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Research Article

Vimentin Intermediate Filaments Stimulate the Neurite Growth Independently of Mitochondria

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Abstract

Vimentin, a type III intermediate filament protein typically found in mesenchymal cells, is also known to function in neurons during neurite formation. While its role in complementing neuron-specific intermediate filaments is unclear, prior evidence that vimentin regulates mitochondrial function and maintains membrane potential led us to hypothesize that its contribution to neuritogenesis is linked to its effects on mitochondria.

Experimental Approach: To test this proposition, we employed vimentin-null CAD cells reconstituted with either wild-type human vimentin or the Vim(P57R) mutant, which lacks mitochondrial binding capacity.

Key Finding

Subsequent analysis showed that vimentin intermediate filaments facilitate neuritogenesis through a pathway that is independent of mitochondria.

Keywords: Intermediate filaments, Vimentin, Neurites, Mitochondria

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INTRODUCTION

Neuritogenesis is an energy-intensive process that demands coordinated cytoskeletal remodeling, intracellular transport, and membrane expansion. Mitochondria are essential for this process, providing the necessary energy in the form of ATP to fuel both the initial formation and continued elongation of neurites. During neuronal differentiation, mitochondria are dynamically redistributed to regions of high-energy demand, resulting in a highly skewed distribution within growing neurites that shows a sevenfold greater density near the growth cone compared to more proximal regions [1].

The cytoskeleton plays fundamental role in neuritogenesis, with its elements acting in concert to mediate mitochondrial transport, positioning, and anchorage. Interactions between mitochondria and various cytoskeletal components, notably intermediate filaments (IFs), have been described [2-4]. A prominent example is the well-documented association with neuron-specific neurofilaments (NFs), which constitute the majority of IFs in mature neurons [1, 4]. However, during early neuritogenesis, or in response to neuronal injury and function recovery after spinal cord injury, another IF protein, vimentin, is actively expressed [5, 6]. The localization of vimentin within growing neurites implies a functional role for this protein in the process of neuronal differentiation [7]. Vimentin is essential for neurite outgrowth in the neuronal cells as its loss impaired neurite outgrowth and subsequent restoration rescued the phenotype [5, 8]. However, its specific role in neuritogenesis remains unclear.

Vimentin is a type III intermediate filament protein predominantly expressed in mesenchymal cells, where it maintains cellular mechanical properties. Previous studies have established that vimentin is a key determinant of mechanical cell stiffness, which is essential for efficient cell migration [9]. Besides, the role of vimentin IFs in cellular physiology was emphasized by demonstration of its interaction with mitochondria [10-12]. Vimentin binds mitochondria directly through its N-terminal domain [10, 11] as well as by using the cytolinker protein plectin 1b [13]. This interaction is critical not only for maintaining intracellular distribution of mitochondria [10] but also for some of their properties [12, 14]. Studies have shown that vimentin-null fibroblasts exhibit a lower mitochondrial membrane potential compared to vimentin-expressing fibroblasts or cells with restored wild-type vimentin [12]. Disrupting mitochondrial binding, through mutation in the N-terminal domain, decreases the membrane potential [12] and make them more sensitive to oxidative stress [14].

Given the above, the role of vimentin IFs in the formation of neurites may be related to their effect on mitochondria. Indeed, we have shown recently that deletion of vimentin caused the noticeable decrease of mitochondrial potential in neural cells [15]. Although this evidence directly links vimentin to neurite formation, it is unclear whether vimentin-dependent regulation of mitochondrial membrane potential influence neurite outgrowth. Based on our prior findings that vimentin is essential for neuritogenesis and may support mitochondrial membrane potential [8, 15], we next asked whether the vimentin-mitochondria interactions are responsible for the promotion of neurite formation and growth. To test this, we employed the CAD cells (Cath.a catecholaminergic neuronal) with knockout of *VIM* gene [15] in which we restored vimentin IFs using either wild-type human vimentin or its mutated form Vim(P57R) capable to form filaments but deficient in the ability to bind mitochondria [10]. Evaluation of the neurite outgrowth in such cells have shown that their length did not differ significantly between cells expressing wild-type and the mutant vimentin.

MATERIALS AND METHODS

Cell culture

CAD(Vim^{-}/$^{-}$) cells generated from the parental CAD cell line (CATCH.a, CRL-3[illegible], ATCC) using CRISPR-Cas9 system [15] were maintained in DMEM/F-12 medium (PanEco, Russia) supplemented with 10% fetal bovine serum (Biolot, Russia) and 1% penicillin/streptomycin (Sigma, United States) at 37°C in a humidified 5% CO_2 atmosphere. Neuronal differentiation and neurite outgrowth were induced by switching the full media to serum-free medium (SFM). For microscopic analysis, cells were plated on glass coverslips and cultured for 48-72 hours in SFM. Following incubation, coverslips with cells were placed in a sealed chamber that contained DMEM/F12 medium and imaged with a PlanApo 63x objective and a 12-bit digital CCD camera (Keyence, Itasca, IL, USA).

Transfection

Cells were transfected with plasmids pVim (wt) and pVim (P57R), encoding human vimentin and its mutant form, respectively (Nekrasova et al., 2011), and/or pVB6-Chromobody encoding the recombinant anti-vimentin antibody (VB6-GFP) (Maier et al., 2015). Transfection was performed according to the manufacturer's protocol (Evrogen, Russia) by mixing 1 μg of plasmid DNA with 2 μL of Transfectin reagent in 100 μL of PBS (phosphate-buffered saline), incubating the complex for 25 minutes, and adding it to cells in 1 mL of full DMEM/F12 medium. Transfections were performed comprising four conditions: (1) Normal Vimentin (hVim): co-transfected with pVim (wt) and pVB 6-Chromobody; (2) Mutant vimentin (Vim (P57R)): co-transfected with pVim (P57R) and pVB6-Chromobody; (3) Control (GFP): transfected with pVB 6-Chromobody only; and (4) Untreated Control (CAD(Vim-/-)): non-transfected cells.

Immunofluorescence

For the immunofluorescence of vimentin IFs cells were fixed with methanol at -20 °C for 10 min. Staining was performed using mouse monoclonal antibody V9 against vimentin (Sigma, USA). FITC-conjugated anti-mouse secondary antibodies (Jackson, West Grove, PA, USA) were used for detection. Microphotographs were acquired with a PlanApo 63x objective and a 12-bit digital CCD camera using a Keyence BZ-9000 microscope (Itasca, IL, USA) (Keyence, Itasca, IL, USA).

Evaluation of neurite length

Following incubation, coverslips with cells were placed in a sealed chamber that contained DMEM/F12 medium and imaged. To detect vimentin intermediate filaments (IFs) in living cells, we used the transfection of cells with the plasmid pVB6-Chromobody (Meyer et al., 2015), encoding a recombinant vimentin-specific antibody fused to GFP. Live-cell imaging was performed using a Keyence BZ-9000 microscope (Itasca, IL, USA) equipped with a temperature-controlled incubator, in which a constant temperature of 37 ± 2°C was maintained. The imaging was conducted with a PlanApo 63x objective and a 12-bit digital CCD camera. The acquired images were transferred to a computer using BZ II Viewer 1.41 software (Keyence, Itasca, IL, USA) and saved as 12-bit graphic files for further analysis. To measure the neurite length, NeuronJ plugin within the ImageJ software was used. All neurites of vimentin-positive CAD cells, control and untreated cells were traced manually in the phase-contrast images, and the average values of the neurite length were calculated.

Statistical analysis

Data presented as mean ± standard error. Statistical comparisons of means between groups were performed using unpaired Student's t-test.

RESULTS

To examine the hypothesis suggesting that vimentin promotes the neurite formation by maintaining the high level of mitochondrial potential we reconstituted the vimentin IFs in CAD(Vim^{-}/$^{-}$) cells using transfection with plasmids encoding wild type human protein or its mutant

Vim(P57R). Expression of both recombinant proteins caused the formation of extended network of IFs with pronounced localization in the neurites (Figure 1). It should be emphasized that substitution of proline-57 for arginine in vimentin does not derogate its ability to form filaments (Figure 1 B).

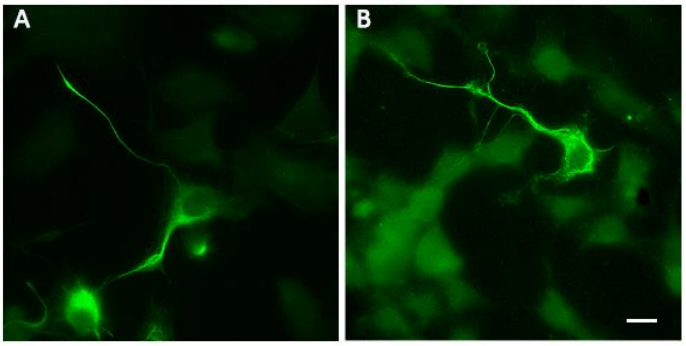

Figure 1: Reconstitution of vimentin IFs in CAD(Vim-/-) cells.

Immunofluorescence of CAD(Vim-/-) cells expressing human vimentin (**A**) and its mutated form Vim(P57R) (**B**). Scale: 10 μm.

We have shown recently that deletion of vimentin IFs in CAD cells impaired the neuritogenesis [8] while their recovery in the CAD(Vim^{-}/$^{-}$) cells using recombinant vimentin restored both the length and the number of neurites. It follows from these data that vimentin somehow promotes the formation of neurites. One of the possible explanations is that vimentin affects mitochondrial functions that are important in the neurite outgrowth. Indeed, we have shown recently that vimentin IFs maintain elevated mitochondrial potential in CAD cells [15] and their deletion by knockout of gene *VIM* causes the decrease of potential. It was also shown that the level of mitochondrial potential could be rescued by expression of human vimentin while the mutated form Vim(P57R) unable to bind mitochondria did not increase the potential [15]. Interestingly, this mutated.

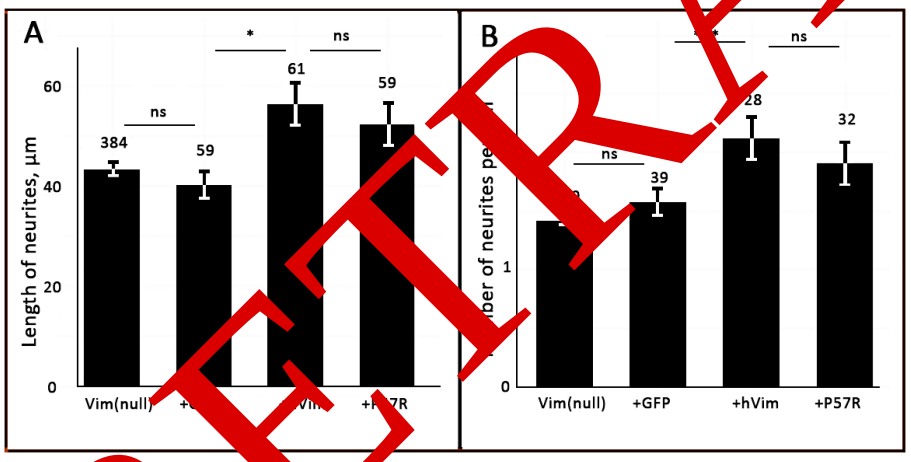

Figure 2: Reconstruction of vimentin IFs in CAD(Vim-/-) cells.

The length of neurites (**A**) or the number of neurites per cell (**B**) in non-transfected vimentin-null cells (Vim(null)), or transfected cells expressing chromobody (+GFP), chromobody and human vimentin (+hVim), and chromobody and mutant vimentin (P57R). The data present the mean values in the indicated number of neurites (A) or cells (B) ± standard error; ns, not significant; * $p < 0.05$; *** $p < 0.001$.

Vimentin had the effect on the neurite growth similar to that of wild type protein. Figure 2 shows that in cells expressing Vim(P57R) the length and the number of neurites were increased similarly to those with wild type vimentin. Thus, despite the fact that the mutant vimentin does not interact with mitochondria and hence does not increase their membrane potential [15] it is still able to affect the growth of neurites.

DISCUSSION AND CONCLUSION

The discovery that vimentin intermediate filaments participate in neurite outgrowth during neural differentiation and wound healing [5-7] raised questions about the underlying mechanism. The simultaneous expression of neurofilaments and vimentin suggests they serve distinct functions. Indeed, the role of neurofilaments is to stabilize neurons while the outgrowth is accompanied by the transient accumulation of vimentin IFs [5, 7]. Based on our prior results demonstrating vimentin's role in sustaining high mitochondrial membrane potential [12], we hypothesized a mechanism for its involvement in neurite outgrowth. This seemed especially likely because we observed vimentin IFs relocating from the cell body to neurites during early neuronal differentiation in CAD cells (Figures 1 and [8]), a time when the most highly energized mitochondria were also located in these vimentin-rich regions [15].

This study determined that vimentin intermediate filaments promote neurite outgrowth through a mechanism independent of mitochondrial function. We restored vimentin in CAD(Vim-/-) cells using a mutated variant, Vim(P57R). This mutant, which is unable to maintain high mitochondrial membrane potential [10, 15], still facilitated neurite growth. This demonstrates that vimentin's role in neuritogenesis is unrelated to mitochondrial energetics.

Given vimentin's established role in cell migration, its stimulatory effect on neurite outgrowth may share mechanistic features with its function in migrating cells. We previously showed that vimentin-null fibroblasts, unlike wild-type cells, are uniformly soft and lack the front-to-back stiffness gradient essential for directional persistence [9]. This led us to hypothesize that vimentin establishes the mechanical polarization required for sustained migration Our recent data on growing neurites supports this: the growth cones of vimentin-null CAD cells exhibit significantly lower directionality than those with vimentin [8]. An alternative mechanism for vimentin's role in neuritogenesis could be the regulation of focal contact dynamics within the growth cone. We propose that, analogous to fibroblast migration, vimentin IFs interact with focal adhesion components to coordinate their turnover [16-18]. Future studies are needed to test this model.

Taken together, our results indicate that the facilitation of neurite outgrowth by vimentin IFs occurs via a pathway that does not involve its effects on mitochondria.

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AUTHOR CONTRIBUTION

A. Minin, B. A. Gebreselase, and O. I. Parfenteva designed and developed an experiment; O.I. Parfenteva and B. A. Gebreselase performed microscopic examinations and the data processing; O.I. Parfenteva, A. Minin and O.I. Parfenteva participated in writing the text of the article. All the authors participated in the discussion of the results.

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