Fibroblast Contraction Occurs on Release of Tension in Attached Collagen Lattices: Dependency on an Organized Actin Cytoskeleton and Serum

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ABSTRACT

The generation of tension in granulation tissue undergoing contraction is believed to be a cell-mediated event. In this study we used attached collagen lattices as a model system for studying the cellular mechanisms of tension generation by fibroblasts in an extracellular matrix. Fibroblasts in attached collagen lattices developed stress fibers, surface associated fibronectin fibrils, and a fibronexus-like transmembrane association interconnecting the two structural components. Release of the attached collagen lattice from its points of attachment resulted in a rapid, symmetrical contraction of the collagen lattice. Rapid contraction occurred within the first 10 minutes after release of the lattice from the substratum, with greater than 70% of the contraction occurring within the first 2 minutes. Rapid contraction resulted in a shortening of the elongate fibroblasts and compaction of the stress fibers with their subsequent disappearance from the cell. Cytochalasin D treatment prior to release disrupted the actin cytoskeleton and completely inhibited rapid contraction. The removal of serum prior to release inhibited rapid contraction, while the re-addition of serum restored rapid contraction. These results demonstrate that fibroblasts can develop tension in an attached collagen lattice and that upon release of tension the fibroblasts undergo contraction resulting in a rapid contraction of the collagen lattice. Fibroblast contraction is dependent upon an organized actin cytoskeleton and is promoted by the presence of serum.

Tissue contraction is a part of normal wound healing. Tissues undergoing contraction can generate tension (Abercrombie et al., 1960; Higton and James, 1964). The generation of tension is believed to be a cell-mediated event; however, how cells generate the forces resulting in tension during tissue contraction is unclear. Fibroblasts have the potential to generate tension. They can exert tension upon a flexible silicon rubber substratum (Harris et al., 1980) and can also generate tension when cultured within a stabilized collagen lattice (Bellows et al., 1982; Farsi and Aubin, 1984; Mochitate et al., 1991). Bundles of actin microfilaments with associated myosin and actin-binding proteins, termed stress fibers (Byers et al., 1983), are present in tension-generating fibroblasts. Stress fibers have been proposed to be organized in response to cell contraction under isometric conditions (Wohlfarth-Boettermann and Fleischer, 1976; Burridge, 1981). They have also been proposed to be contractile (Isenberg et al., 1976; Kreis and Birchmeier, 1980) and may participate in generating the forces responsible for continued development and maintenance of tension (Isenberg et al., 1976; Kreis and Birchmeier, 1980; Farsi and Aubin, 1984; Danowski, 1989).

A specialized fibroblast-like cell, termed the myofibroblast, is present in tissues undergoing contraction (Gabbiani et al., 1972). This cell is characterized by the presence of large intracellular bundles of actin microfilaments (Skalli and Gabbiani, 1988). These actin bundles resemble stress fibers both ultrastructurally (Skalli and Gabbiani, 1988) and by their staining with anti-nonmuscle myosin antibodies (Tomasek et al., 1986; Eddy et al., 1988). These cells are also characterized by fibronexus-like transmembrane associations between actin microfilaments and fibronectin fibrils present at their surfaces (Singer et al., 1984; Tomasek et al., 1987; Tomasek and Haaksma, 1991). Based upon their spatial and temporal distribution and the presence of large bundles of actin microfilaments these cells have been proposed to be contractile and responsible for the production of tension in tissues undergoing contraction (Skalli and Gabbiani, 1988; Schultz and Tomasek, 1990; Ehrlich and Rajaratnam, 1990).

One method to study the development and mainte-
nance of tension by fibroblasts is to examine their contraction when released from isometric conditions. The mechanical release of the trailing portion of a fibroblast migrating upon a nondeformable substratum has been used to examine its development and maintenance of tension (Chen, 1981). The release of an attached collagen lattice from its points of attachment provides a model for studying the generation of tension by fibroblasts in a three-dimensional extracellular matrix (Bellows et al., 1982; Farsi and Aubin, 1984; Mohcitate et al., 1991). In this study we have examined the rapid contraction of collagen lattices at very early time points after release, with particular emphasis on: a) alterations that occur in the shape and cytoskeleton of fibroblasts and the surrounding extracellular matrix upon release; b) how the organization of the actin cytoskeleton relates to development and maintenance of tension and rapid contraction; and c) the dependency on serum of the rapid collagen lattice contraction. A preliminary account of part of this report has been previously presented (Tomasek et al., 1989).

MATERIALS AND METHODS

Cells

Human fibroblasts were obtained from explant cultures of palmar aponeurosis. Normal-appearing palmar aponeurosis was obtained as surgical discard tissue from patients undergoing carpal tunnel release. Pieces of tissue were placed onto 60 mm tissue culture dishes (Falcon, Oxnard, CA), allowed to attach, and cultured in complete media containing M-159 media (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA), 2 mM glutamine, and 1% antibiotic-antimycotic solution. Cells were harvested using trypsin-ethyldiaminetetraacetic acid (EDTA) (GIBCO) and cultured in 75 cm² tissue culture flasks (Falcon). Fibroblasts used in these experiments were between cell passages two and ten.

Preparation of Collagen Lattices

Fibroblasts were cultured within type I collagen lattices as previously described (Tomasek et al., 1982), so that the final collagen concentration was 0.65 mg/ml and the cell concentration was 1.25 × 10⁶ cells/ml. A 250 μl drop of the collagen-cell suspension was placed on a 35 mm plastic tissue culture dish (Falcon). The placement of the drop of collagen/cell suspension onto a dry plastic tissue culture dish insured that the lattice would remain attached for the 5 day culture period. After 1 hour incubation at 37°C, to allow for gelation of the collagen, 1.5 ml of complete media was placed over the collagen lattice. Care was taken not to detach the lattices from the underlying plastic substratum. The attached lattices were then incubated for 5 days. For experiments examining the effect of cell concentration on rapid contraction, fibroblasts were mixed with the collagen solution so that a final cell concentration of 0.625, 1.25, or 2.5 × 10⁶ cells/ml was obtained.

Rapid Contraction Assay

After 5 days of incubation, the attached lattices were mechanically released from the underlying substratum by freeing the edges of the collagen lattice with a scalpel and releasing the rest of the area by gently pipetting media at the lattice-dish interface. Culture dishes were then returned to the incubator. Rapid contraction was analyzed by measuring the diameter of the lattice before and at various times after release, using a Nikon SMZ-1 stereoscope. Lattice diameters were normalized due to variation in the initial diameter of the lattices which ranged from 14–16 mm. The relative lattice diameter was obtained by dividing the diameter of the collagen lattice at each time point by the initial diameter of the lattice.

Microscopy

For light and electron microscopy, collagen lattices were fixed, dehydrated, and embedded in Polybed 812 (Polysciences, Warrington, PA) (Tomasek et al., 1982). Sections (1 μm thick) were stained with 1% toluidine blue and photographed with an Olympus Vanox photomicroscope. Thin sections were stained with uranyl acetate and lead citrate and photographed on a JEOL 100 C transmission electron microscope.

For immunofluorescence, collagen lattices were fixed with paraformaldehyde (Tomasek et al., 1982; Tomasek and Hay, 1984). To visualize the actin cytoskeleton, fibroblasts in the lattice were permeabilized by treatment with acetone for 5 minutes at −20°C, rinsed with phosphate buffered saline (PBS), and stained with bodipy phallacidin (Molecular Probes Inc., Eugene, OR) (Barak et al., 1980). Pieces of lattice were mounted in 80% glycerol in PBS, examined, and photographed with an Olympus Vanox photomicroscope.

For immunoelectron microscopy, collagen lattices were fixed with paraformaldehyde (Tomasek et al., 1982) and incubated with an anti-human plasma fibronectin mouse monoclonal antibody (mAb) diluted 1:50 in PBS followed by a goat anti-mouse IgG antibody conjugated to 5 nm colloidal gold (Janssen Life Sciences Products, Piscataway, NJ) diluted 1:5 in PBS. The mAb was a gift from Dr. Albert Millis (State University of New York at Albany, Albany, NY). This mAb has been previously characterized and described (Millis et al., 1985). As a control, the primary mAb was replaced with PBS. Lattices, after immunostaining, were fixed for electron microscopy as described above. To obtain a three-dimensional view of the distribution of fibronectin, some of the blocks were prepared for viewing in the high voltage electron microscope (HVEM) (Song et al., 1986). Sections, 0.75 μm thick, were examined at an accelerating voltage of 1 million electron volts using the ARI EM 7 HVEM (NIH Biotechnology HVEM Resource, Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY).

Cytochalasin D Treatment of Lattices

Cytochalasin D was added to the culture media of attached collagen lattices after 5 days in culture. Lattices were released 30 minutes after treatment with cytochalasin D. The rate of lattice contraction was determined as described above. Cytochalasin D (Sigma) was kept as a 2 mM stock solution in dimethyl sulfoxide (DMSO) at −20°C and added to the culture media for a final concentration of 6 μM. Control lattices received an equivalent concentration of DMSO (0.3%).
FIBROBLAST CONTRACTION IN COLLAGEN LATTICES

Fig. 1. Dark field photomicrographs illustrating rapid contraction. a: Attached collagen lattice cultured for 5 days is round and symmetrical in outline. b: Ten minutes after release the diameter of the same lattice is dramatically reduced. The circumference of the lattice prior to release is visible as a white line due to the scraping of the plastic surface with a scalpel to mechanically free the attached lattice. Bar, 2 mm.

Fig. 3. Light micrographs demonstrating the alterations in cell shape and extracellular matrix upon release. a: After 5 days in culture, collagen fibrils and fibroblasts in attached collagen lattices become aligned parallel to the underlying planar substratum. b: Two minutes after release the collagen has become compacted and pli-cated. Fibroblasts have withdrawn their processes and appear more rounded. c: Ten minutes after release the collagen is more compacted. Epoxy sections (1 µm) were cut perpendicular to the underlying substratum and stained with toluidine blue. Bar, 20 µm.

Fig. 2. Analysis of rapid collagen lattice contraction. a: Rapid contraction is dependent upon cell number. The number of fibroblasts incorporated into the lattice at the initiation of the culture were none (o), $1.25 \times 10^5$ (c), $2.5 \times 10^5$ (△), or $5 \times 10^5$ (□) cells/ml. b: Most of the rapid contraction occurs in the first 2 minutes after release. The data are averages of quintuplicate cultures. Standard deviations are shown.

Removal of Serum

Attached collagen lattices, after 5 days in culture, were washed 2 times over a 5 minute period with unsupplemented M-199 previously warmed to 37°C. After the final wash lattices were released. Some of the washed lattices received 10% fetal bovine serum immediately prior to release. Control lattices were washed with M-199 supplemented with 10% fetal bovine serum. Washed and released lattices received 10% fetal bovine serum 30 minutes after release. The rate of lattice contraction was determined as described above.

RESULTS

Fibroblasts Rapidly Contract a Released Collagen Lattice

Figure 1 illustrates the rapid contraction that occurred upon release of an attached collagen lattice. Contraction rapidly reduced the diameter of the lattice within the first 10 minutes after release (Fig. 2a,b).
Fig. 4. Electron micrographs demonstrating the alterations in morphological appearance of fibroblasts and collagen upon release. a: Fibroblasts cultured in attached collagen lattices for 5 days are well spread, with a smooth surface and ovoid nucleus. b: Two minutes after release the fibroblasts become rounded with numerous protrusions extending from the cell surface. The collagen fibrils become compacted after release. Bar, 10 μm.

This rapid contraction was followed by a slower contraction of the lattice (Fig. 2a), which continued for at least 3 days after release (not illustrated). Rapid lattice contraction is a cell-mediated process as lattices without cells did not contract (Fig. 2a). Also, increased cell concentration increased the amount of rapid contraction (Fig. 2a).

Rapid contraction, within the first 10 minutes after release, was further analyzed. Figure 2b shows measurements of the lattice diameter during this time. Within 2 minutes after release, lattices were reduced to an average relative diameter of 0.69, a reduction of 31% (Fig. 2b). This was followed by a slower rate of contraction until an average relative diameter of 0.57, a reduction of 43%, was reached 10 minutes after release (Fig. 2b). These results demonstrate that 72% of the rapid retraction that occurs within 10 minutes after release occurs within the first 2 minutes.

Fibroblasts Alter Their Shape and Organization of Surrounding Collagen Fibers Upon Rapid Contraction

The shape of the fibroblasts and the organization of the collagen fibers changed dramatically upon release of the collagen lattice from the substratum. Before release of the lattice the fibroblasts and collagen fibers were aligned parallel to the underlying substratum (Fig. 3a). The cells appeared to be well spread in the lattice with a smooth cell surface (Figs. 3a, 4a). Two minutes after release the fibroblasts appeared rounded as compared to cells in attached collagen lattices (Figs. 3b, 4b). The surface had developed numerous protrusions and nuclear indentations were prominent (Figs. 3b, 4b). Collagen fibers became wavy and compacted, losing their parallel orientation with the underlying substratum (Figs. 3b, 4b). By 10 minutes after release the fibroblasts appeared more rounded and the collagen fibers more compacted (Fig. 3c).

Fibroblasts Alter Their Cytoskeleton Upon Rapid Contraction

Cytoskeleton prior to release

Fibroblasts cultured for 5 days within attached collagen lattices were stained with bodipy phallacidin to visualize the organization of f-actin. Fibroblasts were found to contain abundant bundles of actin microfilaments oriented parallel to the long axis of the cell (Fig. 5a).

Bundles of actin microfilaments formed close transmembrane associations with extracellular filaments at the cell surface (Fig. 6a). These extracellular filaments labelled intensely with anti-fibronectin antibody (Fig. 6b). Collagen fibrils making up the lattice showed little labelling. These transmembrane associations resemble the previously described fibronexus (Singer, 1979).
the stress fibers appeared thickened and intensely stained (Fig. 5b, arrows). Most of the stress fibers had disappeared by 10 minutes after release, with the staining for actin organized into aggregates (Fig. 5c).

Alterations in the cytoskeleton observed with electron microscopy were consistent with those observed by fluorescence microscopy. Two minutes after release, the bundles of actin microfilaments appeared compacted; electron-dense regions along their length were prominent and periodically spaced (Fig. 7a,b). By 10 minutes after release, few of these large bundles of actin microfilaments could be found in the cells; the only remnant being electron-dense regions surrounded by densely packed microfilaments (not illustrated).

**Rapid Contraction Is Dependent Upon an Organized Actin Cytoskeleton**

Cytochalasin D was used to determine the potential role of the actin cytoskeleton in lattice contraction. Fibroblasts cultured within attached collagen lattices for 5 days were treated with 6 μM cytochalasin D for 30 minutes prior to release from the substratum. The effect of the drug upon the actin cytoskeleton was evaluated by staining treated fibroblasts with bodipy phallacidin. The cytoplasm of the cytochalasin D-treated cells contained small aggregates of actin and lacked organized stress fibers (Fig. 8a). Rapid lattice contraction was found to be almost totally inhibited in the cytochalasin D-treated lattices (Fig. 8b).

**Rapid Contraction Is Dependent Upon the Presence of Serum**

The removal of serum from 5 day-attached lattices immediately prior to release resulted in a dramatic reduction in the amount of rapid contraction (Fig. 9). At 10 minutes post-release, lattices from which serum had been removed were reduced to an average relative diameter of 0.79, compared to 0.53 for control cultures (Fig. 9). This reduction is not due to lack of protein, since the addition of 4% bovine serum albumin did not restore rapid contraction (not illustrated). Rather, the reduction in rapid contraction was due to the lack of serum, as the addition of serum to a serum-free lattice immediately prior to release resulted in rapid contraction comparable to that observed for the control (Fig. 9). To determine whether fibroblasts after serum removal and release are still contractile, serum was added back 30 minutes after release. Rapid contraction occurred immediately upon the addition of serum (Fig. 9, arrow). The addition of serum brought the total amount of rapid contraction back to control levels.

**DISCUSSION**

We have used the attached collagen lattice model to examine the cellular mechanisms responsible for the generation of tension by fibroblasts. This model is uniquely suited for studying this phenomenon. First, it allows fibroblasts to interact with the surrounding extracellular matrix in three dimensions. Second, because the collagen fibers are attached to the underlying substratum, fibroblasts can develop tension in the collagen lattice. Third, the attached collagen lattice can be mechanically released from the underlying plastic dish with the immediate loss of tension. Fourth, the release of an attached collagen lattice provides the op-
Fig. 6. Electron micrographs demonstrating the organization of the cytoskeleton within and fibronectin filaments at the surface of fibroblasts cultured within attached collagen lattices for 5 days. a: Large bundles of actin microfilaments (large arrowhead) overlap with extracellular filaments (arrow) at the cell surface forming a transmembrane association. Collagen fibrils (small arrowhead) also are closely apposed to the cell surface. b: Stereo pair of high voltage electron micrographs of fibroblast cultured for 5 days in an attached collagen lattice. Lattice was labelled with anti-fibronectin antibody followed by secondary antibody, as described in Materials and Methods. Gold particles are primarily restricted to extracellular filaments that extend from the cell surface. a: Bar, 0.5 μm; b: Bar, 0.5 μm.

portunity of observing an intact fibroblast population undergoing isotonic contraction. We have observed that attached collagen lattices when released from the underlying substratum undergo a rapid contraction. These results demonstrate that fibroblasts can generate tension within an extracellular matrix. The development and maintenance of tension is an active cell process dependent upon an organized cytoskeleton. Previous studies have also observed that fibroblasts can contract an attached collagen lattice, however they did not or could not observe contraction at the very early time points after release (Bellows et al., 1982; Farsi and Aubin, 1984; Unemori and Werb, 1986; Mochitate et al., 1991). Rapid collagen lattice contraction appears to be primarily dependent upon active fibroblast contraction. Disruption of the actin cytoskeleton with cytochalasin D, just prior to release, inhibited rapid contraction. Active fibroblast contraction would depend on an organized actin cytoskeleton to generate contractile forces. Consistent with active fibroblast contraction was the dramatic change in the shape of the cells upon rapid contraction of the collagen lattice.

Rapid contraction of released collagen lattices was dependent upon the presence of serum. The effect of serum on collagen lattice contraction was immediate. This is most obvious when serum is added to lattices which have been released in the absence of serum. Under these conditions the lattices, which are floating freely in the dish, undergo an immediate contraction upon the addition of serum. Platelet derived growth factor and transforming growth factor beta have both been previously demonstrated to promote the contraction of free-floating collagen lattices (Montesano and Orci, 1988; Clark et al., 1989). Neither could replace serum in promoting rapid collagen lattice contraction (Tomasek, unpublished observations). The mechanism by which serum promotes rapid contraction is currently under investigation.

With the development of tension in an attached collagen lattice, adult human fibroblasts form bundles of actin microfilaments, similar to those observed in other...
Fig. 7. Electron micrographs demonstrating the organization of the cytoskeleton of a fibroblast 2 minutes after release of a 5 day attached collagen lattice. a: Large bundles of actin microfilaments are still present within the cell. b: Higher magnification of a thin section adjacent to that seen in Figure 7a. Prominent periodic electron densities are present within bundles of actin microfilaments. Intermediate filaments are closely associated with bundles of actin microfilaments (arrowhead). a: bar, 2 μm; b: bar, 0.5 μm.
types of fibroblasts (Bellows et al., 1982; Farsi and Aubin, 1984; Mochitate et al., 1991). The formation of stress fibers may be dependent upon the mechanical properties of the collagen lattice. In the attached collagen lattice, the collagen fibrils are tethered to the underlying substratum. The fibroblasts can and do slowly reduce the height of the lattice, however they cannot reduce the diameter of the lattice (Guidry and Grinnell, 1985). Over time, the collagen fibrils become reorganized so that they are oriented parallel to the underlying substratum and become stabilized (Nakagawa et al., 1989a,b). Such a mechanically stable collagen lattice could provide a rigid substratum for the development of tension. Cell contraction under isometric conditions has been shown to promote the development of prominent microfilament bundles by aligning individual microfilaments along lines of stress (Wohlfarth-Bottermann and Fleischer, 1976) and may promote stress fiber formation (Burridge, 1981). The mechanically stable collagen fibrils in the attached collagen lattice could provide a substratum that would resist contractile forces generated by the cell resulting in tension and stress fiber formation.

Stress fibers present in fibroblasts may generate isometric tension and rapidly contract collagen lattices upon release. Isolated stress fibers have been demonstrated to be highly contractile elements (Isenberg et al., 1976; Kreis and Birchmeier, 1980). Upon release of the attached lattice, fibroblasts changed their shape and lost their stress fibers. This is analogous to isotonic contraction which results in a disassembly of bundles of actin microfilaments (Wohlfarth-Bottermann and Fleischer, 1976). A similar loss of stress fibers in fibroblasts cultured within attached collagen lattices and subsequently released has been described, however in these studies the cytoskeleton was not examined until 1 hour after release (Unemori and Werb, 1986; Mochitate et al., 1991) or at an indeterminate time after release (Farsi and Aubin, 1984). We have observed an alteration in stress fibers 2 minutes after release, and by 10 minutes almost all stress fibers have disappeared. The stress fibers that are present 2 minutes after release appear highly condensed and have prominent periodic electron densities along their length. These ultrastructural properties are consistent with contraction of the stress fibers (Langanger et al., 1986).

Rapid contraction is followed by a slower contraction which occurs over a matter of hours and extends to days. During this slow phase of contraction the fibroblasts spread again in the lattice and do not reform stress fibers (Tomasek, unpublished observations). This slower phase of contraction appears to be analogous to the contraction of a free-floating collagen lattice (Bell et al., 1979). Cell traction has been proposed to generate the force responsible for contraction of free-floating lattices (Harris et al., 1980, 1981). Tractional force is distinct from fibroblast contraction (Harris et al., 1980, 1981; Ehrlich and Rajaratnam, 1990). These results suggest that slow contraction of a free-floating collagen lattice is a different process than the rapid contraction of a released collagen lattice.
Adult human fibroblasts cultured within attached collagen lattices acquire stress fibers, fibronectin fibrils, and a fibronexus and develop tension. These cells resemble, both morphologically and functionally, myofibroblasts observed in tissues undergoing contraction (Skalli and Gabbiani, 1988; Schultz and Tomasek, 1990; Ehrlich and Rajaratnam, 1990). Other types of fibroblasts also acquire these characteristics when cultured in attached collagen lattices, including human foreskin fibroblasts (Mochitate et al., 1991), rabbit synovial fibroblasts (Unemori and Werb, 1986), monkey and porcine periodontal ligament fibroblasts (Bellows et al., 1982; Farsi and Aubin, 1984), and the human fetal lung fibroblast cell lines WI-38 and IMR-90 (Tomasek et al., 1982; Farsi and Aubin, 1984). Other types of fibroblasts that acquire myofibroblast-like characteristics are cultured within attached collagen lattices or upon the cutting of splinted granulation tissue, and porcine periodontal ligament fibroblasts (Bellows et al., 1982). Tension does develop in unsplinted wounds (Billingham and Medawar, 1955; Abercrombie et al., 1960). Tension does develop in unsplinted wounds that are small excised wounds (Billingham and Medawar, 1955; Abercrombie et al., 1960), presumably the result of resistance by the surrounding skin to the inward pull of the granulation tissue. It does not develop, however, to the extent observed in splinted wounds (Billingham and Medawar, 1955; Abercrombie et al., 1960). Tension does develop in unsplinted wounds (Billingham and Medawar, 1955; Abercrombie et al., 1960). Tension does develop in unsplinted wounds (Billingham and Medawar, 1955; Abercrombie et al., 1960). Tension does develop in unsplinted wounds (Billingham and Medawar, 1955; Abercrombie et al., 1960).

The forces responsible for the development of tension during tissue contraction may be similar to those described in attached collagen lattices. Abercrombie and coworkers (1960) demonstrated that if an open wound is splinted, tension will develop such that upon cutting a portion of the granulation tissue, contraction occurs at such a rapid rate as to be directly visible. Rapid contraction, as observed in our released collagen lattices or upon the cutting of splinted granulation tissue, does not occur during normal wound healing. The rapid contraction most likely represents an exaggeration of certain aspects of the normal process (Abercrombie et al., 1960). Tension does develop in unsplinted wounds (Billingham and Medawar, 1955; Abercrombie et al., 1954), presumably the result of resistance by the surrounding skin to the inward pull of the granulation tissue. It does not develop, however, to the extent observed in splinted wounds (Abercrombie et al., 1960). As proposed for collagen lattices, tension development in the contracting tissue may promote the formation of stress fibers, which, in turn are highly contractile. These results suggest that the fibroblasts, which acquire stress fibers and a fibronexus, provide the contractile forces responsible for tension development in contracting tissues.

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LITERATURE CITED


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