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The Influence of Caldesmon Suppression on Proliferation and Motilities of Vascular Smooth Muscle Cells

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Abstract

Migration of vascular smooth muscle cells (VSMCs) from the media to intima constitutes a critical step in the development of proliferative vascular diseases. To elucidate the regulatory mechanism of VSMCs motility, the roles of caldesmon (CaD) were investigated previously. CaD is an actin-binding protein dynamically regulating cytoskeleton structure. In this study, the potential role of CaD in mediating proliferation and motility of VSMCs were discussed. First, structural effect of CaD on cytoskeleton integrity was analyzed with CaD knock-down; second, the proliferation of VSMCs was measured in CaD knock-down and control cells; third, the specific role of CaD on VSMCs motilities was evaluated with *in vitro* migration and invasion assays. We found that CaD is an integral component to maintain cytoskeleton integrity of VSMCs. Our data indicated that CaD suppression does not show significant influence on VSMCs proliferation, but negatively modulates the motilities of VSMCs, and CaD depletion would significantly facilitate migration and invasion of VSMCs.

Keywords

1. Introduction

Migration of vascular smooth muscle cells (VSMCs) from media to intima is a critical step in the development of proliferative vascular diseases such as atherosclerosis, and in response to vascular injuries such as angioplasty and organ transplantation [1] [2]. Terminally differentiated VSMCs normally do not proliferate or migrate.

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Upon stimulation, VSMCs undergo remodeling from contractile to synthetic phenotypes, enabling VSMCs to migrate [3] [4]. Under these pathological conditions, a number of smooth muscle-specific contractile proteins are converted to a non-muscle isoform [5]. One of such signature proteins is caldesmon (CaD).

CaD is an actin-binding protein that also interacts with myosin, tropomyosin and calmodulin [6]. In mammalian cells, caldesmon is expressed in two isoforms by differential transcription of a single gene [7]. The higher molecular-weight isoform (h-CaD) is expressed in differentiated, contractile smooth muscle cells, whereas the lower molecular-weight isoform (l-CaD), lacking the central region, is expressed in dedifferentiated vascular smooth muscle cells, and non-muscle cells [8]-[10]. Upon dedifferentiation, h-CaD is rapidly degraded and only l-CaD is expressed in VSMCs, associating with the process of proliferative vascular disease [11] [12].

In this study, we focused on the potential role of CaD in regulating the proliferation and motilities of differentiated VSMCs. First, we analyzed the structural effect of CaD on human VSMCs cytoskeleton via CaD knockdown; second, we used proliferation assay to measure cell proliferation with or without CaD expression; third, the *in vitro* migration and invasion assay was used to measure cell motility dynamics. Furthermore, the detachment assay was used to evaluate VSMCs adhesion on the substrate. Our results indicated that CaD suppression did not show significant influence on proliferation of VSMCs, but negatively regulated the motilities of primary dedifferentiated VSMCs. CaD depletion would significantly facilitate migration and invasion of VSMCs.

2. Material and Methods

2.1. Cell Culture

The primary human aortic smooth muscle cells (HASMCs) were obtained from Sciencell, San Diego, US. Cells were cultured in smooth muscle cell complete medium (Sciencell, San Diego, US) and placed in a humidified incubator at 37°C and 5% CO_2. Cells between passages 3 - 7 were used for all experiments, and they were serum deprived for 24 h before being tested.

2.2. CaD Stable Knock-Down via Lentiviral Infection

CaD shRNA lentiviral particles were purchased from Santa Cruz, CA, US. All experimental procedures were followed with manufacturer's instruction. Briefly, the HASMCs were seeded in 12-well plates till 50% confluent. Then, the medium was replaced with 1 mL complete medium containing 5 μg/mL polybrene (Santa Cruz, CA) and 15 μL CaD shRNA lentiviral particles. The infection control was the lentiviral particles packed with scrambled shRNA sequence (Santa Cruz, CA). The infection medium was replaced with 1 mL complete medium and incubated at 37°C and 5% CO_2 overnight. To select the stable clones expressing shRNA, the Puromycin dihydrochloride (Santa Cruz, CA) was added to each well with the 5 μg/mL concentration. The complete medium containing fresh puromycin was changed every 3 - 4 days till resistant colonies can be identified. Western-blotting was followed to check the knock-down efficiency of CaD in HASMCs.

2.3. Western-Blotting

The expression level of endogenous CaD reduced by infection was evaluated by Western-blotting analysis using an ECL system (Amersham Biosciences). Briefly, after 24 h incubation, culture medium was removed, and cells were rinsed twice with ice-cold PBS. Proteins were extracted by adding to each well 100 μL of lysing buffer (Santa Cruz, CA). The plates were incubated on ice for 30 min and scraped. Total cell extracts were loaded onto sodium dodecyl sulfate (SDS)-polyacrylamide gels and the separated proteins were transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). Blots were incubated with CaD monoclonal antibody (Abcam, Cambridge, MA) diluted in TBST (containing 0.1% Tween 20 and 2% BSA) overnight at 4°C. Then, blots were washed and incubated with monoclonal anti-rabbit secondary antibody and detected. α-actin was used as a control for equal loading.

2.4. Fluorescence Microscopic Imaging

CaD knock-down and control cells were seeded on glass coverslips and incubated overnight, till well-spread. Cells were then starved for 24 h and washed in PBS, fixed for 15 min in freshly prepared 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) and permeabilized with 0.3% Triton X-100 in 4% PFA in PBS for 5 min. F-actin was stained with rhodamine-phalloidin and incubated for 1 h. Finally, cell-loaded cover-slips were

rinsed and mounted on glass slides in Mowiol (Sigma). Images were obtained using Laser Scanning Confocal Microscope (LEICA TCS SP2). Data were acquired and analyzed with Leica TCS SP2 software.

2.5. Cell Proliferation Assay

Proliferation of cultured HTFs was measured by use of the commercially available MTT (3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide) cell proliferation kit (Sigma), according to the manufacturer's instructions. Cells were plated at a density of 10^4 cells per well in 96-well plates and were allowed to adhere for 24 h. After cultures were washed with phosphate buffered saline (PBS), then treated with 5 mg/mL MTT for 4 hours at 37°C. The relative active number of cells was determined by an automated plate reader (Bio-Rad, Hercules, CA).

2.6. *In Vitro* Migration and Invasion Assays

In vitro migration and invasion assays were performed using Transwell insert (24-well insert, pore size 8 μm; Corning, NY). Briefly, 3×10^4 quiescent cells were re-suspended in phenol-red free and serum-free medium and placed in the top-chambers. The lower chambers were filled with complete medium containing 20% FBS for 24 h. For the invasion assay, the inserts were previously coated with extracellular matrix gel (BD Biosciences, Bedford, MA). At the end of the experiments, MTT was added, and cells were incubated for further 2 h. Then cells from the top of the Transwell chambers were removed using cotton swabs (residual cells). Cotton swabs containing residual cells and Transwell chamber migrated or invaded cells were placed in 24-well plates containing 400 μL of DMSO. After 1h of gentle shaking, 100 μL of samples were removed, and the absorbance was measured at 570 nm using an ELISA plate reader. The percentage of migrated or invaded cells was calculated as: percentage of migrated or invaded cells = A/(A + B) × 100, where A is the absorbance of migrated or invaded cells, and B is the absorbance of residual cells.

2.7. Cell Detachment Assays by Trypsinization

When cells reached 75%, Plates were washed twice with 1xPBS, followed by the addition of standard 0.25% trypsin/EDTA solution (Cellgro™). Observations were performed using 100x objective of Nikon Eclipse TE300 fluorescence microscope using fluorescence channel. The extent of transfected green cell rounding in time was estimated manually.

2.8. Statistical Analysis

Data were expressed as the mean ± SD. Statistical difference between groups was examined using the ANOVA and Student's *t*-test. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. The Influence of CaD Suppression on Cytoskeleton Integrity of Dedifferentiated VSMCs

To evaluate how CaD affects the structure of cytoskeleton in primary VSMCs, we used CaD shRNA lentiviral particles to stably knock down CaD expression in HASMCs, the lentiviral particles packed with scrambled shRNA sequence were employed as control. Western-blotting results showed CaD expression was rarely detected in CaD stable knock-down (KD) cells (**Figure 1(a)**). The relative expression of CaD in KD cells was decreased almost 90% compared with control cells (**Figure 1(b)**). Furthermore, The actin filaments were promptly disrupted in CaD knock-down cells, only short-thin stress fibers could be detected in the area of membrane ruffles and lamellipodial extensions, whereas the control cells showed very robust cytoskeleton bundling crossing the entire cytoplasm with long-strong stress fibers (**Figure 2(a)**). This observation was also supported by quantitative image analysis, the relative density of F-actin filaments was decreased almost 60% in CaD knock-down HASMCs compared with control cells (**Figure 2(b)**).

3.2. The Influence of CaD Suppression on Proliferation of Dedifferentiated VSMCs

Cell proliferation was measured by the MTT assay. The assay is based on measuring the intracellular formazan
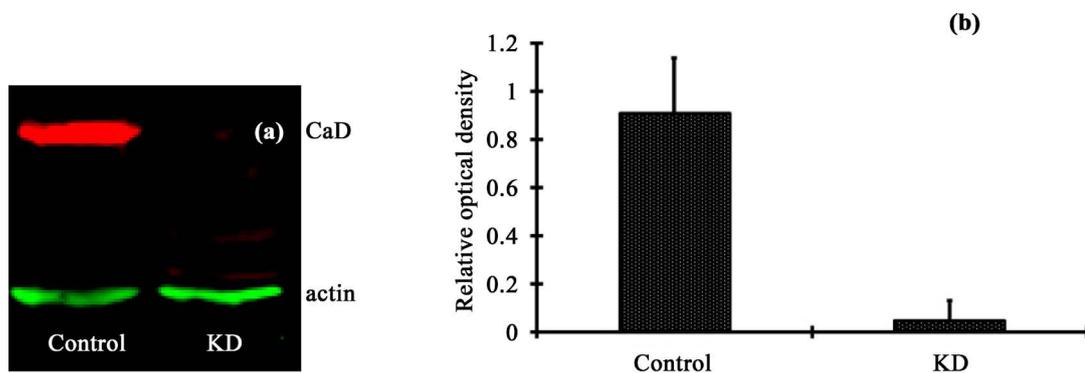
Figure 1. CaD knock-down in VSMCs with virus infection. (a) A blot representative for CaD knock-down in VSMCs. CaD was stained in red and actin, which was a lording reference, was stained in green; (b) The relative expression levels of CaD knock-down and control cells are shown in bar diagram. Data represents the mean of three independent experiments. $P < 0.05$.
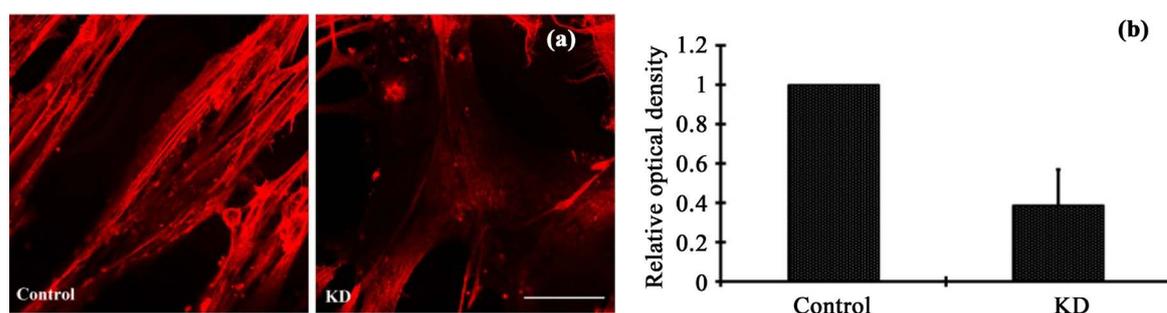

Figure 2. Fluorescence imaging analysis of HASMCs with CaD knock-down. F-actin was stained with rhodamine-phalloidin (red) and the relative F-actin density of CaD knock-down cells was measured and normalized to control cells. (a) A confocal image representative for actin filaments in both CaD knock-down and control cells. Scale bar: 20 μm; (b) Relative density of F-actin in CaD knock-down HASMCs compared with control cells in bar diagram. $P < 0.05$.
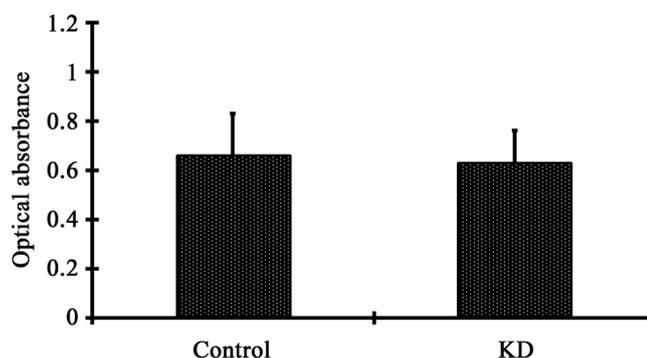

Figure 3. The optical absorbance of VSMCs between CaD knock-down and control.

spectrophotometrically which is facilitated by active cells. The optical absorbance of CaD knock-down cells were 0.63 ± 0.13, while that of control cells was 0.66 ± 0.17 (**Figure 3**). The MTT assay revealed depletion of CaD did not present significant suppression on proliferation of VSMCs.

3.3. The Influence of CaD Suppression on Motility Activities of Dedifferentiated VSMCs

By using Transwell assay, the migration and invasion activities of HASMCs cells were measured. The results showed that the motilities of HASMCs were significantly promoted by CaD depletion: the percentage of migrated cells was increased by about 1.5-fold (**Figure 4(a)**) compared with control cells; for invasion assays, normal control HASMCs rarely invaded through extracellular matrix gel and the percentage of invaded cells was

just about 15%, when the expression of CaD was significantly depleted, cells became more aggressive and the percentage of invaded cells increased by about 2.0-fold compared with control cells (**Figure 4(b)**).

3.4. The Influence of CaD Suppression on Substrate Adhesion of Dedifferentiated VSMCs

Finally, in search of the true cause for the increased motility activities, we attempted to test whether CaD knock-down affects cell detachment, which is another step critical to cell motility. We used a simple assay by quantifying the number of rounded cells (including detached cells) as a function of time following trypsinization to compare the detachment kinetics of VSMCs cells. We found that CaD knock-down cells (squares) showed prompt responses to trypsin digestion as compared to the control cells (triangles) (**Figure 5**).

4. Discussion

It has been well established that CaD binds to actin and stabilizes the filamentous structure. Binding of CaD to actin also inhibits the actomyosin interaction, and results in inhibition on many cellular processes [6]. A number of studies on cell physiology have demonstrated a role of caldesmon in the modulation of cell division [13], migration [14], adhesion [15] *et al.*, by regulating contractility and stability of the actin cytoskeleton in non-muscle cells. In this study, we present new evidence that caldesmon is an integral component of maintaining the integrity of the actin cytoskeleton in primary human aortic smooth muscle cells. Besides, we reported for the first time that CaD knock-down would significantly facilitate the motility activities of dedifferentiated VSMCs.

When we explored the basic function of CaD in cytoskeleton stability of HASMCs, we found actin filaments
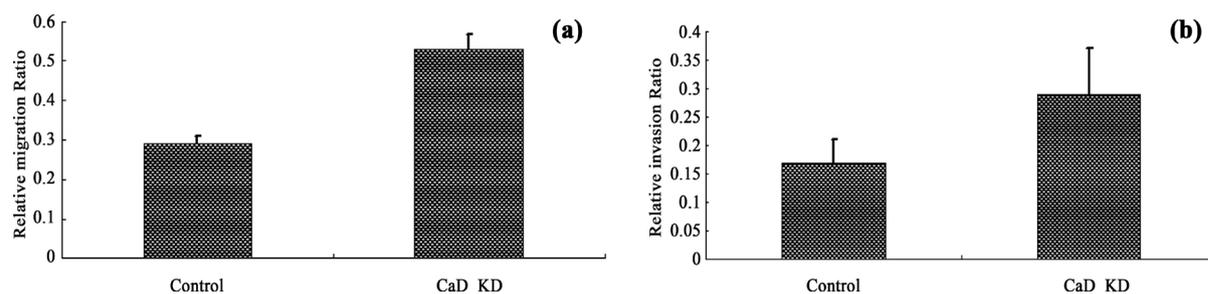

Figure 4. Summaries of HASMCs motilities assays. (a) Percentage of migrated both CaD knock-down and control HASMCs in bar diagrams: the percentage of migrated CaD knock-down cells was increased by about 1.5-fold compared with control cells; (b) Percentage of invaded both CaD knock-down and control HASMCs in bar diagrams: CaD knock-down cells became more aggressive and the percentage of invaded cells increased by about 2.0-fold compared with control cells. Data shown were a mean of three independent experiments. $P < 0.05$.
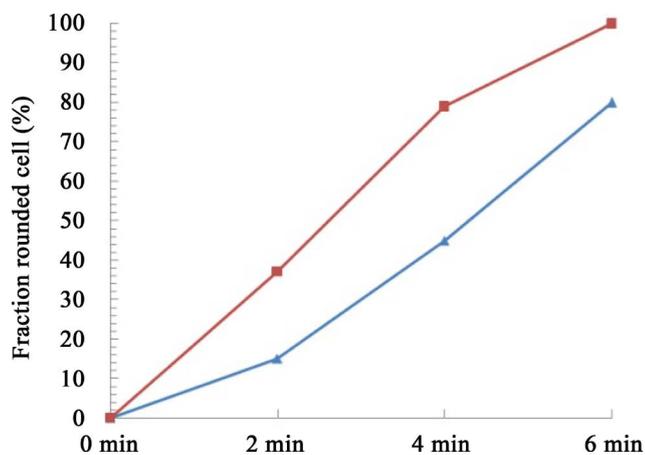

Figure 5. Detachment of VSMCs cells upon trypsinization. KD (squares), control (inverse triangles). Cells from each plate were trypsinized and monitored under the phase-contrast and fluorescence microscope for time-dependent retraction, rounding and detachment. Percentage of round cells at 2, 4, 6 min was plotted for each type of cells and KD cells were more prompt to be rounding at each time point.

were promptly disrupted in CaD depleted cells, only short-thin stress fibers could be detected in the area of membrane ruffles and lamellipodial extensions, whereas the control cells showed very robust cytoskeleton bundling crossing the entire cytoplasm with long-strong stress fibers (**Figure 2**), these results are very consistent with the previous studies that depleting CaD by gene silencing impairs stress fibers in non-muscle cells [16], which may suggest CaD is a critical element for the formation of thick stress fibers in primary cultured dedifferentiated VSMCs.

The migration and invasion of dedifferentiated VSMCs from the media to intima and in situ proliferation, are essential steps for proliferative vascular diseases development [1] [2]. Our data showed that the deletion of CaD did not show significant influence on VSMCs proliferation, which is consistent with previous studies [13]. It's been reported that CaD is actively involved in non-muscle cell migration [14], our previous studies also showed CaD over-expression would inhibit the migration of smooth muscle cell line A7r5, but very few reports directly mention the exact role of CaD in motilities of primary VSMCs. In order to find out how CaD plays in regulating VSMCs motilities, we performed migration and invasion assays with CaD knock-down HASMCs. One of the key steps for cell migration is contraction by which cell will move forward [5]. CaD knock-down cells exhibited much more motility activities than control cells. The relative percentages of migration and invasion were 1.5-fold and 2.0-fold higher in CaD knock-down HASMCs compared with control cells. This result might be interpreted with our detachment assays. Our data showed that detachment of CaD knock-down cells were much easier to round up and detach from the substrate upon trypsin stimulation, which is an essential step for cell to move forward. Our study further suggests that CaD may serve as a more effective therapeutic reagent in d proliferative vascular diseases such as atherosclerosis and restenosis.

5. Conclusion

In this study we have shown that CaD suppression disrupted the actin filamentous structure of VSMCs. Despite of intensive studies over the past three decades, the functional role of CaD in non-muscle cells remains elusive. In this regard, our data shed light onto the following aspects: 1) CaD suppression did not show significant influence on VSMCs proliferation; 2) CaD negatively modulated the motilities of VSMCs, and CaD depletion would significantly facilitate migration and invasion of VSMCs. These new insights not only help us to better understand how CaD works, but also provide useful information on how cell motility is regulated. For VSMCs, in particular, our findings suggest that CaD may serve as a novel therapeutic strategy to combat vascular diseases such as atherosclerosis and restenosis.

Acknowledgements

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