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The Role of Nonmuscle Myosin IIA in Endothelial Cell

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A thesis submitted to the
School of Medicine
at West Virginia University
in partial fulfillment of the requirements
for the degree of

Master of Science
in
Biomedical Science

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Morgantown, West Virginia
2010

Key word: myosin IIA; knock down; endothelial cell; tension; motility

Abstract

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Myosins are molecular motors that convert chemical energy into mechanical force. Vertebrate cells contain three myosin II isoforms, designated as myosin IIA, IIB and IIC, and each isoform has unique biochemical and biophysical characteristics. It is generally accepted that all three isoforms are involved in regulating actin structures and cell contraction. However, it is unknown what role(s) each isoform plays in maintaining actin structures and generating cellular force. Experiments presented in this thesis will begin to document what role myosin IIA plays in endothelial cell contraction and actin dynamics.

Myosin IIA ablated from endothelial cells by infection with adenovirus encoding a short hairpin interfering RNA targeting mouse nonmuscle myosin IIA (shRNAi-IIA) mediated knockdown of myosin IIA, altered endothelial cell shape and induced reorganization of actin filaments. Myosin IIB content was unaltered and myosin IIB remained associated with actin filaments. Cell attachment and spreading occurred faster and there was an increase in cell motility compared to control cultures. Loss of myosin IIA resulted in a 40% reduction in basal force production and a significantly blunted thrombin induced cell contraction. These results show myosin IIA is needed for normal endothelial cell functions and is essential for generation of basal and agonist-induced force.

Acknowledgements

I would like to thank my advisor, Dr. Robert Wysolmerski for his mentorship. This work would not have been possible without his unfaltering support and encouragement. I would also like to thank my committee members, Dr. Fred Minnear and Dr. Michael D. Schaller, for all of their patient guidance, support, and critical reading of my work. I would also like to thank Dr. Zoe Goeckeler and Dr. Karen Martin for their expertise and advice. Finally, I would like to thank Wei Fu and Kimberly Meide for their help and friendship in this wonderful journey.

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Glossary

Ad-shRNAi-IIA adenovirus encoding hair-pin short interfering RNA targeting mouse non-muscle Myosin IIA

DMEM Dulbecco's Modified Eagle Media

DOC Deoxycholic Acid

ECIS Electric Cell-substrate Impedance Sensing

MEM Minimal Essential Media

MHC Myosin heavy chain

MLC Myosin light chain

MLCK Myosin light chain kinase

Myosin IIA Nonmuscle Myosin IIA

Myosin IIB Nonmuscle Myosin IIB

SVEC Mouse vascular endothelial cell

Chapter 1

Introduction

The vascular system is lined by a continuous sheet of endothelial cells. The individual endothelial cell is typically a flattened, extended polygonal cell which interfaces with its multiple neighbors through a modest variety of junctional specializations characteristic of its site in the circulation. *In vivo*, the flattened state of cell abutted to cell is the usual condition. However, under the influence of a variety of stimuli, sizable gaps form between endothelial cells allowing the passage of large molecules otherwise confined to the circulation [1–4]. Evidence that the opening of endothelial cell junctions depending on an active contractile process was first presented by Majno and Palade [5]. These investigators observed in stimulated post-capillary venules that the endothelial cells bulged into the lumen and their nuclei developed numerous superficial pleats. Majno also observed that the endothelial cells expressed a prominent array of fine cytoplasmic filaments, which he proposed could generate the required contractile force [1,5]. This evidence provided a basis for the theory of endothelial cell contractility, while it need further support. In the early 80's several laboratories demonstrated gap formation between cultured endothelial cells upon exposure to thrombin [6–8]. Rotrosen et al. [9] extended the observation to histamine while Shasby et al. [10] described a more severe retraction when endothelial cells were exposed to cytochalasin B. Furthermore, cytokines also induce gap formation that appears indistinguishable from histamine and thrombin.

Our laboratory has reported on the formation of gaps between endothelial cells induced by ethchlorvynol, an agent capable of increasing pulmonary capillary permeability and causing pulmonary edema [2,11]. We extended our studies to show similar

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effects with histamine, thrombin and PAF [12–14]. In our studies, the shape change is reversible and there is no injury to the cells. We have also shown that the change in F-actin associated with shape change of the endothelial cell occurs in an ATP-dependent manner [15]. Our laboratory used the term retraction initially to avoid any a prior implication that it requires actomyosin-mediated contractile event. However, we now know from our permeabilized cell studies [12,15] and from intact preparations [13,14,16] that endothelial cells possess a myosin light chain kinase (MLCK) controlled contractile mechanism. Our laboratory has published a method that allows quantitative measurements of contractile force generated by cells grown within a collagen gel or by cells grown on top of a collagen matrix [14]. We have utilized the phosphorylation hypothesis as a prototype to study the mechanism of endothelial cell contractility [12,17]. Endothelial cells do not appear to contract in the sense of shortening along a single axis as smooth muscle cells do. Instead, peripheral regions of the polygonal cell focally withdraw leaving spaces between adjacent cells which typically remain connected by tenuous processes [11,12,15,17]. Clues to the mechanism of contraction of endothelial cells have been provided by such diverse cell types as smooth muscle cells [18–21] fibroblasts [13,22–24] RBL cells [25,26] and leukocytes [27]. Smooth muscle cells, unlike endothelial cells, contract in a polarized fashion.

The common event causing the stimulation of smooth muscle contraction is the increase in cytosolic free Ca^{2+} . According to the simplest proposed model, Ca^{2+} associates with calmodulin and this complex activates MLCK which in turn results in phosphorylation of myosin light chain (MLC) thus initiating contraction [21,28]. The phosphorylation of the 20 kD MLC results in activation of the actin activated Mg-ATPase activity of myosin which allows myosin to hydrolyze ATP and affect the transformation of chemical energy to mechanical work. Our permeabilized monolayer studies strongly implicated MLCK and MLC phosphorylation as the mechanism responsible for initiation of endothelial cell retraction. Our laboratory as well as Garcia's utilizing thrombin [29] and studies from Shasby's laboratory [30] with histamine have confirmed that stimulation with inflammatory agonists induces MLC phosphorylation.

Myosin light chain kinases are serine/threonine kinases that specifically catalyze the phosphorylation of serine-19 (Ser19) and threonine-18 (Thr18) in the 20 kD regulatory light chain of myosin II [31–33]. Phosphorylation of myosin light chain by MLCK is responsible for initiation of contraction in smooth muscle and leads to potentiation of

contraction in skeletal muscle [34,35] . However, the function of MLCK and myosin II in the control of nonmuscle contractility is not well understood. In vitro studies have suggested that MLCK may control the activity of nonmuscle myosin in nonmuscle cells. Phosphorylation of Ser19 by MLCK regulates both filament formation [14,32,36] and the myosin II actin activated ATPase activity [18,33] while diphosphorylation of MLC at both Ser19/Thr18 by MLCK has been implicated in increased filament stability and has been shown to further increase myosin II ATPase activity in vitro [37] .

So far, investigated vertebrates appear to contain three different isoforms of the conventional nonmuscle myosin II, designated as nonmuscle myosin IIA, myosin IIB and myosin IIC [38,39]. Although the precise functions of these myosins are not well understood, the mRNA encoding each isoform appears to be consistently expressed across many tissue and cell lines [40–42]. Since these isoforms are regulated by phosphorylation of the 20kD light chains, it is plausible that differential targeting of isoform phosphorylation dictates unique cellular distributions and/or functions. It has been well known that myosin IIA is involved in the generation of contraction in nonmuscle cells and this process requires the actin cytoskeleton, specifically the localized polymerization of actin filaments. Myosin IIA is a potent motor protein responsible for cell contraction, but the specific molecular mechanisms are poorly understood.

Recent study indicates that endothelial barrier integrity depends on the actin and myosin cytoskeleton [43,44]. One possible mechanism of increase of vascular permeability is endothelial cell contraction. However, it is not completely understood how myoactin cytoskeleton is involved in endothelial cell contraction and results in an alteration in cell permeability. Understanding the molecular mechanisms of myosin IIA function in endothelial cells will identify novel therapeutic targets for human diseases that arise due to defects in endothelial barrier integrity.

In this study, the subject is to explore the role of nonmuscle myosin IIA in endothelial cell by knocking down nonmuscle myosin IIA expression in mouse vascular endothelial cells.

Chapter 2

Materials And Methods

2.1 Cell Culture and Reagents

Mouse endothelial cell line obtained from mouse lung tissue was a generous gift of Dr. Troy Stevens, University of South Alabama.

Cells were cultured in minimal essential media (MEM) supplemented with 10% heat-inactivated fetal bovine serum, 50 unit/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin at 37 °C under the humidified atmosphere of 5% CO₂ and 95% air. Cells were split at a ratio of 1:4 every 7-8 days.

2.2 Immunofluorescence Microscopy

Control and infected endothelial cells were washed with pre-warmed PBS 3 times, fixed with freshly made 37 °C fixative (1% formaldehyde in stabilization buffer, pH 6.5) for 1 minute at 37 °C, followed by incubation with fixation and permeabilization buffer (2% formaldehyde, 0.2% triton X-100, 0.5% DOC in stabilization buffer, pH 6.5) for 1 hour at 37 °C. Before staining, cultures were washed once with PBS and then permeabilized in stablization buffer containing 0.5% triton X-100, 0.6% DOC, pH 6.5 for 10 minutes at room temperature. After permeabilization, cell cultures were washed with PBS 3 times and flooded with 2 ml of blocking buffer (PBS containing 0.8% BSA, 0.5% fish gelatin, 10 $\mu\text{g}/\text{ml}$ IgG) for 1 hour. Blocking buffer was removed and cultures were incubated with primary antibody in diluted blocking buffer (1:10 dilution) for 3 hours at room temperature. After primary antibody incubation, cell cultures were washed with diluted blocking buffer and then incubated with secondary antibody in diluted

blocking buffer for 2 hours at room temperature. Cultures were washed 3 times with PBS and coverslipped with 90% glycerol/10% PBS containing 0.1 M n-propyl gallate. Pictures were taken on a Zess 510 confocal microscope and Z series images were built into composite micrographs.

For staining of myosin IIA + actin or myosin IIB + actin, myosin IIA or myosin IIB were stained in 0.25 $\mu\text{g}/\text{ml}$ affinity purified rabbit polyclonal anti-myosin IIA or anti-myosin IIB primary antibody and followed by Alexa 488 labeled goat anti rabbit secondary antibody. Actin filaments were stained with 1.0 $\mu\text{g}/\text{ml}$ rhodamine phalloidin (Sigma, Cat No. P1591) for 1 hour at room temperature. For double staining of myosin IIA + myosin IIB, myosin IIA was stained with 1.0 $\mu\text{g}/\text{ml}$ working concentration of rabbit polyclonal anti-myosin IIA, followed by Alexa 488 labeled donkey anti rabbit secondary antibody. Myosin IIB was stained with 1.5 $\mu\text{g}/\text{ml}$ working concentration of affinity purified goat polyclonal primary antibody, followed by Alexa 568 labeled donkey anti goat secondary antibody.

2.3 Virus Amplification

Adenovirus encoding short hairpin interfering RNA targeting a mouse nonmuscle myosin IIA (shRNAi-IIA) was amplified in 293T cells. Cells were seeded into T75 cm^2 flask 24 hour prior to infection. After achieving 70-80% confluence, 293T cells were washed 3 times with serum free MEM media without penicillin/streptomycin, incubated with media containing virus at 37 °C for 2 hours. After infection, cell cultures were fed with media (without penicillin/streptomycin) with 10% fetal bovine serum and incubated at 37 °C. After most of the cells become round (about 24 hours post infection), cells were dislodged by pipeting up and down, media and cells were collected and spun down at 1000 $\times$ g (2000 rpm), 4 °C for 20 minutes. Cell pellets were re-suspended in 5 ml of sterile 100 mM Tris buffer (pH8). Cell pellets were frozen in a dry-ice-methanol slurry and immediately thawed in a 37 °C water bath. Cell pellets were processed through 3 freeze-thaw cycles. Cell lysates were spun down at 1000 $\times$ g (2000 rpm) at 4 °C for 20 minutes, supernatant was saved and pellet was discarded.

2. MATERIALS AND METHODS

2.4 CsCl Purification

CsCl gradients were prepared in SW-40T tubes (Beckman ultra clear tubes, Cat344059): First, 2.12 ml of 12 g/cc CsCl (26.8 g CsCl in 92 ml of 100 mM Tris buffer PH 8, 10 mM EDTA) was placed at bottom of SW-40T tube. Then CsCl layer containing 2.82 ml of 1.4 g/cc CsCl (53 g CsCl in 87 ml of 100 mM Tris pH8, 10 mM EDTA) was pipeted onto the top of 12 g/cc CsCl. 7 ml viral supernatant was gently added on top of CsCl cushion and spun down at $210,000\times g$ (35,000 rpm) at 4 °C for 1hr in the SW-40 Ti rotor, by using max-start and low brake.

After centrifugation, two distinct bands form the CsCl gradient interfaces. The bottom band which is white and fluffy at the interface between the 1.4 g/cc and 1.2 g/cc layers was collected with an 18G gauge needle attached to a 5 ml sterile syringe. The tube was punctured below the band for withdrawing only the viral particles.

A second CsCl gradient in polyallomar quick seal tubes was prepared (Beckman 13x51mm, Cat342412).

Tubes were placed on ice and virus was added to the bottom of each tube, then 1.34 g/cc (13.5 g of CsCl in 30 ml of 100 mM Tris buffer pH 8, 10 mM EDTA) was added to the top of the dome.

Sealed tubes were spun at $320,000\times g$ (60,000 rpm) at 4 °C overnight (more than 8 hours) in the rotor VTi65.2.

PD10 columns (PD10 sephadex G25, pre-packed column) were washed twice with 25 ml of sterile 30% glycerol in PBS. After removing tubes from centrifuge, the top of each tube was punctured to release pressure. A white and fluffy band containing virus was collected with an 18G gauge needle attached to a 5 ml sterile syringe.

Virus was placed on PD-10 column. 10 ml of 30% glycerol/PBS solution was added to the column, and 500 μ l fractions were collected. All samples were analyzed by using Nanodrop spectrophotometers at OD 260 nm to determine which fraction contain purified virus. Virus was stored at -80 °C.

2.5 Adenovirus Infection

50,000 cells were seeded onto 35 mm dish pre-coated with 20 μ g/ml collagen 24 hours before infection. For each infected dish, cell cultures were incubated with 1ml MEM media containing adenovirus (without penicillin/streptomycin and serum) for 2 hours

at 37 °C, then fed with 1 ml MEM media (without penicillin/streptomycin) plus 20% fetal bovine serum. After 48 hours, old media was removed and cultures were fed with fresh MEM media (without penicillin/streptomycin) with 10% fetal bovine serum containing same amount of adenovirus as initial infection. The total infection duration was 96 hours.

2.6 Western Blot Analysis

Cell cultures were washed 3 times with PBS before harvest. Each culture dish was flooded with 100 μ l of 100 °C 1.5 $\times$ SDS sample buffer containing 10 μ g/ml DNase I. Cells were collected by scraping from the dish with a rubber policeman and transferred to microfuge tubes.

Cells were sonicated and heated at 100 °C for 10 minutes to denature proteins and samples were stored frozen at -80 °C.

SDS samples were run on 10% SDS-PAGE gels and then transferred to PVDF membrane. 0.1% SDS was included in transfer buffer to help facilitate protein transfer.

Membranes were incubated with the affinity purified primary antibodies against NMHC IIA (rabbit polyclonal anti-myosin IIA, 0.025 μ g/ml), NMHC IIB (rabbit polyclonal anti-myosin IIB, 0.025 μ g/ml), and GAPDH (Sigma, Cat. No. G8795, mouse monoclonal, 1:10,000 dilution) at room temperature for 3 hours, followed by incubation with horseradish peroxidase-conjugated secondary antibodies at 1:5000 dilution (goat anti rabbit) at room temperature for 1 hour. The immunoblotted proteins were incubated with ECL reagent and exposed to X-ray film.

2.7 Isometric Tension Measurements

Cells were resuspended in appropriate amount of collagen solution containing fibrinogen (1 mg/ml rat tail collagen I, 3 mM NaOH, 0.2 mg/ml fibrinogen in DMED media plus 10% fetal bovine serum) to achieve final concentration of 1×10^6 cell/ml. 2 unit/ml thrombin was added to collagen/cell suspension before being poured into a Teflon mold with central pole. Molds were incubated at 37 °C for 2 hours to allow collagen polymerization, which initiated the collagen/cell matrix forming a ring.

2. MATERIALS AND METHODS

Molds were fed with MEM media (with penicillin/streptomycin) plus 10% fetal bovine serum 2 hours after casting and incubated at 37 °C for 24 hours prior to adenoviral infection.

After 24 hours, collagen/cell gels were washed twice with MEM media without penicillin/streptomycin and serum before being infected with adenovirus within molds. For each infected mold, collagen/cell rings were infected by incubating with 1 ml adenovirus-containing MEM media (without penicillin/streptomycin and serum) for 2 hours at 37 °C. Molds were then fed with 2 ml MEM media (without penicillin/streptomycin) plus 15% fetal bovine serum. After 48 hours infection, media were replaced with fresh MEM media (without penicillin/streptomycin) with 10% fetal bovine serum plus same amount of adenovirus as initial infection and incubated at 37 °C. Infections were carried out for a total of 96 hours. After 96 hours incubation, the collagen/cell rings were removed from molds and hanged on isometric force transducer (model 52-9545; Harvard Apparatus, South Natick, MA). Rings were bathed in 50 ml HEPES-buffered MEM media supplemented with 10% BSA in a thermoregulated bath at 37 °C and pH 7.4. After baseline force was developed, cell rings were stimulated with 2 unit/ml thrombin. After achieving stable tension, the cell rings were treated with 2 μ M cytochalasin D.

2.8 Attachment and Spreading Measured by ECIS

Control or infected cells were seeded onto ECIS cultureware (100,000 per well, 0.8 cm² per well) pre-coated with collagen (5 μ g/well). The ECIS measurement was started immediately after cells were seeded into chambers. Cells attached and spread on the small gold electrode on the bottom of the chamber. A current passed between the small gold-film electrode and a larger gold counterelectrode. As cultured cells attach and spread onto the electrodes, the current has to flow in the spaces under and between the cells which causes the increase in the electrical resistance, as the cell membrane is essentially an insulator. The total time duration of measurement is 24 hours.

2.9 Cell Motility

Control or infected cells were seeded onto 35 mm dish (50,000 per dish) and subjected to cell motility measurement by Nikon Swept Field confocal system 24 hours after cast-

ing. The total time duration of measurement is 5.5 hours. The centroid position (x, y) of each cell was tracked at each 60-second interval for the entire duration of the experiment. Cells that migrated out of the image field as well as divided during the experiment were excluded from the calculation. The speed of migrating cell was calculated by using the distance that the centroid position of the cell moved over each 30 minute interval.

Chapter 3

Results

3.1 Adenovirus Infection

In order to determine the optimal condition for reducing the level of myosin IIA in mouse endothelial cells, experiments were undertaken to assess the appropriate incubation dose and time needed to reduce nonmuscle myosin IIA using adenovirus encoding an shRNAi to nonmuscle myosin IIA in cells.

Figure 3.1: Dose-dependent experiment - Figure presents the result of western blot of cell lysate from mouse endothelial cell cultures infected with different amount of ad-shRNAi-IIA (1=MOI 12.5 per cell, 2=MOI 25 per cell, 3, 3'=MOI 50 per cell, 4, 4'=MOI 100 per cell, 5, 5'=MOI 200 per cell, prime indicates duplicate loading). Increasing virus dosage resulted in a steady decrease in myosin IIA expression reaching maximal myosin IIA knockdown at MOI 100 per cell.

Figure 3.1 is a representative western blot of cell lysate from mouse endothelial cell cultures infected with different amount (MOI 12.5 per cell, MOI 25 per cell, MOI 50 per cell, MOI 100 per cell, MOI 200 per cell) of adenovirus encoding sh-RNAi-IIA. Western Blots were probed with affinity purified antibody to myosin IIA. As shown in figure 1,

the shRNAi-IIA probe is effective in reducing endogenous endothelial cell myosin IIA. Low dosage of virus had a minimal effect on myosin IIA levels, while increasing doses resulted in a steady decrease in myosin IIA, reaching maximal IIA knockdown at MOI 100 per cell. For quantitative analysis, the myosin IIA knockdown was ratioed to the level of GAPDH in cell extracts.

Myosin IIA levels were reduced by 26% in cultures incubated with MOI 12.5 per cell, 56% in cultures incubated with MOI 25 per cell and 62% in cultures incubated with MOI 50 per cell, while in cultures incubated with MOI 100 per cell and MOI 200 per cell a maximal 82% reduction occurred. From these results, a dose of MOI 100 per cell ad-shRNAi-IIA was chosen for use in all experiments.

After establishing the appropriate dose, experiment was undertaken to determine the time duration for maximal reduction of myosin IIA levels. Mouse endothelial cells were infected with MOI 100 per cell for 48 hours, 72 hours and 96 hours. After appropriate time intervals, both control and infected cell cultures were harvested, and cell lysates were analyzed by western blot for myosin IIA expression level.

After 48 hours incubation with virus, the myosin IIA level was reduced by 48%. Further increasing the incubation time to 72 hours and 96 hours, we achieved a reduction in myosin IIA of 71% and 81% respectively (figure 3.2). Maximal reduction of myosin IIA occurred after 96 hours incubation with ad-shRNAi-IIA.

Figure 3.2: Time-course experiment - Mouse endothelial cells were infected with MOI 100 per cell for 48 hrs, 72 hrs and 96 hrs. Western blot of cell lysates from mouse endothelial cell cultures showed maximal reduction of myosin IIA occurred after 96 hrs incubation with ad-shRNAi IIA.

To confirm the dose response and time course experiments, cells were infected with a dose of MOI 100 per cell for 96 hour. Cells lysates were analyzed by western blot as shown in Figure 3.3.

3. RESULTS

Figure 3.3: Confirmation of optimal condition for infection - Mouse endothelial cells were infected with a dose of MOI 100 per cell for 96 hour and a 90% reduction in myosin IIA content occurred in respond.

After 96 hours, a 90% reduction in myosin IIA content occurred in respond to a dose of MOI 100 per cell as shown in figure 3.3 A.

Since myosin IIA and myosin IIB heavy chains have similar structural characteristics, cell lysates were probed with affinity purified myosin IIB antibody to ensure that our IIA probe had no effect on myosin IIB content. As shown in figure 3.3 B, incubation of cells with adenovirus encoding shRNAi-IIA had no effect on myosin IIB content while reducing myosin IIA levels by 90% (figure 3.3 B).

3.2 Myosin II and Actin Localization in Preconfluent Endothelial Cells

It's well-known that nonmuscle myosin II and F-actin have a fundamental role in regulating cell shape, motility and force development. In tissue culture dishes preconfluent endothelial cells assume various shapes with different cytoskeletal organization. As cells grow, divide and contact one another, their cytoskeleton continuously changes until reaching a stable configuration 5-6 day after being seeded onto culture dishes. At confluence endothelial cells form a sheet of polygonal cells tightly attached to one another forming a semi-permeable membrane that regulates the movement of solutes across the monolayer. Since, endothelial cells change shapes as they grow, we sought to determine the structural differences in myosin II and actin organization in endothe-

3.2 Myosin II and Actin Localization in Preconfluent Endothelial Cells

lial cells 24 hours after seeding from endothelial cells within a confluent monolayer 5 day post seeding. Figure 3.4 illustrates the distribution of myosin IIA and actin and figure 3.5 myosin IIB and actin within representative cell morphologies identified in culture. Actin filaments for the most part were bundled into stress fibers that ran parallel to the long axis of the cell. In one group of cells (Fig. 3.4A and 3.5A) there was a complex array of paranuclear actin filaments with small filaments emanating radially for this complex. In a second group (Fig. 3.4B and 3.5B) the actin filaments varied in length, ran parallel to one another but had no paranuclear actin complex. In the last group identified Fig. 3.4C and 3.5C actin filaments appeared to arise from a paranuclear array of filaments and coursed toward the periphery of the cell in a radial spoke like pattern. Both myosin IIA (Fig. 3.4) and IIB (Fig. 3.5) were associated with the underlying actin filaments however myosin IIA was more concentrated within the paranuclear actin arrays.

To determine if myosin IIA has a role in forming and maintaining these structures, experiments were undertaken to knock down myosin IIA in endothelial cells and determine what effects loss of myosin IIA had on the cellular cyto-architecture. Endothelial cells were infected with a adenovirus encoding a shRNAi to myosin IIA, cells were harvested 96 hours post infection and seeded at preconfluent densities fixed 24 hrs after seeding and stained for either myosin IIA/actin (Fig. 3.6) or myosin IIB/actin (Fig. 3.8) staining. Parallel dishes were harvested for SDS gels analysis to determine the extent of myosin IIA knock down (Fig. 3.7A) and to show shRNA-IIA constructs did not alter myosin IIB protein levels (Fig. 3.7B). Treatment of cultures with our adenovirus encoding shRNAi-IIA at MOI 100 per cell resulted in 90% reduction of myosin IIA with no significant affect on myosin IIB levels (Fig. 3.7B). Immunofluorescent localization studies showed major reductions in myosin IIA levels within all cells compared to controls (Fig. 3.4). However, the total amount of IIA loss varied somewhat from cell to cell. Two levels of NMHC-IIA knock down cells were identified. Fig. 3.6A illustrates a representative cell where negligible amounts of myosin IIA remain within the cytoplasm. The lingering myosin IIA is randomly dispersed throughout the cytoplasm taking on no distinct pattern. Cells with this phenotype were larger in size and display large protruding lamella from different regions of their cell body. Co-staining these cells with rhodamine phalloidin showed loss of NMHC-IIA has a striking effect on underlying actin stress fibers. The ordered stress fiber organization within the cytoplasm was

3. RESULTS

Figure 3.4: Myosin IIA and actin localization in control cells - Preconfluent endothelial cells were stained for myosin IIA and actin. Endothelial cells were fixed 24 hours post seeding. Bar, 20 μm .

3.2 Myosin II and Actin Localization in Preconfluent Endothelial Cells

Figure 3.5: Myosin IIB and actin localization in control cells - Preconfluent endothelial cells were fixed 24 hours post seeding and stained for myosin IIB and actin. Bar, 20 μm .

3. RESULTS

Figure 3.6: Myosin IIA and actin localization in preconfluent cells infected with ad-shRNAi-IIA - Loss of myosin IIA induced loss of stress fibers, which cause significant changes in actin organization and cell shape. Bar, 20 μm .

3.2 Myosin II and Actin Localization in Preconfluent Endothelial Cells

Figure 3.7: Myosin content in ad-shRNAi-IIA infected endothelial cells - Myosin II A level was dramatically decreased in ad-shRNAi IIA infected cells, while myosin IIB was not affected.

lost. Stress fiber that remain were short filaments randomly orientated concentrated near the nuclear region while membrane protrusions were filled with a fine reticular meshwork of actin filaments (Fig. 3.6A). The majority (>85%) of infected cells exhibited this phenotype. About 15% of the cells observed still had detectable myosin IIA present within the cytoplasm. Myosin IIA localized at the cell periphery and associated with underlying actin filaments. Actin filament structure was disorganized, however, the number of actin filaments present in cells and the degree of disruption did not directly correlate with the extent of myosin IIA knock down. The content of myosin IIB minimally changed in NMHC-IIA knock down cells (Fig. 3.7B). Myosin IIB co-localized with the actin stress fibers and the fine actin meshwork that formed in myosin IIA knock down cells (Fig. 3.8A-C). These results confirmed the specificity of Ad-shRNAi knock down construct and showed that myosin IIB association with actin appeared unaltered in NMHC-IIA knock down cells.

In order to directly compare the localization of myosin IIA and myosin IIB in control and NMHC-IIA knockdown endothelial cells, cultures were fixed and double stained for myosin IIA and myosin IIB. As shown in figure 3.9 myosin IIA and myosin IIB exhibited a sarcomeric like distribution associated with the underlying actin filaments. This was the same distribution found in untreated control cells stained for myosin IIA/actin and

3. RESULTS

Figure 3.8: Myosin IIB and actin localization in preconfluent cells infected with ad-shRNAi-IIA - Cell shapes were changed dramatically while myosin IIB still associated with actin. Bar, 20 μm .

3.3 Myosin II and Actin Localization in Confluent Monolayers

IIB/actin alone (Fig. 3.4 and 3.6). NMHC-IIA knock down resulted in disruption but not complete loss of actin stress fibers. Myosin IIB associated with stress fibers as well as the fine actin network that formed within the cell cytoplasm. Staining for myosin IIA/IIB/actin in control and myosin IIA knockdown cultures confirmed our previous results.

Figure 3.9: Double staining for myosin IIA and myosin IIB - Control and ad-shRNAi-IIA infected cells were double stained for both myosin IIA and myosin IIB (myosin IIA is in green and myosin IIB is in red in merge picture). Bar, 20 μm .

3.3 Myosin II and Actin Localization in Confluent Monolayers

Since the confluent endothelial cell monolayer is believed to be a model representative of endothelial cells lining blood vessels, we sought to determine if loss of myosin IIA alters endothelial cell monolayer cytoskeletal structure. Result from myosin IIA / actin staining of control and ad-shRNAi-IIA infected confluent endothelial monolayers is presented in figure 3.10. In untreated controls, myosin IIA was localized to the cell margins outlining individual cell within monolayers as well as within the cytoplasm as small droplets or rods like structures. F-actin concentrated at cell borders and formed stress fibers throughout the cytoplasm. Loss of myosin IIA did not cause monolayers to lift off the culture dishes nor cause large gaps to form between individual cells. Besides a slight change in cell shape, monolayers appeared unaffected as examined by light microscopy. In contrast, monolayers infected with ad-shRNAi-IIA and stained for

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myosin IIA/actin showed loss of myosin IIA from the cell margins while small aggregates of myosin IIA staining material formed within the cytoplasm. The actin localization at the cell periphery was also lost while the cytoplasmic stress fibers appeared shorter and more disorganized. In control, monolayers myosin IIB localized to the boarders between cells similar to that seen with myosin IIA (Fig. 3.11). Small droplets of myosin IIB were also distributed throughout the cytoplasm. F-actin localized to the cell margins and central stress fibers and myosin IIB was found decorating their structures. In myosin IIA knocked down cells, myosin IIB appeared minimally altered. Myosin IIB remained localized at cell margins. However, myosin IIB appeared to associate more prominently with central stress fibers.

Figure 3.10: Myosin IIA and actin localization of control and ad-shRNAi-IIA infected confluent monolayers - The result presented that loss of myosin at cell periphery affected organization of actin fiber. Bar, 20 μ m.

3.3 Myosin II and Actin Localization in Confluent Monolayers

Figure 3.11: Myosin IIB and actin localization of control and ad-shRNAi-IIA infected confluent monolayers - Myosin IIB localization was not affected by loss of myosin IIA, even though cells appeared larger and more spread. Bar, 20 μ m.

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3.4 Cell Attachment and Spreading

Knocking down of myosin IIA alter both the actin and myosin II cytoskeleton in both pre-confluent and post confluent endothelial cells. Since, the cytoskeleton is critical for cell attachment and spreading and we wanted to determine if reorganization of the cytoskeleton altered the rate and degree of endothelial cell attachment and spreading. For these experiments we used Electric Cell-substrate Impedance Sensing (ECIS) measurements to follow changes in cell attachment and spreading in control and infected endothelial cells.

Collagen coated ECIS culture chambers were placed in the ECIS apparatus. Control and ad-shRNA-IIA infected mouse endothelial cells were seeded into chambers and measurements started immediately. Figure 3.12 shows representative cell attachment and spreading tracings for control (uninfected), negative control (cell treated with non-targeting virus) and myosin IIA knockdown cells. No significant difference in cell adhesion was detected within the first 15 minutes. By 30 minutes, the myosin IIA knockdown cells showed a greater increase in electrical resistance suggesting that either more cells had adhered to the substratum or the myosin IIA knockdown cells spread faster. Myosin IIA knockdown cells continued to show an increase in resistance over the ensuing 12 hour compared to controls suggesting knockdown cells spread faster or were bigger than the control cells. After 12 hours, knockdown cells exhibited a stable resistance while the control cell lines continued to develop resistance, suggesting control cells were forming a tighter or more cohesive layer. These experiments suggested that loss of myosin IIA initially accelerated cell spreading allowing for greater coverage of the ECIS chamber and resulting in an increase in resistance compared to the paired controls. However, as the cells continued to spread/grow, it appeared that myosin IIA was needed for development of a cohesive endothelial cell monolayer.

3.5 Myosin IIA and Endothelial Cell Motility

Our result showed myosin IIA was involved in modulating cell adhesion and spreading, which were early events in cell migration. To determine the role of myosin IIA in endothelial cell migration, experiments were undertaken to directly measure the rate of endothelial cell movement in control and myosin IIA knockdown endothelial cells. 50,000 control or myosin IIA knockdown cells were seeded onto dishes and allowed to

3.5 Myosin IIA and Endothelial Cell Motility

Figure 3.12: Endothelial cell attachment and spreading - Myosin IIA knockdown cells (black curve) initially had a larger slope than that of control curves. With time, the slope decreased, suggesting a lower cell adhesion function or cell division rate in infected cells than control cells.

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adhere and spread for 12 hours before motility measurements were performed. Cultures were washed with media containing 0.25% BSA 30 minutes prior to the start of an experiment to remove virus. Parallel cultures were maintained under identical conditions to assess the effects of the adenovirus constructs on the levels of myosin IIA. Western blot analysis showed that cells treated with adenovirus encoding shRNAi-IIA showed a 80% reduction of myosin IIA levels (Fig. 3.15). Figure 3.13 depicts a representative control motility movie showing the movement of endothelial cell every 30 minutes over a 5.5 hour time frame. After starting motility movie, pictures were captured every 60 seconds and the rate and path of cell migration were tracked at thirty minutes intervals for a total of 5.5 hours. The red dot placed on a cell at time zero allows one to follow the motility of an individual cell over the time frame outlined in specific protocols. Migration of control endothelial cell is depicted in Fig. 3.13 while the migratory path of myosin IIA knockdown cells is shown in Fig. 3.14. The rate of cell movement ($\mu\text{m/hr}$) is shown in Table 3.1. Myosin IIA knockdown cells (Fig. 3.14) were more spread and bigger than control cells (Fig. 3.13), which was consistent with the results we observed in static cultures. In addition, myosin IIA knockdown cells moved 35% faster ($13.54 \pm 5.34 \mu\text{m/hr}$) than control cells ($8.69 \pm 5.41 \mu\text{m/hr}$). These results suggested myosin IIA limited the rate of endothelial cell motility and loss of this molecule resulted in unfettered motion.

	Average Velocity ($\mu\text{m/hr}$)
Control	8.69 ± 5.41
IIA knockdown	13.54 ± 5.34

Table 3.1: Effect of loss of myosin IIA on endothelial cell motility - Result indicated that loss of myosin IIA increase cell motility.

To further analyze the cell motility, Fig. 3.16 illustrated the positions of cells at 30 minute interval for the duration of experiment. One axis is angular data (degree), another axis is radial data (μm). The control endothelial cells tended to stay near the point of origin which indicated that most control cells barely moved during the 5.5 hour time lapse experiment. On the other hand, endothelial cells infected with ad-shRNAi-IIA displayed greater movement with variable directions and considerable distance. This phenomenon of increased cell migration suggested that loss of nonmuscle myosin IIA might increase endothelial cell motility.

3.5 Myosin IIA and Endothelial Cell Motility

Figure 3.13: Motility of control cells - Pictures were taken every 30min for the first 5.5 hours. Red dot was added to help track one cell movement. No apparent net movement was observed as shown.

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Figure 3.14: Motility of ad-shRNAi-IIA infected cells - Pictures were taken every 30min for 5.5 hours. Red dot was added to help track one cell movement. It showed that cell shapes were changed apparently compared to control.

3.6 Effects of Myosin IIA on Isometric Tension

Figure 3.15: Effect of ad-shRNAi-IIA infection on myosin IIA content - Myosin II A level was clearly decreased in ad-shRNAi-IIA infected cells.

3.6 Effects of Myosin IIA on Isometric Tension

Since, actin and myosin are responsible for generating endothelial cell contractile forces [48], we sought to determine if loss of myosin IIA alters the contractile force produced by endothelial cells. In this last set of experiments, we directly measured the contractile force produced by endothelial cell depleted of myosin IIA. Isometric tension measurements were performed as outlined by Goeckeler and Wysolmerski [14]. Representative force tracings are shown in Fig. 3.17. Depletion of myosin IIA (green tracing) resulted in about 41% reduction in baseline tension compared to control (red tracing). Both myosin IIA knockdown and control monolayers rapidly increased their isometric tension upon stimulation with thrombin. However, myosin IIA depleted monolayers produced about 33% less force than control. Taken together, these results indicate that myosin IIA is essential for endothelial cells to generate basal tension and agonist-stimulated isometric tension.

3. RESULTS

Control

IIA KD

Figure 3.16: Path of cell movement - angular data (degree) vs radial data (μm).

3.6 Effects of Myosin IIA on Isometric Tension

Figure 3.17: Force measurement of control and ad-shRNAi-IIA infected cells
- Red line presented control cells, the green line presented ad-shRNAi-IIA infected cells. After the baseline force was reached, the cells were stimulated with 2 unit/ml thrombin, which induced a rapid increase in tension. After achieving peak tension, tension dropped to establish a new baseline force. Application of cytochalasin D (Cyto D) depolymerized actin filaments and abolished force production. Western blot result indicated the myosin levels in cells.

Chapter 4

Discussion

This study analyzed the role of nonmuscle myosin IIA in regulating endothelial cell shape, motility and contraction by knocking down nonmuscle myosin IIA in endothelial cells with ad-shRNAi-IIA. The western blot result presented that about 90% percent of nonmuscle myosin IIA expression was decreased after MOI 100 per cell infection for 96 hours and it also showed that that nonmuscle myosin IIA expression could be effectively depleted without affecting the levels of the nonmuscle myosin IIB isoform.

Jeon et al. [49] provide evidence that nonmuscle myosin II is responsible for establishing cell polarity and retaining the cellular structure. The regulatory site of nonmuscle myosin regulatory light chain (MRLC) is directly phosphorylated by AMP-kinase, which is an enzyme activated in response to energy deprivation. These investigators observed that the defect of AMP-kinase can lead to failure of activation of nonmuscle myosin II and dramatic abnormalities in cellular structure. However, after activation of AMP-kinase, they observed actin cytoskeleton polarization and dramatic changes in cell shape. Our morphology analyses were consistent with their observations that knocking down nonmuscle myosin IIA dramatically changed the cell shape and myoactin cytoskeleton. Our results provide further evidence that knocking down nonmuscle myosin IIA in endothelial cells causes decrease in number of F-actin bundles and increase of fine actin meshwork, which is also consistent with the observations of Sandquist et al [50].

Cell motility was analyzed in several experiments here, involving initial attachment and spreading measured by ECIS and random migration after attachment, measured by live cell imaging by Nikon swept field confocal system. In the ECIS result, myosin

IIA knockdown cell presented a higher resistance during initial attachment, which indicated a faster attachment in myosin IIA knockeddown cells than control cells. This result is consistent with earlier conclusions Jay et al. [51] presented that myosin II contributes to cellular detachment and retraction, and Wylie and Chantler [52] deduced that nonmuscle myosin IIA drive neurite retraction in neuronal cells. Consistent with these conclusions, in the following random migration experiment by using Nikon swept field live cell confocal system, cells were observed more spread and larger in myosin IIA knockeddown cells and the moving rate of random migration was slightly higher than control cells.

Normally, contraction force helps detach the cell tail from the substratum. Since previous result demonstrated that the cellular detachment and retraction were inhibited in myosin IIA knockdown cells, we predicted that the contraction force will be decreased in myosin IIA knockeddown cells. In our laboratory, its observed that loss of nonmuscle myosin IIA not only cause morphology changes, it can also affect isometric tension production in endothelial cell. Our force measurement data showed that loss of myosin IIA can induce decrease in both baseline force and agonist-stimulated isometric tension produced in endothelial cells compared to control cells (Fig. 3.17). And the parallel western blot result also indicated that myosin IIA level was significantly decreased in ad-shRNAi-IIA infected cells while myosin IIB level was not affected by myosin IIA knockdown. These results suggested that the baseline force and agonist-stimulated isometric tension produced in myosin IIA knockdown cells may result primarily from nonmuscle myosin IIB. In the absence of myosin IIA, myosin IIB can still produce considerable baseline force and agonist-stimulated isometric tension force. We concluded that both myosin IIA and IIB contribute to the cell contractility of endothelial cell in normal condition. Loss of myosin IIA will result the decrease in the total baseline force produced as well as agonist-stimulated isometric tension force produced. In the absence of myosin IIA, can myosin IIB maintain contractile force to the same degree as in control cells or increase force production to partially compensate for the loss of the myosin IIA needs to be determined.

In our laboratory, we are also interested in the effect of loss of myosin IIB on endothelial cells. More surprising was our finding that in myosin IIB knockdown cells, actin filaments within the cytoplasm were not disrupted or lost like what we observed in myosin IIA knockdown cells previously stated. Myosin IIB deficient cells displayed

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regular cell shape and cytoskeleton, and actin filaments were bound into stress fibers running through cytoplasm. In addition, cells maintained their regular shapes, no exaggerated membrane protrusion occurred. Taken together, loss of nonmuscle myosin IIA in endothelial cells leads to disruption of stress fibers resulting in apparent changes in cell shape and membrane protrusions. Meanwhile, different from those phenotypes in myosin IIA knockdown cells, in endothelial cells with ablation of nonmuscle myosin IIB while exclusively express nonmuscle myosin IIA isoform, cells displayed normal cell shape and actin filament. One hypothesis is that the stabilization of stress fiber as well as maintenance of cell shape depends heavily on nonmuscle myosin IIA, which is responsible for interacting with actin filaments and other cross-link proteins to form high order stress fibers in cells. In myosin IIA knockdown cells (Fig. 3.8), myosin IIB colocalized with short and disorganized stress fibers, suggesting that nonmuscle myosin IIB also contributes to stress fiber development, but might not be sufficient in maintenance the high order structure when nonmuscle myosin IIA is ablated from cells.

In summary, our data have shown that knocking down the expression of nonmuscle myosin IIA in mouse endothelial cells causes a dramatic decrease of stress fiber as well as alteration of actin cytoskeleton organization with concomitant changes in cell shape, migration and contractile force. Our data consistent with previous work by other investigators provided strong evidence supporting the idea that nonmuscle myosin IIA plays an important part in regulating cell shape, motility and contraction. More work is required for a further understanding of how these activities are monitored by the cell and the role myosin IIA interfere in endothelial cell monolayer permeability by regulating cytoskeleton.

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