig. 1 Movement of Fn containing fibres imaged in real time. Frames shown are at 0, 40, 90, and 140 min. A series of fluorescence images were taken of live CV-1 cells incubated with Fn-AF568 for 48 h prior to imaging. Fifteen images were taken every 10 min for a total of 140 min. The *dotted circle* indicates position of a nucleus and *arrow A* points to unpolymerized Fn-AF568. *Arrows B and C* identify fibres that have been pulled straight, *arrow D* points to a fibre that has shortened, and *arrow E* points to a fibre that snaps and retracts over time. *Arrow F* points to a portion of a fibre that does not move significantly during imaging, and *arrow G* identifies the end of the same fibre which is pulled downward over time (scale bar = 50 μm)

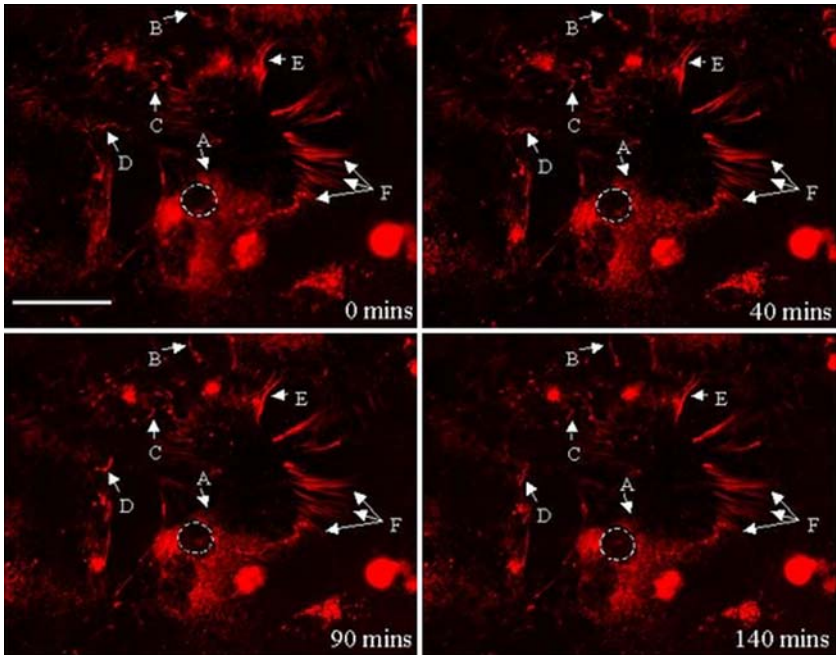

Fig. 2 Movement of Fn containing fibres imaged in real time. Frames shown are at time 0, 40, 90, and 140 min. A series of fluorescence images were taken of live CV-1 cells incubated with Fn-AF568 for 48 h prior to imaging. Fifteen images were taken every 10 min for a total of 140 min. The *dotted circle* indicates position of a nucleus and *arrow A* points to unpolymerized Fn-AF568. *Arrows B, C, and D* identify fibres that have changed significantly over time. *Arrow E* points to fibres whose movements are subtle and difficult to detect unless all 15 frames are viewed as a sequence. *Arrows F* points to parallel fibres that are static and no movement is observed over the 140 min of observation (scale bar = 50 μm)

of the fibre at point 1 is brighter than the small segment of the fibre at point 2. The segment at point 2 becomes fainter in the image as it is stretched and therefore more difficult to distinguish after 90 min. The change in length of the fibre from 90 to 140 min is more easily distinguished in the complete movie of 15 frames (S1). The gap between the remaining end of the fibre and previous point of attachment indicates that in this case the fibre retracted after the break. This suggests that the fibre was under tension and stretched over time until a threshold of extension was reached. The retraction of the fibre is consistent with the elastic nature of Fn (Ohashi et al. 2002; Erickson 2002).

The orientation of the fibre between arrows F and G in Fig. 1 changes over 140 min of observation. Arrow F marks the position of one end of the fibre which changes very little while the end at arrow G is steadily moved in the downward direction by 13.6 μm. The fibre is not visibly thinned or broken suggesting that the force acting on the fibre is transmitted uniformly across the fibre. A concurrent change in orientation and shape of Fn fibres is observed in Fig. 2. Arrows B, C, and D identify three fibres that reorient over time by as much as 90°. The uppermost end of the fibre identified with arrow B does not move significantly over time while its lower end is moved toward the bottom of the image. It appears to be anchored at one end and rotates to its final position after 140 min. The fibre at arrow C is sharply bent at 0 min but has been straightened after 140 min. The brightness of these two fibres remains more or less constant over the 140 min of observation. The final position of the fibre at arrow D after 140 min is approximately 90° to its original position at 0 min. After 40 min, this fibre is relatively straight but after 90 min the fibre is noticeably kinked and brighter. The final conformation of the fibre after 140 min is an uneven V shape. Viewing the images in sequence (S2), suggests that a force acts perpendicular to this fibre at the point of the arrow after 0 min, not at the ends of the fibre. The position where the force was applied becomes the point of the V in the final image (140 min).

Movement of other fibres, arrow E for example, is more subtle and can be viewed best when the series of 15 frames is played in sequence (S2). In some cases however, prominent Fn fibres remain stationary while change in the length and position of other fibres is quite pronounced. The most striking distinction in the series of images in Fig. 2 is the absence of movement of the prominent fibres indicated by the arrows at F. These fibres are aligned approximately parallel to each other and no significant movement is observed over the 140 min of observation.

Analysis of fixed cells shows that the fibres have different orientations under and outside the cell

CV-1 cells were fixed, *permeabilized* and fluorescently labelled with an anti-Fn primary antibody and a fluorescein conjugated secondary antibody. Confocal images at the adherent surface of the cells show diffuse staining in the area covered by the cell. Bright filamentous staining is observed at the edges or junctions of the cells (Fig. 3a, arrow 1) and in areas beneath the cell when it is no longer adhered (Fig. 3b). The regions under the nuclei are less bright (Fig. 3a, arrow 2). Figure 3b demonstrates that the filaments may be aligned in parallel (arrow 1) or oriented randomly (arrow 2).

Immunofluorescent labelling identifies the location of Fn containing networks, but does not reveal where the edges of the cell are relative to the brightly stained filamentous networks. AFM images of the same areas of the cell imaged by confocal microscopy reveal cellular structures in addition to fibres. Figure 4 shows the confocal (a), the AFM height (b), and the AFM deflection (c) images of the same region at identical magnification (a–c). It is evident that the brightly stained filamentous network seen in the confocal image can be correlated with structures observed in the AFM images, with particularly good contrast and detail in the deflection images. Careful comparison of the confocal and the AFM images shows that there is a one-for-one correspondence between the Fn containing fibres in the confocal image and the filaments observed by the AFM *outside the cell* (arrows 1 and 2). The fibres identified by fluorescence as containing Fn under thick areas of the cell are seldom observed in the AFM images (Fig. 4a, arrows). Fn containing fibres imaged clearly by AFM outside the cell are primarily aligned in parallel structures rather than being randomly oriented. Presumably, structures outside the cell periphery were laid down by the cell but were left behind as it moved. Fn left behind may be remodelled by other cells during subsequent movement.

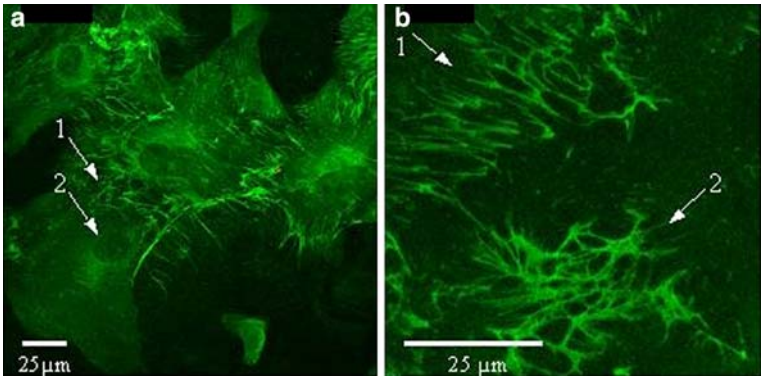

Fig. 3 Representative confocal images of CV-1 cells following fixation by paraformaldehyde, permeabilization and immunolabelling for Fn. The images in **a** and **b** illustrate the bright filamentous staining at the edges (**a**, *arrow 1*) and the less bright, diffuse staining below the nucleus (**a**, *arrow 2*). The images in **c** show the difference between the parallel (**c**, *arrow 1*) and random (**c**, *arrow 2*) orientation of the filaments (scale bars = 25 µm)

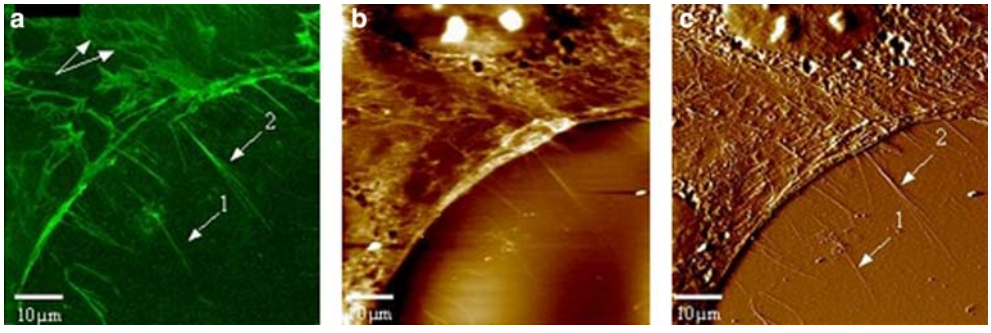

Fig. 4 Selected images from confocal (**a**), AFM height (**b**), and AFM deflection (**c**) measurements of CV-1 cells following fixation by paraformaldehyde, permeabilization and immunolabelling for Fn. Images **a**–**c** show images of part of two cells collected at identical magnification. In **a** and **c**, *arrows 1 and 2* indicate Fn containing fibres located outside the cell. Arrows in **a** indicate Fn networks observed beneath the cell (scale bars = 10 µm)

Fn containing fibres observed in the confocal image are not always seen in the AFM image (Fig. 4a, arrows) because they are under the cell. To confirm this, we used fixed but *non-permeabilized* cells since the anti-Fn antibody will only have access to and bind to Fn on the dorsal surface of the cell or on the glass surface. It is evident from comparing Fig. 5a with 5b, c, that there is no Fn on the top surface of the cell. The cell boundary is clearly identifiable in the AFM height (b) and deflection (c) images and it is clear from the outline of the cell drawn in Fig. 5a that the labelled Fn is primarily located outside the cell (Fig. 5a, arrows 1 and 2). Figure 5 shows bright staining of Fn containing fibres that extend past the edge of the cell. Very little fluorescence is observed in areas on or beneath non-permeabilized cells. Therefore, to obtain fluorescence images or AFM images of Fn containing fibres adhered to the substrate beneath the cell, the cell must be permeabilized (Fig. 4) or removed.

Figure 6 shows areas where cells were mechanically removed from the cover slip prior to labelling. Randomly oriented networks of Fn containing fibres remained adhered to the substrate (Fig. 6a, arrow, note scale). AFM height (b) and deflection images (c) confirm that the cell body was removed, leaving the Fn containing networks behind as a "footprint" of where the cell was attached to the substrate (Fig. 6b, c, arrows). The same area was imaged several times with no observable change in the structure or location of the fibres. Therefore, the attachment of Fn to the glass cover slip is greater than the lateral forces that are produced by the AFM tip during imaging in contact mode.

Fn containing fibres are distinct from intracellular fibrous structures

Fn containing fibres are frequently observed protruding nearly perpendicular to the edge of the cell (Fig. 4b, c, arrows 1 and 2). In some cells there is a nearly one-to-one correlation of fibres that are either *inside or under* the cell with the Fn containing fibres protruding from the edge of the cell (Fig. 5c, arrow 2).

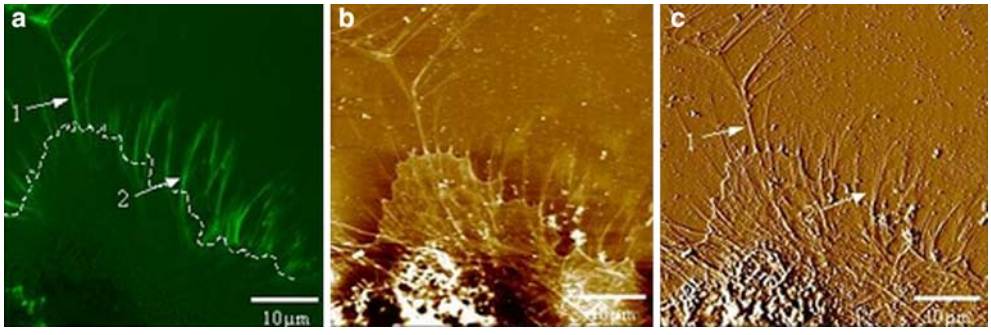

Fig. 5 Confocal (**a**), AFM height (**b**), AFM deflection image (**c**) of a fixed, but non-permeabilized CV-1 cell immunolabelled for Fn. The cell boundary as determined by the AFM images is represented by a *white dotted line* in the confocal image (**a**). Anti-Fn antibody will not have access to the adherent side of the cell of non-permeabilized cells and therefore labelling will only occur on the outside of the cell. In the AFM height (**b**) and deflection images (**c**), Fn containing fibres extend past the edge of the cell. *Note*: the confocal image is shifted upward relative to the AFM images (scale bars = 10 μm)

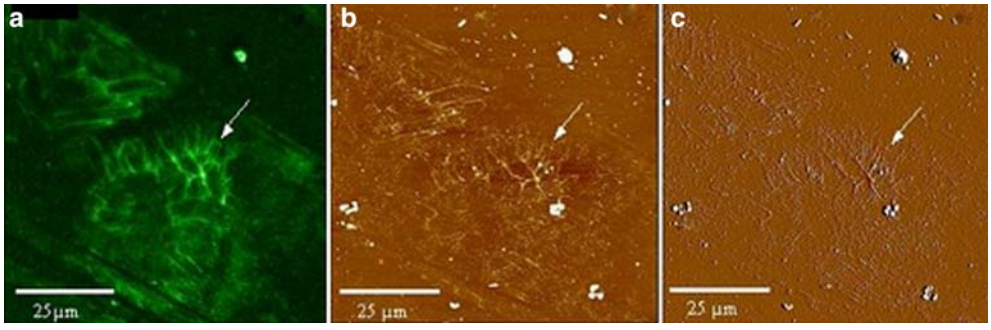

Fig. 6 Confocal (**a**), AFM height (**b**), and AFM deflection image (**c**) of an area where cells have been scraped off the cover slip prior to fixing and labelling for Fn. After scraping, randomly oriented networks of Fn containing fibres remain adhered to the substrate as observed in the confocal image (**a**, *arrow*). In AFM height (**b**) and deflection images (**c**), only a network of fibres remains (*arrow*) as the cell has been removed by scraping (scale bars = 25 μm)

The fibres are most likely continuous Fn containing fibres originating from beneath the cell and continuing to protrude from the edge of the cell. Alternatively, if the fibres are intracellular, the one-to-one correlation would suggest a strong interaction between the Fn fibres outside the cell and an intracellular component, such as one of the three main cytoskeletal components.

Since microfilaments can be imaged with the AFM (Henderson et al. 1992), a dual labelling experiment was used to determine if interaction between Fn and microfilaments occurred in a one-to-one correlation. Confocal images of cells labelled for both microfilaments (Fig. 7a) and Fn (Fig. 7b) show that these two fibres exist in the same areas of the cell (Fig. 7c), but that only Fn is observed outside the cell. Microfilaments observed in confocal images (Fig. 7c, arrow 1) correspond to fibres imaged in AFM height (Fig. 8a, arrow 1) and deflection (Fig. 8b, arrow 1) images taken at a higher magnification of the exact same region of the same cell. Fn containing fibres (Fig. 7c, arrow 2) also correspond to fibres in AFM images (Fig. 8a, b, arrow 2). The images confirm that the actin and Fn containing fibrous structures appear as distinctly different objects and structures. It is also evident that the microfilaments are not intracellular extensions of the extracellular Fn fibres.

Discussion

A model for Fn remodelling

To account for Fn reorganization observed during cell motility and the distinct organization of the fibres in

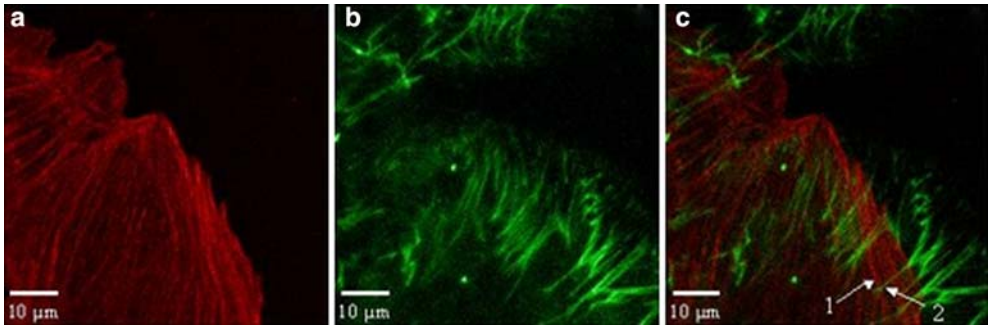

Fig. 7 Confocal images of paraformaldehyde fixed and permeabilized CV-1 cells simultaneously labelled for microfilaments and Fn. The image of microfilaments (**a**) and Fn (**b**) are overlaid in image (**c**). The overlay (**c**) shows that patterns of microfilaments and Fn fibres are markedly different. Microfilaments (**c**, *arrow 1*) appear to interact with Fn containing fibres (**c**, *arrow 2*) only at certain points along their length (scale bars = 10 μm)

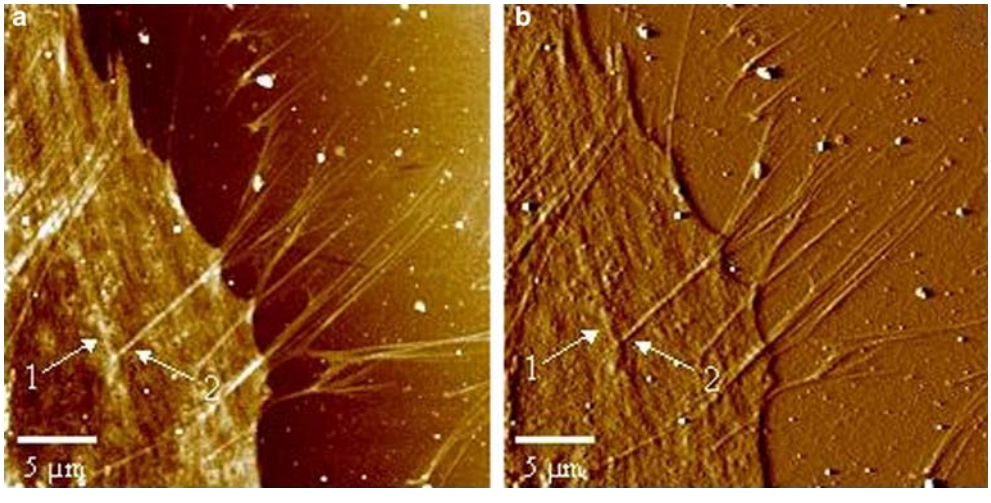

Fig. 8 AFM height (**a**) and deflection images (**b**) of cells shown in Fig. 7. Microfilaments observed in the confocal images in Fig. 7c, *arrow 1* is also observed in the AFM height and deflection images (*arrow 1*). Fn containing fibres identified in the confocal image (Fig. 7c, *arrow 2*) are observed in AFM height and deflection images as well (*arrow 2*). The deflection image **b** shows that Fn containing fibres (*arrow 2*) show greater contrast than microfilaments (*arrow 1*). *Note*: AFM images were taken at a higher magnification than the confocal images in Fig. 7 (scale bars = 5 μm)

parallel arrays outside of the cell, we propose that the tension produced by the cell normal to the fibre is the origin of the reorganization when the cell moves. The direction of the tension relative to the fibre is critical since the mechanical stability of proteins depends on the direction in which the force is applied (Brockwell et al. 2003; Carrion-Vazquez et al. 2003). Specifically, if the net force acting on the Fn fibre is perpendicular to the fibre itself, the Fn will move in the direction of the force (Fig. 9a). The binding between the Fn modules and the integrins will remain intact and the fibres will detach from the substrate along the fibre because the Fn–integrin bond is stronger than the local Fn–substrate bond.

In contrast, if the net force is parallel to the Fn fibre, the multiple attachments along the fibre will collectively resist movement. A net force pulling parallel to an Fn containing fibre would have to release multiple contacts to the substrate along the length of the fibre in order to move the fibre as a whole. Therefore, the Fn is more likely to stretch in response to the tension. As the Fn stretches, the affinity of the binding loop to the integrin is reduced (Krammer et al. 1999) which should lead to the release of Fn from the integrin and hence the cell leaving the fibres adhered to the substrate after the cell has moved (Fig. 9b).

Figure 10 illustrates the application of the principles of this model in a retrospective series of drawings that impose hypothetical Fn fibres and forces sequentially on the known AFM image. The same AFM image is used for Fig. 10a–e to illustrate the possible progression of cell retraction and Fn remodelling at the tail of the cell. The white dotted line indicates proposed positions of the cell edge during retraction. In Fig. 10a, red and green lines represent a force pulling perpendicular to a hypothetical Fn fibre (yellow dotted line). The force is perpendicular to the Fn fibre and the modules that bind the integrin remain intact. The fibre is detached from the substrate and pulled in the direction of the net force. This is illustrated further in Fig. 10b, c where the hypothetical Fn fibres (yellow) continue to be pulled in the direction of cell movement as the cell edge recedes (white dotted line).

Eventually, the net force will be parallel to the fibre and this will stretch the Fn fibre and release it from the cell because of the decrease in the integrin–Fn interaction at the 10th type III module. In Fig. 10d, e, once the Fn fibre is outside the cell, it remains adhered to the substrate and is no longer remodelled, while the portion of the fibre still beneath the cell continues to be pulled in the direction of cell movement. If the cell

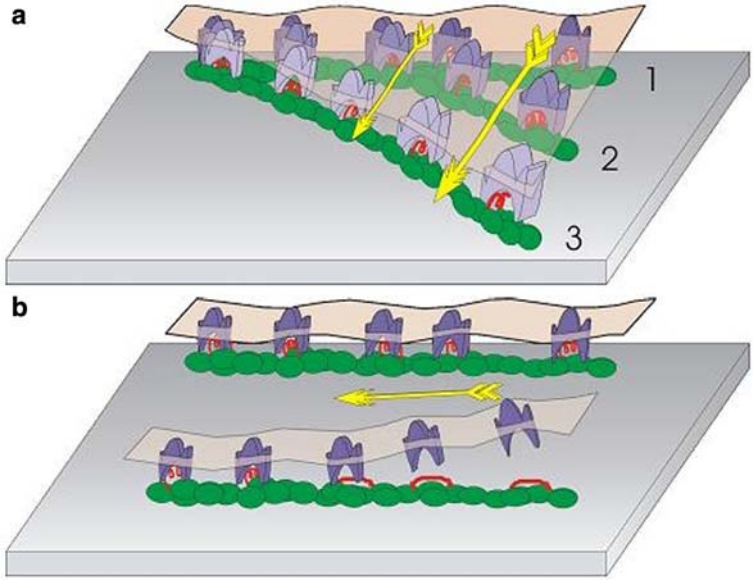

Fig. 9 Model illustrating Fn remodelling and Fn release from the cell under different net forces acting on Fn through integrins. In **a**, the net force (*yellow arrows*) acting through the integrins (*purple*) on the Fn fibre (*green*) is perpendicular to the fibre itself. The RGD loops (*red*) on Fn modules which bind integrins will remain intact and the fibres will detach from the substrate. As a result, the fibres will be pulled in the direction of the net force leading to remodelling as illustrated by three successive stages (1, 2, and 3) in **a**. In **b**, the net force is parallel to the Fn fibre and therefore the multiple attachments along the fibre will resist movement. The Fn will be stretched and the affinity of the binding loop to the integrin will be reduced. Presumably, the loss of affinity of the binding loop to the integrin will result in the release of Fn from the cell and the fibres will remain adhered to the substrate after the cell has moved

changes the direction of its movement, the fibres that are remodelled subsequent to the change will reflect the new direction.

Release of the cell from Fn may occur even if the force acting on the Fn fibre is not completely parallel to the fibre as long as there is also an opposing tension that leads to a net force along the length of the fibre beyond the threshold level. In Fig. 10f, green arrows represent forces acting on Fn on an angle as a result of contraction or tension in the cytoskeleton (Ingber 2003). Purple arrows represent opposing tension due to cell retraction. These are greatest at the cell edge (Munevar et al. 2001; Fig. 10f). We propose that when the net force (black arrow) is parallel to the Fn fibre, the Fn fibre is stretched. In this case, the Fn modules unfold causing the Fn–integrin bond to weaken. The integrin will release the Fn fibre and as the cell continues to retract (white dotted line), the Fn will be left behind adhered to the substrate, but outside the cell. Although no quantitative force measurements have been presented, the dynamic nature of the matrix and analysis of the patterns of Fn containing fibres and cell morphology infer the presence of forces affecting Fn remodelling.

Fn networks are dynamic

Fn secreted by cells in a soluble form is polymerized into insoluble fibres and incorporated into an Fn matrix. In the body, there are two sources of the Fn available for polymerization. One source is cellular Fn which is locally synthesized by the cell itself and a second source of Fn is originally synthesized by the liver and exists as plasma Fn carried by the circulatory system (Pickering et al. 2000). When cells are grown in culture, plasma Fn is available to the cell from the growth medium. Although cellular and plasma Fn differ slightly in their molecular makeup, both are secreted in a soluble form and contribute to matrix assembly. In addition, both forms of Fn can be visualized in live cells. Fn-GFP (green fluorescent protein) and Fn-YFP (yellow fluorescent protein) chimeric proteins have been used to study cellular Fn matrix assembly in living cultures (Ohashi et al. 1999, 2002; Erickson 2002). In such experiments, live cells were transfected with the Fn-GFP or Fn-YFP resulting in the synthesis of fluorescent Fn by the cells. Fluorescent Fn was secreted by the cells, assembled into networks, and the living cultures were observed by confocal microscopy to monitor the dynamics of the Fn matrix.

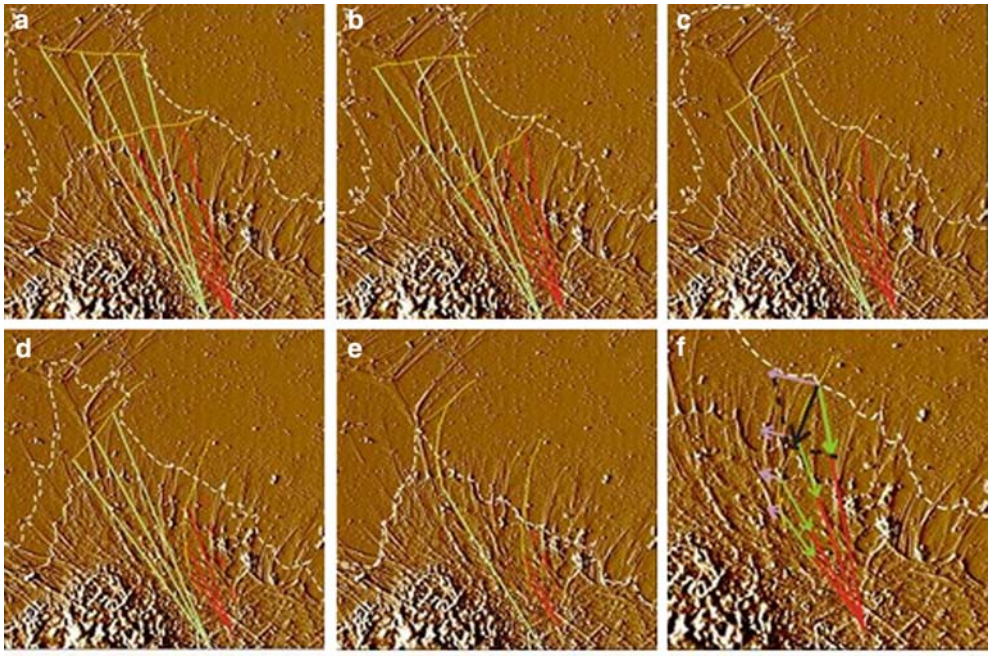

Fig. 10 AFM deflection image shown in Fig. 5 is used to illustrate a model for Fn remodelling. In each image, *white dotted lines* represent the receding cell edge and *yellow dotted lines* represent Fn as it is remodelled. *Green and red solid lines* indicate cellular structures which may pull on Fn. If the net force acting on Fn is approximately perpendicular to the fibre, the Fn fibre will detach from the substrate and be remodelled as the cell moves (**a**–**e**). In **f**, *purple arrows* represent opposing tension due to cell retraction, which is greatest at the cell edge, and *green arrows* represent forces acting on Fn at an angle as a result of contraction or tension in the cytoskeleton. The *black arrow* represents the net force that is parallel to the Fn fibre. A net force parallel to the fibre will stretch it and result in the release of the cell from Fn. The Fn fibre will remain behind adhered to the substrate rather than remodelled

Visualization of plasma Fn is also possible with chemical modification of soluble Fn protomers (Baneyx and Vogel 1999; Baneyx et al. 2002; Pickering et al. 2000). Immunolabelling results in the tagging of both cellular and plasma fibronectin and the images appear brighter compared to samples using modified protomers (data not shown). Unlike modified Fn protomers however, immunolabelling cannot be used in living cultures. The experiments presented here involved the modification of Fn protomers with Alexa Fluor® 568. The modified protomers were incorporated into networks and the pattern of Fn containing networks observed using the modified protomers is similar to those observed by others using Fn-GFP and Fn-YFP. Thus, modifying protomers is an acceptable technique for fluorescent labelling and incorporation of Fn into the matrix of living cells.

The movement of Fn containing fibres observed in the series of images in Figs. 1 and 2 indicates that the Fn matrix is a dynamic network affected by cell movement. Changes in the Fn matrix observed using modified Fn protomers, such as the straightening of bent fibres (arrows B and C in Fig. 1, and arrow C in Fig. 2) are similar to movements observed in living cultures using Fn-GFP (Ohashi et al. 1999, 2002; Erickson 2002). The conformation of a fibre in the matrix may be affected by any of the fibres to which it is connected due to the interconnectedness of the matrix and therefore the origin of Fn attachments within the matrix determines the extent of movement. In fact, a fibre can be changed to a different extent at a variety of points along its length which suggests that forces of varying magnitudes and directions must be at work. For example, the fibre at arrow B, Fig. 2 appears to rotate around the uppermost part of the fibre, which remains stationary throughout the 140 min of observation. Similar movements have been observed in studies using Fn-GFP where Ohashi et al. have shown that Fn fibres can be fixed at one end while the other end rotates, suggesting that the moving part of the fibre must be attached to the cell while the stationary end is attached to the substrate (Ohashi et al. 2002). Fn attachment to cells is sensitive to stretching and therefore the direction of the force applied to the fibre is also important (Krammer et al. 1999, 2002). Fn matrix reorganization will depend then, on the magnitude and direction of the strain applied by the cell and the strength of attachment of the fibre to the surface.

In some cases, entire fibres remain stationary for the duration of observation. In Fig. 2, the arrows at F mark numerous parallel fibres that do not change over time. Previous studies suggest that stationary fibres are attached to the substrate and not to the cell (Ohashi et al. 2002; Erickson 2002). In studies by Erickson (2002) and by Ohashi et al. (2002), cytochalasin B was used to disrupt the cytoskeleton to study the response of Fn fibres when released from the cell (Ohashi et al. 2002; Erickson 2002). After treatment, the cells contracted toward the cell centre but the majority of Fn fibres showed no movement, especially those toward the cell edge. The study suggests that older segments which have elongated and are toward the cell edge appear static because they are attached to the substrate. It is possible then, that the fibres observed in our studies at arrow F are not attached to the cell but are adhered to the substrate only although we cannot confirm that the cell has indeed released the Fn. If the cell were still attached to the stationary Fn fibres, this would indicate that any forces acting on these parallel fibres were not great enough to overcome the forces holding the fibres in their present position.

Evidence of prior cell motion: Fn containing fibres extend from the uropod perpendicular to the edge

Analysis of patterns of Fn containing fibres and cell shape supports the model of Fn remodelling described in this paper. The direction of fibroblast-like cell motion can be inferred from its morphology. A motile cell frequently appears as an elongated triangle where the smaller side of the triangle forms the front or leading edge of the cell (Chen 1981; Bray 2001). The flat section of cell spreading at the leading edge is referred to as the lamellipod (DiMilla et al. 1991). The point of the triangle opposite to this side is the elongated tail of the cell, or uropod, which forms the last point of attachment as the cell moves. The two sides of the triangle leading to the uropod are typically smooth and under tension (Bray 2001). In Fig. 4c, a curved edge leading to a developing uropod is observed. The curved edge suggests a direction of motility towards the left side of the image. Fibres extend from this edge indicating that Fn containing fibres were left behind by the cell as it moved. In Fig. 5c, the upper portion of the cell resembles a point and may be forming a uropod. Fn containing fibres are imaged extending from both sides of the point and from the point itself. In each of these images, cells at one point in time were adhered in the areas where the fibres are imaged outside the cell but the fibres are exposed as the cell moved. Although membrane ripping has been observed by fibroblast cells (Chen 1981), we do not observe integrins outside CV-1 cells after immunofluorescent labelling (data not shown). The Fn containing fibres observed in AFM images vary in width which may result form aggregates of integrins that initiate a number of Fn polymerization

reactions in close proximity resulting in larger fibre widths in some areas. Such an event would strengthen adhesion to the substrate in that particular area.

The model we have proposed describes how Fn is remodelled during cell motion. A corollary of this model is that the pattern of Fn containing fibres left behind will provide a "history" of cell motion. The distribution of external fibres and their pattern will contain information about the forces exerted by the cell during motility.

Our confocal images show that Fn containing fibres exposed by individual cells are organized in similar patterns in which the fibres are aligned in arrays perpendicular to the retracting edges. Figure 3b illustrates two distinct patterns of fibre organization. In the upper half of the image, the fibres appear to be organized in arrays similar to the arrangement of fibres trailing behind moving cells. Nearly all Fn containing fibres imaged outside of a cell edge are found in an array-type pattern. We believe these are fibres left on the surface after the cell has moved away.

In the lower half of the image, fibres are organized in criss-crossed networks of randomly oriented directions. Generally, Fn containing fibres in randomly oriented patterns cannot be imaged by AFM unless the cell is removed and are not observed in fluorescence images unless the cell is permeabilized. In Fig. 6, cells were removed by scraping and randomly oriented networks of fibres were imaged by AFM (Fig. 6b, c). Thus, we conclude that the randomly oriented networks occur predominately under the cell where they are undetectable to AFM and are not susceptible to staining in non-permeabilized cells. We attribute this pattern to the fact that the cell may polymerize Fn in various directions as it adheres and spreads, resulting in randomly oriented networks beneath the entire cell.

Remodelling during motion

If the fibre remains attached to the substrate at one end and to the cell at the other, Fn containing fibres release their attachment to the substrate at various locations between the ends, and the fibre will be pulled in the direction of motion as the cell moves. The fibres may reattach to the substrate in a new location, resulting in arrays of fibres in the direction of motility. Therefore, it is possible to distinguish Fn matrices which have been remodelled from those which remain as polymerized networks as the result of adhesion.

For example, in Fig. 5c, Fn containing arrays are oriented in different directions indicating that parts of the cell were under different stresses to allow the cell to move in one particular direction. In Fig. 5c, several Fn containing fibres collimate at a junction point creating a fan shaped array of fibres (Fig. 5c, top left). Fibres of different orientations meeting at a point of intersection may indicate the point of origin or the source of stress. In Fig. 5c, Fn is aligned in arrays beneath the cell suggesting that the force originated at the front or middle of the cell.

Stresses at the cell–substrate interface

In a study of stresses at the cell–substrate interface, Dembo and Wang (1999) observe strong traction under the lamellipod. Using traction force microscopy, Munevar et al. (2001) determined that the strongest traction was located at the leading and trailing ends of the cell whereas no significant traction was present around the nucleus. The source of the traction may originate from sites of attachment such as focal adhesions (Burridge et al. 1988) that mechanically connect the substrate and the ECM to the cytoskeleton (Choquet et al. 1997; Riveline et al. 2001; Schmidt et al. 1993; Wang et al. 1993). The cytoskeleton generates tension within the cell which determines cell shape and drives cell movement (Davies et al. 1997; Ingber 1997; Galbraith and Sheetz 1998). Mathur et al. (2000) have shown that a local stress applied to a cell results in a global effect due to the transmission of forces by the cytoskeleton. Ingber's (2003) tensegrity model suggests that when a force is applied to adhesion receptors, the mechanical load will be transferred to cytoskeletal components, which will either distort or break. Microfilaments, a key component of the cytoskeleton, span the entire cell and are believed to be primarily responsible for transmission of stress due to their contractile and flexible nature (Mathur et al. 2000). Microfilaments are therefore the likely sources for fibres under tension stretched between the sites of attachment (focal adhesion) and sites of force generation needed to drive the leading edge forward. As the cell moves forward, attachment sites remain fixed to the substrate requiring new adhesions and microfilaments to be produced at the leading edge (Wang 1985). It is believed that the organization of microfilaments relates to the organization of Fn fibrils (Hynes and Destree 1978) since microfilaments are necessary for Fn assembly into fibres (Shaub 1999). In addition, microfilaments are believed to generate tension by pulling on the ECM (Hynes and Destree 1978). Therefore, the force acting on the Fn resulting in its remodelling may be primarily due to microfilament contraction in the direction of cell motility. If the force is due to microfilament contraction, the ends of the fibres closest to the centre of the cell will be pulled in the general direction of motion.

Conclusion

In this work, we have shown that fibronectin can be identified in fibres and studied in live cell cultures by confocal microscopy and that these same fibres can be studied in detail in fixed cells by AFM at higher resolution. The combination of the techniques provides new insights into the distribution and reorganization of the ECM fibres by the cells. During adhesion of stationary cells, the fibres appear randomly oriented under the cell, but regions that the cell appears to have traversed contain fibres that are oriented in parallel arrays in the direction the cell has moved. These observations lead to the conclusion that the fibres are reorganized by the forces of cellular movement when these forces are perpendicular to the length of the fibre. Moreover, when the net force is along the length of the fibre, the interaction between the cell and fibre is weakened and the fibre is left behind. This model is consistent with the theoretical conjecture that the binding between the 10th segment of the fibronectin polymer and the membrane bound integrin is weakened by tension applied along the fibre. The proposed mechanism provides for a balance between attachment and detachment dependent on the direction of tension applied to the fibres by the cell.

The reorganization observed in this work in cell culture may also be relevant to cellular motility in tissues. The organized network of ECM proteins must be laid down by the cells in the first place in tissues. The orientation of fibres in such a network could be determined by the movement of the cells on the matrix, leading to primarily parallel arrays at the local level. Once in place, the orientation of the fibres in the network may guide the cell movement along the direction of the fibres since the collections of fibres would be harder to reorganize.

Tension in the tissue as a whole would provide another level of organizing principle at a larger scale, but would presumably be dependent on the local order at the cellular level. While the data presented in this work are clearly suggestive of the model proposed here, the real test will depend on measurement of the binding interaction while the fibronectin fibres are subjected to a stress. We are currently working on such experiments.

Acknowledgements The authors would like to thank Dr. Niki Boyd for fruitful discussions, the Petersen and Norton group members, and the Sandig lab in the Department of Anatomy and Cell Biology for their technical assistance. We would also like to acknowledge financial support from NSERC for strategic grants and CFI for infrastructure grants to NOP and PRN as well as OGSST and NSERC for postgraduate scholarships for KLD.

References

Adams JC, Watt FM (1990) Changes in keratinocyte adhesion during terminal differentiation: reduction in fibronectin binding precedes $\alpha_5\beta_4$ integrin loss from the cell surface. Cell 63:425–435

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2002) Molecular biology of the cell, 4th edn. Garland, New York

Anderson EH, Ruegsegger MA, Murugesan G, Kottke-Merchant K, Marchant RE (2004) Extracellular matrix-like surfactant polymers containing arginine–glycine–aspartic acid (RGD) peptides. Macromol Biosci 4(8):766–775

Baneyx G, Vogel V (1999) Self-assembly of fibronectin into fibrillar networks underneath dipalmitoyl phosphatidylcholine monolayers: role of lipid matrix and tensile forces. Proc Natl Acad Sci USA 96(22):12518–12523

Baneyx G, Baugh L, Vogel V (2001) Coexisting conformations of fibronectin in cell culture imaged using fluorescence resonance energy transfer. Proc Natl Acad Sci USA 98(25):14464–14468

Baneyx G, Baugh L, Vogel V (2002) Fibronectin extension and unfolding within cell matrix fibrils controlled by cytoskeletal tension. Proc Natl Acad Sci USA 99(8):5139–5143

Baugh L, Vogel V (2004) Structural changes of fibronectin adsorbed to model surfaces probed by fluorescent resonance energy transfer. J Biomed Mater Res 3:525–534

Bray D (2001) Cell movements from molecules to motility, 2nd edn. Garland, New York

Brockwell DJ, Paci E, Zinober RC, Beddard GS, Olmsted PD, Smith DA, Perham RN, Radford SE (2003) Pulling geometry defines the mechanical resistance of a beta-sheet protein. Nat Struct Biol 10:731–737

Burridge K, Fath K, Kelly T, Nucko G, Turner C (1988) Focal adhesions: transmembrane junctions between the extracellular matrix and cytoskeleton. Annu Rev Cell Biol 4:487–525

Carrion-Vazquez M, Li H, Lu H, Marszalek PE, Oberhauser AF, Fernandez JM (2003) The mechanical stability of ubiquitin is linkage dependent. Nat Struct Biol 10:738–743

Chen WT (1981) Mechanism of retraction of the trailing edge during fibroblast movement. J Cell Biol 90:187–200

Chen CS, Mrksich M, Huang S, Whitesides G, Ingber DE (1997) Geometric control of cell life and death. Science 276:1425–1428

Choquet D, Felsenfeld DP, Sheetz MP (1997) Extracellular matrix rigidity causes strengthening of integrin–cytoskeleton linkages. Cell 88:39–48

Craig D, Gao M, Schulten K, Vogel V (2004) Tuning the mechanical stability of fibronectin type III modules through sequence variations. Structure 12:21–30

Davies PF, Barbee KA, Volin MV, Robotewskyj A, Chen J, Joseph L, Griem ML, Wernick MN, Jacobs E, Polacek DC, DePaola N, Barakat AI (1997) Spatial relationships in early signaling events of flow-mediated endothelial mechanotransduction. Annu Rev Physiol 59:527–549

Dembo M, Wang Y-L (1999) Stresses at the cell-to-substrate interface during locomotion of fibroblasts. Biophys J 76:2307–2316

DiMilla PA, Barbee K, Lauffenburger DA (1991) Mathematical model for the effects of adhesion and mechanics on cell migration speed. Biophys J 60:15–37

Erickson HP (2002) Stretching fibronectin. J Muscle Res Cell Motil 23:575–580

Galbraith CG, Sheetz MP (1998) Forces on adhesion contacts affect cell function. Curr Opin Cell Biol 10:566–571

Gao M, Craig D, Lequin O, Campbell ID, Vogel V, Schulten K (2003) Structure and functional significance of mechanically unfolded fibronectin type III1 intermediates. Proc Natl Acad Sci USA 100(25):14784–14789

Geiger B, Bershadsky A, Pankov R, Yamada KM (2001) Transmembrane crosstalk between the extracellular matrix–cytoskeleton crosstalk. Natl Rev Mol Cell Biol 11:793–805

Henderson E, Haydon PG, Sakaguchi DS (1992) Actin filament dynamics in living glial cells imaged by atomic force microscopy. Science 257:1944–1946

Hynes RO (1990) Fibronectins. Springer, Berlin Heidelberg New York

Hynes RO (1999) Cell adhesion: old and new questions. Trends Cell Biol 9(12):M33–M37

Hynes RO, Destree AT (1978) Relationships between fibronectin (LETS protein) and actin. Cell 15:875–886

Ingber DE (1997) Tensegrity: the architectural basis of cellular mechanotransduction. Annu Rev Physiol 59:575–599

Ingber DE (2003) Tensegrity II. How structural networks influence cellular information processing networks. J Cell Sci 116:1397–1408

Krammer A, Lu H, Isralewitz B, Schulten K, Vogel V (1999) Forced unfolding of the fibronectin type III module reveals a tensile molecular recognition switch. Proc Natl Acad Sci USA 96:1351–1356

Krammer A, Craig D, Thomas WE, Schulten K, Vogel V (2002) A structural model for force regulated integrin binding to fibronectin's RGD-synergy site. Matrix Biol 21:139–147

Mathur AB, Truskey GA, Reichert WM (2000) Atomic force and total internal reflection fluorescence microscopy for the study of force transmission in endothelial cells. Biophys J 78:1725–1735

Munevar S, Wang Y-L, Dembo M (2001) Traction force microscopy of migrating normal and H-ras transformed 3T3 fibroblasts. Biophys J 80:1744–1757

Ohashi T, Kiehart DP, Erickson HP (1999) Dynamics and elasticity of the fibronectin matrix in living cell culture visualized by fibronectin-green fluorescent protein. Proc Natl Acad Sci USA 96:2153–2158

Ohashi T, Kiehart DP, Erickson HP (2002) Dual labelling of the fibronectin matrix and actin cytoskeleton with green fluorescent protein variants. J Cell Sci 115:1221–1229

Pickering JG, Chow LH, Li S, Rogers KA, Rocnik EF, Zhong R, Chan BMC (2000) alpha5beta1 integrin expression and luminal edge fibronectin matrix assembly by smooth muscle cells after arterial injury. Am J Pathol 156(2):453–465

Potts JR, Campbell ID (1994) Fibronectin structure and assembly. Curr Opin Cell Biol 6:648–655

Renner BL, Jorgensen M, Markowski K, Salchert C, Werner TP (2004) Control of fibronectin displacement on polymer substrates to influence endothelial cell behaviour. J Mater Sci Mater Med 15:387–390

Riveline D, Zamir E, Balaban NQ, Schwarz US, Ishizaki T, Narumiya S, Kam Z, Geiger B, Bershadsky AD (2001) Focal contacts as mechanosensors: externally applied local mechanical force induces growth of focal contacts by an mDia1-dependent and ROCK-independent mechanism. J Cell Biol 153:1175–1186

Schmidt CE, Horwitz AF, Lauffenburger DA, Sheetz MP (1993) Integrin–cytoskeletal interactions in migrating fibroblasts are dynamic, asymmetric, and regulated. J Cell Biol 123:977–991

Shaub A (1999) Unravelling the extracellular matrix. Nat Cell Biol 1:E173–E175

Sheetz MP, Felsenfeld DP, Galbraith CG (1998) Cell migration: regulation of force on extracellular-matrix–integrin complexes. Trends Cell Biol 8:51–54

Wang Y-L (1985) Exchange of actin subunits at the leading edge of living fibroblasts: possible role of treadmilling. J Cell Biol 101:597–602

Wang N, Butler JP, Ingber ED (1993) Mechanotransduction across the cell surface and through the cytoskeleton. Science 260:1124–1127

Zhong CL, Chrzanowska-Wodnicka M, Brown J, Shaub A, Belkin AM, Burridge K (1998) Rho-mediated contractility exposes a cryptic site in fibronectin and induces fibronectin matrix assembly. J Cell Biol 141:539–551