学 位 論 文 の 要 旨

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学　位　論　文　名　iTRAQ-Based Proteomic Analysis of APP Transgenic Mouse Urine Exosomes

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論 文 内 容 の 要 旨
INTRODUCTION

Alzheimer's disease (AD) is a common dementia disease in the elderly population. The presence of extracellular amyloid plaques and intraneuronal neurofibrillary tangles in certain brain areas are the main pathological hallmarks of the disease. The amyloid plaques mainly contain aggregated amyloid β (Aβ) peptide, which was demonstrated their importance in AD pathology.

Increased production or decreased clearance is considered the cause of Aβ deposition. Increased production of the peptide is seen in familial AD. In sporadic AD, Aβ is deposited mainly around the vessels due to the disturbance of vessel-dependent Aβ clearance. However, intracellular aggregated Aβ can also be found in a widespread area of brain regions in AD. How this aggregated peptide is deposited intracellularly and affects such wide areas of the brain is still under investigation. It is speculated that Aβ is transferred from cell to cell by certain mechanisms. In this respect, exosome-mediated transport could be the reason for such widespread distribution of Aβ in the central nervous system. Indeed, recent reports showed the importance of exosome-mediated transport of Aβ in AD pathology. Exosomes are extracellular vesicles with a diameter of 30-150 nm, which carry cargo like signaling molecules, RNAs, and proteins. They are released from various types of cells into the extracellular space and transport the cargo to the target cells to modulate their functions.

AD is largely diagnosed clinically since there are no commonly available laboratory tests, and tissue biopsies are not recommended for the disease. Recently, positron emission tomography (PET)-based diagnosis and analysis of CSF has been developed. However, PET-based diagnosis systems have limitations regarding availability, cost, and maintenance, and the main drawback of analysis of CSF is its invasiveness. Since exosomes have the ability to cross the blood-brain barrier,

it is conceivable that neuron-derived exosomes isolated from peripheral fluid compartments could be a tool for disease diagnosis. Hence, to find valuable information about AD pathology and suitable targets for disease diagnosis, we isolated exosomes from the urine of an AD model mouse. Then, neuron-derived exosomes were separated from urine exosomes, and comprehensive proteomic and bioinformatic analyses were performed.

MATERIALS AND METHODS

Male mice with the human amyloid precursor protein (hAPP) transgene (J20) were used as an AD model and their wild-type (WT) littermate as a control. Urine was collected every 24 hours for 1 month from J20 mouse (n=9) and WT mouse (n=9) at 3-month age. Subsequently, the urine exosomes (U-exo) were isolated from urine by polymer precipitation, neuron-derived exosomes (N-exo) were then isolated from urine exosomes by immunoprecipitation with neuron-specific L1CAM antibody. Afterwards, exosomes were characterized by transmission electron microscopy and western blot of exosome markers. And the exosomes were then used for proteomic analysis based on iTRAQ coupled with MALDI TOF MS/MS. Next, the proteomics data were analyzed with online bioinformatics tools, Metascape for Gene Ontology enrichment analysis and protein-protein interaction analysis, WoLF PSORT for subcellular localization prediction, GenAtlas for brain expression, CellMarker 2.0 for neuronal markers. Finally, the most important proteins were confirmed by western blot.

All experiments with animals in this study were approved by the Animal Care and Use Committee of Shimane University (approval number: IZ29–28).

RESULTS AND DISCUSSION

We isolated U-exo and N-exo from urine. During the characterization of exosomes, both U-exo and N-exo were positive for exosome markers (Tsg101, ALIX, CD9, and CD63), and exhibited typical exosomal spherical bilayer membrane structure and were also in the exosome diameter range of 30-150 nm, suggesting the success of exosomes isolation. Next, we performed proteomic analysis, a total of 659 and 481 proteins with intensity values were detected in U-exo and N-exo, respectively. To minimize the false positive identification of proteins, 79 and 117 proteins with high confidence were screened as identified proteins in U-exo and N-exo, respectively. As most biochemical methods are prone to include technical variance, we considered an additional cutoff of 1.2-fold change in iTRAQ ratio (J20/WT) to select for different proteins. A total of 61 and 92 proteins were more abundant in the U-exo and N-exo of J20 mice compared to WT mice, respectively. Among them, 41 different proteins are common in both U-exo and N-exo, while 20 and 51 different proteins are exclusively in U-exo and N-exo, respectively. For the quality control analysis, protein subcellular localization analysis showed that most of the U-exo and N-exo proteins were located in extracellular and membrane,

indicating the high purity of our samples. Besides, enrichment analysis of cellular component (CC) displayed enriched terms closely related with exosomes, such as extracellular exosome, extracellular matrix, brush border membrane, secretory vesicle, further supporting the high purity of our exosomes. To evaluate the efficacy of L1CAM antibody to separate N-exo from U-exo, we checked the brain expression of the identified proteins. Our results confirmed that N-exo contains more proteins that can be expressed in the brain, 24 U-exo proteins and 41 N-exo proteins, suggesting a CNS origin of N-exo. Furthermore, after checking the neuron markers, we found that there were 15 neuron markers in N-exo, suggesting the neuron origin of N-exo.

To investigate the functions of U-exo and N-exo proteins, we performed Gene Ontology enrichment analysis and characterized in terms of biological process (BP), CC, and molecular functions (MF). In case of MF, all the common different proteins, the identified U-exo and N-exo proteins were highly enriched in enzyme activity terms, especially hydrolases, suggesting that U-exo and N-exo mainly mediate the degradation process of proteins. For BP, 41 common different proteins in both U-exo and N-exo were enriched in the terms related to protein metabolism, especially the regulation of enzyme activity, further implying the importance of U-exo and N-exo in protein degradation. Interestingly, the identified proteins in U-exo and N-exo are mostly clustered under the terms of lipid metabolic processes, Aβ metabolism and clearance processes, which is consistent with previous reports that lipids and Aβ have important functions in AD. After comparing the proteins participating in the highly enriched BP terms in both U-exo and N-exo, we found acid ceramidase is the key protein in lipid metabolism, and clusterin, ApoE, neprilysin, and angiotensin converting enzyme are important regulators in Aβ metabolism and clearance. Notably, we performed protein-protein interaction analysis to further explore the association among the proteins identified in U-exo and N-exo, and detected four protein complexes in U-exo and N-exo. Importantly, ApoE and clusterin are partner proteins in protein complex 1, suggesting that they act as protein interactors in the metabolism and clearance of Aβ. Given that clusterin has markedly higher peptide coverage with 49 and 50 peptides in U-exo and N-exo, respectively, it was chosen to verify the efficacy of the iTRAQ-based quantification. Our results showed that clusterin existed both in U-exo and N-exo and presented as three different molecular weights (about 80, 70, and 50 KDa in size). Interestingly, the band distributions of clusterin in U-exo and N-exo are different, which deserve further exploration.

CONCLUSION

In conclusion, we have established a method of purification of neuron-derived exosomes from urine, done a comprehensive proteomics analysis, and demonstrated the enrichment of AD-related proteins in both urine and neuron-derived exosomes. Such analysis is important to understand the pathophysiology of the disease, identification of new therapeutic targets as well as the identification of novel diagnostic markers for the disease.