

Mechanisms of MDK Upregulation in Early Alzheimer's Disease and Its Impact on A β Clearance Through Microglial Regulation

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Abstract. Recent studies have demonstrated upregulation of midkine (MDK) in the brains of 5xFAD mice, and MDK knockout exacerbates amyloid plaque formation and microglial activation. However, the molecular mechanism of MDK upregulation in Alzheimer's disease (AD) pathology and its effect on microglial-mediated A β clearance remain unclear. The study hypothesizes that the co-localization of MDK with A β and the MDK-A β interaction may alter microglial recognition and processing of plaques, which contributes to AD pathology. In this study, 5xFAD transgenic mice will be utilized to determine the distribution of MDK, analyze the MDK expression and subcellular localization in microglia, characterize the binding affinity of MDK A β and evaluate the effects of MDK A β complexes on microglia. Consequently, this study is expected to elucidate the molecular mechanism of MDK accumulation in the AD brain and its regulatory role in microglial phagocytosis, providing an experimental basis for the development of MDK-targeted therapeutic strategies.

Keywords: Alzheimer's Disease, midkine, Microglia phagocytosis

1. Introduction

A central hypothesis in AD research is that disease progression is driven by an imbalance between the production and clearance of amyloid- β 42 (A β 42) [1], with microglial phagocytosis which plays a pivotal role. In Alzheimer's disease, microglia have two roles: they protect the brain by phagocytosing A β plaques, however, with ageing and genetic risk factors, they become dysregulated and shift toward engulfing synapses and neurons, thereby exacerbating neurodegeneration [2]. However, emerging evidence indicates that amyloid plaques are complex pathological structures composed not only of A β , but also of extracellular matrix (ECM) proteins, inflammatory mediators, and other plaque-associated factors [3, 4]. These plaque-associated molecules may not be mere passive markers; rather, they may actively modulate plaque morphology, impair microglia clearance mechanisms, and thereby represent potential therapeutic targets. Therefore, defining how plaque-associated cofactors influence microglial phagocytosis is essential for understanding AD pathogenesis and for the development of targeted therapies. Heparin-binding growth factor MDK

has been identified as an important component of the A β plaque proteome among plaque-associated molecules [5, 6].

However, the role of MDK in plaque pathogenesis is highly controversial. Early studies showed that MDK promotes A β accumulation [6, 7], but this statement was recently contradicted by Zaman et al. showing that the genetic ablation of MDK in 5xFAD mice significantly aggravated amyloid burden, indicating that MDK is a physiological chaperone inhibiting A β fibrillization [8]. This contradiction, in which MDK has been reported to function both as a pathological driver and as a protective inhibitor, underscores the urgent need to decipher the cellular origins and spatiotemporal dynamics that determine its role in the AD brain. In addition, the functional consequence of MDK on microglia in the context of its presence in A β plaques remains unknown. Current studies have been focused on the effect of the MDK-A β interaction on plaque burden. However, the pathogenicity of A β plaques is not limited to plaque accumulation but importantly depends on their interactions with neighboring neurons and glial cells. While MDK has been shown to bind A β and accumulate in plaques, its functional consequences on microglial activities such as phagocytosis, inflammation or plaque compaction have not been systematically studied. Therefore, elucidating how MDK-modified plaques are recognized by Alzheimer's disease and responded to by microglia is critical for understanding MDK's dual function in the pathophysiology of Alzheimer's disease.

It is unclear whether MDK accumulation is the result of passive redistribution driven by plaque structure affinity, or active upregulation by neuronal signaling and glial reactivity. Determining whether MDK is a specific responder is important for defining its pathological significance, as this distinction determines whether plaque-associated MDK is simply a pathological marker, or also an active modulator of microglial responses to A β plaques, and thus a contributor to AD pathogenesis. We hypothesize that MDK co-localization with A β , and the ensuing MDK-A β interaction may change how microglia recognize and process plaques. Our study combines quantitative biochemistry, spatial transcriptomics, and functional microglial assays to determine how MDK accumulation modulates the neuroimmune microenvironment in the AD brain.

2. Materials and methods

2.1. Mice

5xFAD transgenic mice and wild-type C57BL/6J mice will be obtained from Jackson Laboratory. Use 5xFAD transgenic mice as the experimental group and wild-type littermate controls as the control group for *in vivo* experiments. Primary microglia will be isolated from P0–P2 neonatal C57BL/6J pups for *in vitro* experiments. Each experimental group will consist of 10 mice. Neonatal C57BL/6J pups used for primary microglial culture will be obtained either from commercial suppliers or from wild-type breeders colonies maintained in-house. Every mouse will be housed in a particular pathogen-free habitat with a 12-hour light/dark cycle and unlimited access to food and drink. The mice will be allocated into several groups utilizing a random number table. All animal experimental procedures need to be approved by IACUC.

2.2. DEA/FA sequential extraction and sequential immunoblotting

Mechanically homogenize mouse brain hemispheres in ice-cold Tissue Homogenization Buffer (THB) supplemented with protease inhibitors. Mix brain homogenates 1:1 with 0.4% diethyl amine (DEA) and ultracentrifuge the samples [9–11]. Collect and neutralize the supernatant. Resuspend the remaining pellets in 95% formic acid (FA) [9, 10] and ultracentrifuge the samples [11]. Determine

protein concentrations in both fractions using a BCA assay. Perform Western blot analysis on DEA and FA fractions. On the same PVDF membrane, sequentially detect MDK followed by A β using a stripping and reproving strategy. For sequential immunoblot analysis, membranes will be first probed with rabbit anti-MDK antibody (ABclonal, A0251) Incubated overnight at 4°C and subsequently treated with an HRP-conjugated secondary antibody for 1 hour. The membranes will be then stripped using a mild stripping buffer, blocked again, and reprobed with mouse anti-A β antibody (6E10) overnight at 4°C. Following a second stripping step, β -actin is detected as a loading control.

2.3. Cycloheximide chase assay

Digest hippocampal tissues with trypsin. Purify primary microglia using immunomagnetic bead sorting. Seed purified cells onto poly-L-lysine-coated culture plates and culture in DMEM/F12 complete medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂ incubator. After 24–48 hours of attachment, add cycloheximide to a final concentration of 50–100 μ g/mL [12]. Samples will be collected at 0, 2, 4, 8, and 12 hours. RIPA lysis buffer supplemented with protease inhibitors should be added right away after removing the culture medium and rinsing the cells or slices with ice-cold PBS at each time interval. The resulting lysates will be centrifuged at 12,000 $\times$ g for 15 min at 4°C.

2.4. Protease protection assay

Hippocampal tissues will be homogenized in ice-cold Tris-buffered saline (TBS). The homogenates will be centrifuged at 3,000 $\times$ g for 5 min at 4°C to remove cellular debris and large particulate materials, and the supernatant is collected. The collected supernatant is further subjected to ultracentrifugation at 100,000 $\times$ g for 1 h at 4°C to separate soluble and insoluble fractions. The insoluble pellet obtained from ultracentrifugation is resuspended in 95% formic acid [13] and sonicated to facilitate the solubilization of protein aggregates, followed by neutralization with Tris base. Protein concentrations will be determined using a BCA assay, and protein amounts will be normalized across samples. Proteinase K is added to the samples to achieve final concentrations of 0, 1, 5, and 10 μ g/mL, and the mixtures will be incubated at 37°C for 30 min to perform limited proteolysis. The reaction is terminated by adding PMSF to a final concentration of 2 mM. Samples will be then mixed with 5 $\times$ SDS-PAGE loading buffer containing reducing agents and heated at 95°C for 10 min for protein denaturation. Finally, all samples will be separated by SDS-PAGE, transferred onto membranes, and analyzed by Western blotting for target protein detection.

2.5. Dual-epitope immunoblotting

Sequential extraction with DEA and FA is conducted to isolate the soluble fraction and the insoluble plaque-enriched fraction. Proteins of equivalent quantity will be resolved by SDS-PAGE and subsequently transferred to two separate PVDF membranes for parallel analysis. Dual-epitope immunoblotting is performed using two antibodies that recognize distinct MDK epitopes. The first antibody targets an epitope within the N-terminal region of MDK, whereas the second antibody recognizes the C-terminal region.

2.6. Flow Cytometric Quantification

Whole-brain mononuclear cells will be isolated by enzymatic dissociation and Percoll gradient centrifugation. Microglia is identified and sorted as CD11b⁺CD45^{int} cells by flow cytometry. Total protein is extracted from the sorted microglia and subjected to Western blot analysis to determine MDK expression. In parallel, intracellular staining with anti-MDK antibody is performed to assess MDK expression in microglia by flow cytometry.

2.7. Immunofluorescence colocalization

Brain sections will be subjected to immunofluorescence staining using antibodies against MDK, LAMP1, and Iba1. Appropriate Alexa Fluor–conjugated secondary antibodies will be used for visualization, and nuclei is counterstained with DAPI. Colocalization of MDK with the microglial marker Iba1 and the lysosomal marker LAMP1 is analyzed by fluorescence microscopy.

2.8. qPCR

Extract total RNA using TRIzol reagent. Sort microglia as described in Flow Cytometric Quantification of MDK in Microglia. Collect in buffer with RNA protectant and immediately extract total RNA using TRIzol reagent. Treat 2 µg RNA with DNase I and reverse-transcribe into cDNA. MDK primers from OriGene (Cat# MP208185). Use Gapdh and Actb as dual reference genes. Perform qPCR with SYBR Green detection. Run each sample in triplicate with no-template controls per plate. Thermal cycling: 95°C for 5 min; 40 cycles of 95°C for 10 s and 60°C for 30 s.

2.9. RNAscope in situ hybridization

RNAscope analysis is performed using the RNAscope Multiplex Fluorescent Reagent Kit v2 and an MDK-specific probe, with probe hybridization conducted at 40°C. Following signal amplification, sections will be immunolabeled with a goat anti-Iba1 antibody and an Alexa Fluor–conjugated anti-goat secondary antibody. A probe targeting the bacterial gene dapB serves as a negative control for RNAscope specificity.

2.10. Co-immunoprecipitation

Hippocampal lysates will be incubated with anti-MDK antibody or anti-Aβ antibody for immunoprecipitation, while isotype-matched IgG is used as a negative control. Antibody-bound complexes will be collected using Protein A/G agarose beads and analyzed by Western blot after washing and elution. Reciprocal co-immunoprecipitation is performed to examine the association between MDK and Aβ.

2.11. Prediction for Aβ-MDK structure

Superimpose the MDK structural model with the Aβ peptide structure using PyMOL and structural modeling will be used as a preliminary approach to identify potential interacting surface.

2.12. Truncation mutagenesis

Construct the following Flag-tagged expression plasmids: full-length MDK (amino acids 21–143), an N-terminal deletion mutant (Δ N; amino acids 70–143), and a C-terminal deletion mutant (Δ C; amino acids 21–69). Co-transfect HEK293T cells with each of the above plasmids and A β expression plasmids. Perform anti-Flag immunoprecipitation as described in Co-Immunoprecipitation and detect co-precipitated A β as described in Sequential Immunoblotting for MDK and A β .

2.13. Primary microglia culture

Isolate primary microglia from neonatal mouse cortices using mixed glial culture. Digest with 0.25% trypsin for 10–15 min at 37°C, stop digestion with medium-containing serum, filter through 70 μ m cell strainer, collect cells by centrifugation, resuspend in DMEM supplemented with 10% FBS, seed into poly-L-lysine-coated flasks, maintain at 37°C under 5% CO₂ for 10–14 days, change medium every 2–3 days. After formation of the mixed glial layer, shake at 180–230 rpm at 37°C for 2–3 h, collect suspended cells, and centrifuge to collect microglia.

2.14. Microglial activation assay

Divide primary microglia into four groups, 4 replicate wells each: vehicle control, A β 42 alone, MDK alone, MDK-A β 42 complexes. Keep final A β 42 concentration consistent across groups.

Collect culture supernatants from each well. Measure TNF- α , IL-1 β , IL-6, and IL-10 levels in supernatants using commercial ELISA kits.

Aspirate culture supernatants. Rinse adherent cells twice with ice-cold PBS. Add RIPA lysis buffer containing protease and phosphatase inhibitors. Lyse on ice for 30 min. Perform Western blot as described in Sequential Immunoblotting for MDK and A β . The following primary antibodies will be used: anti-phospho-p65, anti-total p65, anti-phospho-p38, anti-total p38, anti-phospho-ERK1/2, anti-total ERK1/2, anti-phospho-JNK, and anti-total JNK.

Aspirate culture supernatants. Add TRIzol reagent and pipette repeatedly to lyse cells. Extract total RNA, reverse-transcribe, and perform qPCR as described in qPCR Analysis of Mdk mRNA in Brain Homogenates and FACS-Sorted Microglia. Use Gapdh as internal reference.

2.15. Phagocytosis assay

Add HiLyte Fluor 488-labeled A β 42 to primary microglia to 1 μ M final concentration. Incubate at 37°C for 2 h. Use 4°C incubation as negative control. Fix with 4% PFA and counterstain with DAPI. After phagocytosis assay, immunostain with anti-LAMP1 antibody and counterstain with DAPI. Immunostain fixed cells with anti-LAMP1 antibody.

2.16. Receptor blockade assay

Divide primary microglia into control group and blocker treatment groups. One hour before phagocytosis assay, add poly I, TREM2 neutralizing antibody, or LRP1 neutralizing antibody to respective treatment groups. Add equal volume of vehicle to control group. Perform phagocytosis assay as described. Add HiLyte Fluor 488-labeled A β 42 to 1 μ M final concentration and incubate at 37°C for 2 h.

3. Results

3.1. Characterization of MDK expression, solubility, and stability in 5xFAD brains

In 5xFAD mice, MDK is detected at high levels in the DEA-soluble and FA-insoluble fractions, whereas in WT mice, it is present only in the soluble fraction and is undetectable in the insoluble fraction. Consistently, A β is detected in both DEA and FA fractions of 5xFAD brains, showing higher abundance in the FA fraction, whereas it is undetectable in both fractions of WT mice. Furthermore, the half-life of MDK in the 5xFAD group is significantly prolonged compared to the WT group, and MDK in 5xFAD mice exhibits higher resistance to Proteinase K digestion than in WT mice.

If future experiments reveal that MDK remains PK-resistant even after A β is enzymatically cleared, this would suggest that MDK stabilization results from intrinsic protein misfolding rather than being solely dependent on A β binding. To address this, *invitro* aggregation assays with purified MDK will be performed to assess its intrinsic aggregation propensity.

3.2. Microglial MDK enrichment and altered lysosomal distribution

Flow cytometric analysis and Western blot of sorted cells show that MDK protein levels are specifically upregulated in microglia of 5xFAD mice compared to WT mice. Confocal immunofluorescence imaging shows significantly decreased colocalization of MDK with the lysosomal marker LAMP1 in Iba1+ microglia in the 5xFAD group, where instead of being trafficked to the degradative pathway, MDK localizes to discrete vesicle-like structures that do not colocalize with LAMP1.

The sequestration of MDK into non-lysosomal vesicles suggests disruption of the endo-lysosomal trafficking pathway. If the vesicle-like structures are secretory exosomes rather than stalled endosomes, this would suggest that microglia try to clear trapped MDK-A β complexes via extracellular secretion rather than intracellular degradation. To test this possibility, exosomes will be isolated from the interstitial fluid and their MDK and A β content analyzed.

3.3. Structural basis of the binding interface between MDK and A β

Co-IP shows that the anti-MDK antibody co-precipitates A β from 5xFAD mouse brain lysates, whereas the anti-A β antibody reciprocally co-precipitates MDK. No corresponding bands are observed in the IgG isotype control or lysates from WT mice, confirming a specific *in vivo* interaction. Dual-epitope immunoblotting further reveals that MDK in the 5xFAD insoluble fraction is detected by both N-terminal and C-terminal antibodies, indicating the presence of full-length MDK. AlphaFold2 predictions identify a stable binding interface between the C-terminal domain of MDK and A β . This prediction is supported by truncation mutagenesis, in which the mutant retaining the C-terminal domain preserves A β -binding capacity, whereas the N-terminal-only mutant fails to bind A β .

If the N-terminal-only mutant is still able to bind A β but the C-terminal-only mutant is not, that would be in contradiction with our original AlphaFold2 prediction. We would then revisit the structural model and determine whether the interaction is driven by the specific heparin-binding motifs in the N-terminal domain. If neither truncation mutant shows significant binding it would suggest that the interaction is conformation-dependent and requires the two domains to fold in a cooperative manner. To address this possibility, photo-leucine cross-linking combined with mass

spectrometry would be used to identify the specific residues of contact in the context of the full-length protein rather than isolated protein fragments.

3.4. Cell-type specific upregulation of MDK mRNA in microglia

Quantification of MDK mRNA by qPCR in whole brain homogenates shows no significant difference between 5xFAD and WT mice. RNAscope in situ hybridization shows that the number of MDK mRNA puncta within Iba1⁺ microglia is significantly increased in 5xFAD mice compared to WT mice.

If the increase of MDK protein in 5xFAD mice cannot be explained by an increase of mRNA levels alone, regulatory mechanisms such as increased translation efficiency or increased mRNA stability should be considered. To determine if MDK is more actively translated in the 5xFAD environment, we will perform Ribo-seq to determine the ribosome occupancy on MDK mRNA.

3.5. Functional modulation of microglial inflammation and A β clearance by MDK-A β complexes

MDK-A β complexes exhibit higher phagocytic uptake and enhanced colocalization with the lysosomal marker LAMP1 compared to A β alone. Phagocytosis enhanced by MDK is significantly reduced by pre-treatment with poly I, TREM2 neutralizing antibodies or LRP1 neutralizing antibodies. MDK-A β treatment reduces pro-inflammatory cytokine levels, inhibits activation of NF- κ B and MAPK pathways and up-regulates endogenous MDK mRNA.

If endogenous MDK mRNA levels are unchanged this would indicate that the neuroprotective effect of MDK is only mediated by the exogenous protein and not by a transcriptional feedback loop. Alternatively, if MDK-A β treatment leads to increased levels of pro-inflammatory cytokines, it would suggest that MDK is a pro-inflammatory stimulus that enhances A β -induced inflammatory injury.

4. Conclusion

We expect this study to demonstrate a significant upregulation of MDK protein levels in 5xFAD brains, potentially due to enhanced conformational stability and microglial-specific transcriptional activation. The results are expected to provide evidence that MDK directly binds to A β through its C-terminal domain, leading to their co-accumulation in amyloid plaques. The results are expected to show that MDK-A β complexes attenuate A β -induced microglial inflammatory responses while promoting microglial uptake of A β , although delivery of internalized A β to lysosomes remains impaired. This study is expected to reveal a dual role of MDK in AD pathogenesis and provide a theoretical basis for the development of MDK-targeted therapeutic strategies.

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