Shuttling Protein Nucleolin Is a Microglia Receptor for Amyloid Beta Peptide 1-42

Daisuke Ozawa, Takashi Nakamura, Masanori Koike, Kazuya Hirano, Yuichi Miki,* and Masatoshi Beppu

School of Pharmacy, Tokyo University of Pharmacy and Life Sciences; 1432–1 Horinouchi, Hachioji, Tokyo 192–0392, Japan.

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Alzheimer’s disease (AD) is one of several neurodegenerative diseases in which patients develop progressive cognitive impairment. Pathologically, AD is characterized by deposits of senile plaques (extracellular plaques that consist of aggregated amyloid-beta [Aβ] peptides), formation of neurofibrillary tangles, and synapse and focal neuronal loss. This pathological picture indicates the critical necessity of clearing Aβ from brain.1

In particular, senile plaques are extracellular deposits of Aβ found in the gray matter of the brain. Aβ is generated by cleavage of the amyloid precursor protein (APP), an integral membrane protein expressed in many tissues which is concentrated in the synapse of neurons by the secretase family. Aβ is composed of 38–43 amino-acid sequences; amino acids 11–15 of the C-terminus originate from the transmembrane domain of APP, therefore making Aβ highly liposoluble.2–4 Since Aβ was first found to be a main constituent of senile plaques it has been shown that it accumulates in the encephalic parenchyma and cerebral blood vessels, and that at least two kinds of Aβ peptide exist: Aβ1-40 (Aβ40) and Aβ1-42 (Aβ42).2–4 Although Aβ in general has high coherence, specific coherence levels differ for each molecule.5 While Aβ42 aggregates quickly within one second, Aβ40 aggregates more slowly.6 Additionally, Aβ42 facilitates the aggregation of Aβ40, serving as the seeding for Aβ40 aggregation.6 Aβ42 easily forms deposits, is highly toxic, and its increase is considered to be involved in the development of AD (amyloid hypothesis).7 According to this hypothesis, Aβ42 deposits form in the cortex when the balance between its production and decomposition is disrupted. Deposited Aβ42 induces the accumulation of phosphorylated tau protein in neurons, which in turn causes the formation of neurofibrillary tangles and results in dementia.8 A means of clearing Aβ42 is therefore considered a critical priority in AD.

A number of studies have reported on Aβ clearance. Neprilysin and insulin-degrading enzyme are rate-limiting peptides in the physiological degradation of Aβ in the brain.9,10 In addition to limiting Aβ production, microglia (brain phagocytes) remove Aβ by phagocytosis. Indeed, several receptors for monomeric and polymeric (fibril Aβ) have been identified on microglia cell membranes. The serpin–enzyme complex receptor and insulin receptor bind to monomeric Aβ, while the α-7 nicotinic acetylcholine receptor, integrins, receptor for advanced glycosylation end-products (RAGE), and formyl peptide receptor-like 1 (FPRL1) bind to both monomeric and fibril Aβ. Further, the N-methyl-D-aspartate receptor, P75 neurotrophin receptor, collagen-like Alzheimer amyloid plaque-component precursor/collagen XXV, scavenger receptors A, B1, and CD36, aβ1-integrin, and CD47 have all been reported to bind to fibril Aβ. Heparan sulfate proteoglycans have also been described as cell-surface binding sites for Aβ.11 The abundance of receptors that recognize Aβ indicates that its clearance is an important function for microglia. Further research into the mechanisms by which Aβ is removed is therefore critical to the better understanding and treatment of AD.

Nucleolin is present in the nucleus, cytoplasm, and on the surface of some types of cells including macrophages.12,13 Previous studies have shown that macrophages utilize nucleolin to recognize and phagocytose apoptotic and oxidized cells,14,15 as well as living cells that have been treated with cytotoxic agents.16,17 Nucleolin-mediated recognition is therefore simple and powerful, as indicated by the indiscriminant removal of cells. Nucleolin is also a receptor for lipoproteins,18 coxsackie B virus,19 human immunodeficiency virus,20 human parainfluenza virus type 3,21 and enterohemorrhagic Escherichia coli O157:H7.22 Further, nucleolin can bind to...
anionic molecules such as DNA and RNA.13,23) Together, these observations suggest that macrophages with nucleolin on their surface may have a general scavenger-like ability. Nucleolin might therefore also be involved in the phagocytosis of Aβ, albeit that the function of nucleolin on microglia is poorly understood. Here, we investigated whether nucleolin is involved in the phagocytosis of Aβ by microglia.

MATERIALS AND METHODS

Materials Monomeric Aβ40, monomeric Aβ42, carboxyfluorescein (FAM)-labeled monomeric Aβ40, FAM-labeled monomeric Aβ42, Aβ1-16, and Aβ1-42 were obtained from Ana Spec (San Jose, CA, U.S.A.). PKH 26 red fluorescent cell linker kit was obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Control-rabbit immunoglobulin G (IgG) and control-rat IgG were obtained from Santa Cruz Biotechnology (Delaware, CA, U.S.A.). rNUC284, a truncated recombinant human nucleolin containing residues 284–710 and corresponding to the C-terminal two-thirds of the molecule, was produced in E. coli and purified as previously described.14 Anti-nucleolin antibodies (anti-NUC295, an antibody against nucleolin amino-acid residues 295–302, and anti-rNUC284, an antibody against rNUC284) were raised in rabbits and affinity purified, as previously described.14,15

Preparation of Fibril Aβ40 and Aβ42 Fibril Aβ was prepared by long-incubation of monomeric Aβ.24 Fifty microgram per milliliter monomeric Aβ40 or Aβ42 were incubated in Ca2+, Mg2+-free Dulbecco’s phosphate-buffered saline (DPBS(−)) for 5 d at 37°C. Aggregation of Aβ was confirmed using thioflavin T as previously described.25,26

Phagocytosis of Monomeric and Fibril Aβ by EOC2 Cells Immortalized mouse-microglia EOC2 cells (American Type Culture Collection, VA, U.S.A.) were suspended at 5×105 cells/mL in RPMI 1640 medium buffered with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 0.2% bovine serum albumin (RPMI 1640–HEPES–0.2% bovine serum albumin (BSA)) by scraping the tissue flask. EOC2 cells were then incubated with 0–10 µg/mL FAM-labeled monomeric or fibril Aβ for 2 h, and immediately analyzed using a flow cytometer (FACS Calibur, Becton-Dickinson, Franklin Lakes, U.S.A.) with CELLQUEST software and gating for FSC and SSC regions of intact EOC2 cells.

Confocal Images of Phagocytosed Aβ40 and Aβ42 EOC2 cells in Dubbecco’s modified Eagle’s medium (DMEM) medium with 10% fetal bovine serum were plated in 24 well plates at 1×104 cells/well in which round coverslips (15-mm diameter) were placed and cultured at 37°C in 5% CO2 atmosphere for 3 d. The monolayers of EOC2 cells on coverslips were washed in DPBS(−) before use, and incubated with 5 µg/mL FAM-labeled monomeric or fibril Aβ’s at 37°C for 2 h in RPMI 1640–HEPES–0.2%BSA. After which EOC2 cell membranes were stained with the fluorescent cell-linker compound PKH 26 (red) kit. Aβ’s taken up by EOC2 cells were identified using confocal laser scanning fluorescence microscopy (FV1000D, Olympus, Tokyo, Japan).

Surface Plasmon Resonance (Biacore) Analysis of Aβ40 and Aβ42 binding to nucleolin was conducted using a Biacore 2000X (Biacore Life Sciences, Tokyo, Japan). This biosensor can directly measure the binding of a recombinant protein to its natural biological ligand in real time in a quantitative and highly reproducible manner.27) In brief, HBS running buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM ethylenediaminetetraacetic acid (EDTA), and 0.005% Tween 20 (pH 7.4)) was used for sample dilution and analysis. The research-grade CM5 dextran sensor-chip was activated with equal amounts of 0.2 µg N-ethyl-N[3-diethylamino-propyl]-carbodiimide and 0.05 µM N-hydroxysuccinimide. rNUC284 was produced in E. coli and purified as previously described15 at 20 µg/mL, and immobilized in 10 mM sodium acetate buffer (pH 4.0) followed by 1 mM ethanolamine-hydrochloride (pH 8.0) to deactivate excess N-hydroxysuccinimide-esters. This coupling resulted in ca. 10 ng protein/mm2 of immobilized proteins per flow-cell. To evaluate binding, each Aβ was diluted in HBS buffer and analyzed at different concentrations and passed over the sensor chip at a flow rate of 20 µL/min. An activated and blocked flow-cell without immobilized ligand was used to evaluate nonspecific binding. Results were calculated using BIAscanner 4.0 software (Biacore).

Detection of Cell-Surface Nucleolin on EOC2 Cells and Rat Primary Microglia For measurement of cell-surface nucleolin, EOC2 cells were detached from culture flask, and resuspended in RPMI 1640–HEPES–3%BSA, as described above. Freeze stock rat primary microglia (TaKaRa Bio Inc., Shiga, Japan) were thawed at 37°C, and resuspended in RPMI 1640–HEPES–3%BSA. Before treatment with primary antibody, microglia in suspension were pretreated with 10 µg/mL human IgG Fc fragment15 (EOC2 cells) or control-rat IgG (rat primary microglia) in RPMI1640-HEPES-3% BSA at 4°C for 10 min to block Fc receptors. Then EOC2 cells and rat primary microglia were treated with 10 µg/mL anti-NUC295 or control-rabbit IgG in RPMI–HEPES–3% BSA at 0°C for 30 min. Bound antibody was detected by treating the cells with 10 µg/mL Alexa Fluor-488 goat anti-rabbit IgG conjugate (Molecular Probe, Tokyo, Japan) in RPMI–HEPES–3% BSA at 0°C for 30 min. After washing with DPBS(−), the cells were re-suspended in phenol red-free RPMI–HEPES and immediately subjected to flow-cytometric analysis, as described above.

Pretreatment of EOC2 Cells with Anti-nucleolin Antibody for the Phagocytosis Assay EOC2 cells were preincubated with 20 µg/mL anti-rNUC or control-rabbit IgG in RPMI1640-HEPES containing 0.2% BSA at 4°C for 30 min, washed, and subjected to phagocytosis assay using monomeric Aβ42.

Phagocytosis of Monomeric and Fibril Aβ by Nucleolin-Transfected HEK Cells Human nucleolin cDNA was transfected into the monolayer of HEK 293 cells (HEK cells) (Health Science Research Bank, Osaka, Japan) as previously described.14) Sixteen hours after transfection, adherent HEK cells were detached from the bottom of the culture well by incubation with Puck’s EDTA solution (5 mM HEPES, 0.1 mM NaCl, 5 mM KCl, 4 mM NaHCO3, 1 mM EDTA, 5.6 mM glucose) for 10 min and gentle pipetting. HEK cells were then incubated with 5 µg/mL FAM-labeled monomeric or fibril Aβ at 37°C for 2 h, and immediately analyzed using a flow cytometer with CELLQUEST software and gating for FSC and SSC regions of intact HEK cells.

Statistical Analysis Data are presented as the mean±S.D. of at least three experiments and were analyzed with Student’s t-tests.
Phagocytosis of Monomeric and Fibril Aβ40 and Aβ42 by EOC2 Cells

We investigated whether mouse microglial EOC2 cells phagocytosed Aβ40 and Aβ42. EOC2 cells phagocytosed both monomeric and fibril Aβ42 dose dependently (Figs. 1A, C), but only slightly phagocytosed monomeric and fibril Aβ40 (Figs. 1B, D). Figures 1E–I were shown that the confocal images of phagocytosed Aβ42 and Aβ40. Monomeric and fibril Aβ42 were phagocytosed by EOC2 cells (F and G), but only slightly phagocytosed monomeric and fibril Aβ40 (H and I).

Association of Nucleolin with Four Types of Monomeric Aβ (Aβ40, Aβ42, Aβ1-16, and Aβ17-42) We next investigated whether nucleolin is a receptor for Aβ. First, we tested whether nucleolin binds to Aβ by observing the interactions between nucleolin and Aβ using surface plasmon resonance. Aβ was applied to a CM5 dextran sensor-chip, which was immobilized with rNUC284. The sensorgram demonstrated that Aβ42 had high concentration-dependent affinity and did not easily dissociate by washing (Fig. 2A), while Aβ40 had a weak affinity for rNUC284 (Fig. 2B). The kd, ka and KD values between rNUC284 and Aβ42 were 7.36×10^{-5}, 4.76×10^{3}, and 1.55×10^{-8}. To investigate the binding site for nucleolin on Aβ42, we also investigated the association of artificial peptide Aβ1-16 and Aβ17-42 to rNUC284. However, neither of the artificial Aβs showed an association (Fig. 2C). These results indicate that Aβ42 binds to nucleolin but Aβ40 weakly binds to nucleolin, and that the association between nucleolin and Aβ42 may not only require the primary structure of Aβ42, but
also higher-order structures.

**Nucleolin-Expression on Cell-Surface of Microglia** A previous study showed that monocytes and macrophages express nucleolin on their cell surfaces.\(^{14}\) In flow cytometric analysis, anti-nucleolin antibody (anti-NUC295) also bound to EOC2 cells (Fig. 3A) and rat primary microglia (B), producing small but distinct shifts of the fluorescence peaks as compared with control IgG. These results were indicating that nucleolin is expressed on the cell surfaces of microglia.

**Involvement of Microglia-Surface Nucleolin in the Phagocytosis of Aβ40 and Aβ42** We next investigated whether cell surface-expressed nucleolin is a phagocytic receptor for Aβ42 and Aβ40. Phagocytosis of monomeric Aβ42 by microglia was inhibited by anti-nucleolin antibody (anti-rNUC antibody) but not by control-rabbit IgG (Fig. 4A). Although monomeric Aβ40 was only slightly phagocytosed by EOC2 cells (Fig. 1B), the assay was repeated with Aβ40, but no inhibitory effect was observed (data not shown). Next we blocked nucleolin using AGRO, an oligodeoxynucleotide aptamer that binds specifically to nucleolin.\(^{28}\) This inhibited phagocytosis of monomeric and fibril Aβ42 by microglia, whereas control CRO did not (Figs. 4B, C). Additionally, we expressed recombinant nucleolin on the surface of non-microglia HEK cells by transiently transfecting nucleolin on HEK cells as described previously.\(^{14}\) Monomeric and fibril Aβ42 bound more to these HEK cells than to non-transfected HEK cells (Figs. 4D, E), while Aβ40 did not (Figs. 4F, G). Theses results indicate that nucleolin is a receptor of Aβ42, but not Aβ40.

**DISCUSSION**

The aggregation state of Aβ (monomeric or fibril) has been known to affect binding activity. For example, FPRL1, β1 integrin, and RAGE all bind both monomeric and fibril Aβ, whereas scavenger receptor A and scavenger receptor BI only bind monomeric Aβ.\(^{11}\) Here, we showed that nucleolin on the surface of microglia is a receptor for both of monomeric and fibril Aβ42.

The aggregation process of Aβ from monomer to oligomer to fibril (polymer) induces toxicity in neurons and other brain cells that strongly suggests involvement in AD. Reduction of Aβ in brain tissue is therefore one of the key strategies for AD treatment.\(^{29}\) These strategies include the inhibition of Aβ generation, inhibition of Aβ aggregation, and promotion of Aβ clearance. Because Aβ is generated when β- or γ-secretase...
Fig. 4. Involvement of Microglia-Surface Nucleolin in the Phagocytosis of Aβ40 and Aβ42

(A) Pretreatment of anti-nucleolin antibody (anti-rNUC) inhibits phagocytosis of 5μg/mL monomeric Aβ42. (B and C) Co-incubation with 10μM nucleolin aptamer AGRO inhibits phagocytosis of 5μg/mL monomeric (B) and fibril (C) Aβ42. (D and E) Phagocytosis of 5μg/mL monomeric (D) and fibril Aβ40 (E) by nucleolin-transfected HEK cells. (F and G) Phagocytosis of 5μg/mL monomeric (F) and fibril Aβ40 (G) by nucleolin-transfected HEK cells. Each bar represents the mean±S.D. of at least triplicate experiments. *p<0.05; **p<0.01; and ***p<0.001.
cleaves APP, secretase-inhibiting drugs are being investigated and progress is expected.\textsuperscript{30} Research of low molecular, such as cystamine, compounds that suppress \(\text{A}\beta\) aggregation is also advancing.\textsuperscript{11,32} Phagocytosis of \(\text{A}\beta\) by microglia or its degradation by proteolytic enzymes such as neprilysin and insulin-degrading enzyme has been suggested as one means of promoting clearance.\textsuperscript{9,10} Additionally, research into the membrane-protein mediated discharge mechanism of \(\text{A}\beta\) from brain tissue to the periphery through the blood–brain barrier and the blood–cerebrospinal fluid barrier has also been suggested.\textsuperscript{9,10} Interestingly, in addition to microglia, nucleolin is also expressed on the cell surface of blood vessel endothelia.\textsuperscript{33} Therefore, nucleolin may contribute to the clearance of \(\text{A}\beta\) not only through microglia-mediated phagocytosis as shown here, but also through the discharge from brain tissue through endothelium cells. Investigating the function of cell-surface nucleolin will contribute to the clarification, prevention, and medical treatment of AD.

The C-terminal of \(\text{A}\beta/42\) of 41 aa is Ile and of 42 aa is Ala. Hou \textit{et al.} reported that the hydrophobic domain of \(\text{A}\beta/40\) is only Leu17-Phe20. In contrast, \(\text{A}\beta/42\) has three hydrophobic domains: Leu17-Phe20, Ile31-Val36, and Val39-Ile41.\textsuperscript{34} In \(\text{A}\beta/42\), the hydrophobic interaction between molecules in these domains is produced by the turn structure of the Phe20-Ala30 domain, and results in the tendency for \(\text{A}\beta/42\) to aggregate and form fibrils.\textsuperscript{34} Therefore, although 90\% of secreted \(\text{A}\beta\) is \(\text{A}\beta/40\), the \(\text{A}\beta/40\)-deposition in senile plaque is mainly composed of \(\text{A}\beta/42\).\textsuperscript{7} We speculate that this superstructure of \(\text{A}\beta/42\) is important for binding with nucleolin. Surface plasma resonance analysis revealed that the binding of rNUC284 and \(\text{A}\beta/42\) did not dissociate with NaCl or detergent treatments. In contrast, treatment with the protein-denaturing agent guanidine did dissociate the binding, indicating that the bond between nucleolin and \(\text{A}\beta/42\) is neither ionic nor hydrophobic. The superstructure of \(\text{A}\beta/42\) that contributes to its aggregation was also necessary for binding between nucleolin and \(\text{A}\beta/42\). Therefore, the C-terminal of \(\text{A}\beta/42\) of 41 aa Ile and 42 aa Ala is the important region needed for binding with nucleolin. A special role in the specific removal of \(\text{A}\beta/42\) suggests that nucleolin has an important physiological role in AD.

Nucleolin is a phagocyte-receptor that removes various discarded elements.\textsuperscript{12–23,35} This study showed that \(\text{A}\beta/42\) is also a ligand for nucleolin. These observations support the idea that nucleolin on the surface of phagocytes has a general scavenger-like ability. However, the mechanism of nucleolin-mediated phagocytosis, particularly the ligand-internalizing process, remains poorly understood. Although nucleolin can be detected on the cell surface, how it exists there remains unknown due to its lack of a transmembrane domain.\textsuperscript{12,13} Several other \(\text{A}\beta\)-binding proteins such as milk-fat globule epidermal growth-factor 8 and calreticulin also lack transmembrane domains.\textsuperscript{36,37} These proteins require additional scaffold proteins with transmembrane domains such as integrin or CD91 to phagocytose \(\text{A}\beta/42\).\textsuperscript{36,37} We have found that an unidentified protein is needed as a scaffold protein for nucleolin to exist on cell surfaces (Hirano K., unpublished data). Therefore, nucleolin may be associated with membrane-protein with transmembrane-domains as a scaffold protein.

Activated microglia have been observed around senile plaques in the brains of AD patients\textsuperscript{38,39} and in fact microglia are activated by fibril \(\text{A}\beta\).\textsuperscript{40} Although microglia are usually in a state of rest and do not damage neurons and tissue, activated microglia can induce neuronal cell and tissue injury by inducing inflammation via the release of inflammatory cytokines, including tumor necrosis factor \(\alpha\) and interleukin-1 (IL-1).\textsuperscript{11} The possibility therefore exists that phagocytosis of \(\text{A}\beta\) by microglia may lead to the exacerbation of AD.\textsuperscript{11} Therefore, it is possible that nucleolin on the surface of microglia also participates in the inflammatory reaction seen in AD. Further study is needed to clarify the action of microglia after nucleolin-mediated phagocytosis of \(\text{A}\beta/42\).

Here, we showed that nucleolin is a receptor for \(\text{A}\beta/42\). Additional comprehensive evaluation of nucleolin, including its molecular composition, as a microglia receptor for \(\text{A}\beta/42\), will provide important new information in our understanding of AD.

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