Melatonin attenuates β-amyloid-induced inhibition of neurofilament expression

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KEY WORDS Alzheimer disease; amyloid beta-protein; neurofilament proteins; melatonin

ABSTRACT

AIM: To explore the effect of β-amyloid (Aβ) on metabolism of cytoskeletal protein neurofilament, and search for effective cure to the lesion. METHODS: Wild type murine neuroblastoma N2a (N2awt) and N2a stably transfected with wild type amyloid precursor protein (N2aAPP) were cultured. Sandwich ELISA, immunocytochemistry, and Western blot were used respectively to measure the level of Aβ, the expression and phosphorylation of neurofilament proteins. RESULTS: The immunoreactivity of neurofilament protein was almost abolished in N2aAPP, which beard a significantly higher level of Aβ. Melatonin effectively decreased the level of Aβ, and restored partially the level of phosphorylated and non-phosphorylated neurofilament in N2aAPP. CONCLUSION: Overproduction of Aβ inhibits neurofilament expression, and melatonin attenuates the Aβ-induced lesion in cytoskeletal protein.

INTRODUCTION

Alzheimer’s disease (AD), a progressive neurodegenerative disorder affecting the cognitive function of brain, is a highly prevalent dementia in aging. It is characterized by the presence of numerous senile plaques and neurofibrillary tangles (NFTs). The former are composed of β-amyloid (Aβ), a peptide of 39-43 amino acid. Aβ is derived from proteolytic processing of type I transmembrane glycoprotein, called amyloid precursor protein (APP), which is cleaved at two sites by two secretases, β- and γ-secretase, then yield short peptide Aβ[1]. On the basis of genetic research, neuropathology, cell biology, and transgenic animal model, Aβ deposition is indicated to play a central role in AD development[2]. Accumulating evidence suggests that the degree of dementia is associated with rather NFTs than Aβ deposition[3]. NFTs are intraneuronal cytoplasmic lesions, consisted of bundles of paired helical filaments (PHFs)[4]. The subunit protein of PHF is cytoskeleton protein tau. Tau, a microtubule associated protein, participates in resembling and remaining the stability of microtubule[5]. In this process, another cytoskeleton protein, neurofilament (NF) plays an important role. NF belongs to the intermediate filament of neurons, contributing to maintain cytoskeleton and stabilizes cell morphology and axonal transport[6]. It is also hyperphosphorylated and accumulated in the plasma and elevated in AD cerebrospinal fluid[5,8].

Increasing data implies that the formation of tangles represents one of several cytological responses by cells to the gradual accumulation of Aβ and Aβ associated...
protein\textsuperscript{[9]}. But there is no direct evidence to explain the relationship between the two lesions. Additionally, Melatonin is a hormone that not only participates in the regulation of the circadian rhythms but may also exerts neuroprotective effect in AD\textsuperscript{[10]}. Melatonin is decreased during the aging process and patients with AD have more profound reductions in this hormone\textsuperscript{[11]}. It has also been reported that melatonin protected neuronal cells from Aβ-mediated oxidative damage and inhibited the formation of amyloid fibrils \textit{in vitro}\textsuperscript{[12, 13]}. However, a direct relationship between melatonin and the biochemical pathology of AD has not been demonstrated. The present study was aimed to clarify the effect of Aβ overproduction on metabolism of neurofilament proteins and to elucidate whether melatonin has protective effect against Aβ-induced lesion in cytoskeletal protein.

**MATERIALS AND METHODS**

**Cell culture** Wild type murine neuroblastoma N2a (N2awt) and N2a transfected with human APP695 (N2aAPP) were obtained from Dr XU (Rockefeller University, NY, USA). The cells were maintained in a medium containing 50 % Dulbecco’ modified Eagle’s medium (DMEM) and 50 % Opti-MEM, supplemented with 5 % fetal bovine serum (Gibico BRL, Gaithersburg, USA) in 5 % CO\textsubscript{2} at 37 ºC, and stably transfected cells were selected in medium in the presence of 0.2 g/L G418.

**Immunocytochemistry** Cells were cultured in the presence or absence of 10 µmol/L melatonin (Sigma, St Louis, MO, USA) for 24 h. The cells on the coverslip were fixed for 7 min in ice-cold acetone after rinse with PBS, then permeabilized in 0.3 % Triton X-100 in PBS. Endogenous peroxidase was blocked with 0.3 % H\textsubscript{2}O\textsubscript{2} for 10 min and non-specific binding sites were blocked with 10 % normal goat serum for 10 min at room temperature. The cells were exposed to primary antibodies SMI31, SMI32, SMI33, and SMI34 (1:5000 diluted in 3 % BSA in PBS, containing 0.02 % NaN\textsubscript{3}) for overnight at 4 ºC, then to peroxidase-labeled goat-anti-mouse secondary antibody at 37 ºC for 2 h and developed with diaminobenzidine tetrachloride (5 g/L).

**ELISA** The plates (Nunc Maxisorp, Denmark) were coated with antibody G\textsubscript{2-10} specific for Aβ40 (Abeta GmbH, Deutsch) overnight at 4 ºC and blocked with 5 % BSA in PBS, containing 0.02 % NaN\textsubscript{3}) for overnight at 4 ºC, then to peroxidase-labeled goat-anti-mouse secondary antibody at 37 ºC for 2 h and developed with dianinobenzidine tetrachloride (5 g/L).

**Statistical analysis** All data were presented as mean±SD and Student’s two-tailed t-test was used for the comparison.

**RESULTS**

**Overproduction of Aβ induced neurite retraction and abolished the immunoreaction of NF protein** The level of Aβ1-40 both in culture medium and in cell lysate were significantly higher in N2aAPP than in N2awt (Tab 1). The morphology of N2awt showed abundant and long neurites with clear profile. Much less and shorter neurites were observed in N2aAPP (Fig 1), which bear significantly high level of Aβ (Tab 1). In N2awt, the phosphorylated NF proteins recognized by SMI31 and SMI34 were detected mainly on cell surface, whereas non-phosphorylated NF proteins bound to SMI32 and SMI33 were stained in cytoplasm and neurites when the dilution of the antibodies was 1:5000 (Fig 2A-D). Surprisingly, NF was not detected in N2aAPP with same panel of antibodies at the same dilution.

**Tab 1. Effect of melatonin on Aβ in medium and in cell lysate of N2awt and N2aAPP measured by Sandwich ELISA. n=3. Means±SD. *P<0.05, **P<0.01 vs N2awt. †P<0.05, ‡P<0.05 vs control cells.**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>Aβ40/µg·L\textsuperscript{-1}</th>
<th>In medium</th>
<th>In cell lysate</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2awt</td>
<td>Control</td>
<td>26.12±1.06</td>
<td>0.578±0.015</td>
<td></td>
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<tr>
<td></td>
<td>Melatonin-treated</td>
<td>26.00±0.92</td>
<td>0.569±0.039</td>
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<tr>
<td>N2aAPP</td>
<td>Control</td>
<td>47.95±1.73</td>
<td>3.403±0.382</td>
<td></td>
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<tr>
<td></td>
<td>Melatonin-treated</td>
<td>47.38±2.11</td>
<td>2.945±0.362</td>
<td></td>
</tr>
</tbody>
</table>

**Fig 1. Morphology of N2awt (A) and N2aAPP (B). Bar: 30 µm.**
dilution. Positive staining was only obtained when the dilution of antibodies was decreased to 1:500 in this cell line (Fig 3A-D). No NF was detected in N2aAPP even when dilution of the antibodies was 1:500 in Western blot (data not shown).

Melatonin inhibited Aβ overproduction and enhanced the immunoreactivity of NF In N2awt, no significant change was seen in the level of Aβ1-40 both in medium and in cell lysate after treatment of the cell with 10 µmol/L melatonin. In N2aAPP, remarkable reduction of Aβ level in cell lysate but not in culture medium was observed by melatonin (Tab 1).

Using SMI31, SMI32, SMI33, and SMI34, no obvious change in either staining pattern or strength was seen in N2awt by treatment with 10 µmol/L melatonin (Fig 2E-H). On the other hand, the immunoreactivity with the same panel of the antibodies was significantly enhanced in N2aAPP after treatment with melatonin (Fig 3E-H). However, no NF band could be detected by Western blot even after treatment with melatonin in N2aAPP (data not shown).

DISCUSSION

Although it has not been elucidated clearly about the etiology and pathophysiology of AD till now, abundant evidence suggest that abnormal process of cytoskeleton and Aβ deposition were both key events in AD. Is there any interaction between the two events or any cure to inhibit the lesion? To answer this question, we use cell line N2aAPP to explore the effect of overproduction of Aβ on cytoskeleton and search for desirable protective strategies.

We confirmed Aβ level in the cell lines used for

**Fig 2.** Effect of melatonin on NF. Under phase contrast microscope N2awt cells were stained by DAB. A, B, C, D: control group; E, F, G, H: treated group. A, E: SMI31; B, F: SMI32; C, G: SMI33; D, H: SMI34 diluted in 1:5000. Bar: 30 µm.

**Fig 3.** Effect of melatonin on NF. Under phase contrast microscope N2awtAPP cells were stained by DAB. A, B, C, D: control group; E, F, G, H: treated group. A, E: SMI31; B, F: SMI32; C, G: SMI33; D, H: SMI34 diluted in 1:500. Bar: 30 µm.
the study by Sandwich ELISA. It was demonstrated that not only in medium, but also in cell lysates, Aβ level in N2aAPP was much higher than that in N2awt. We observed a dramatic decrease of neurites both in number and in length in N2aAPP, which beared a significantly elevated Aβ level. Neurite outgrowth is associated closely with cytoskeleton protein, such as NF. NF is composed of a triplet protein, namely light chain (NF-L), middle chain (NF-M), and heavy chain (NF-H). It interacts with microtubule-associated proteins to maintain and stabilize structure of cytoskeleton, regulates axonal diameter, transportation, outgrowth and repairment[14]. In present study, overproduction of Aβ almost abolished the expression of NF in N2aAPP cells. Therefore, abnormality in NF may be one of the key mechanisms in AD neurofibrillary degeneration, and we have demonstrated for the first time overproduction of Aβ affects NF. In vivo studies based on this experiment will gain more insight into the relationship of abnormal process of cytoskeleton and Aβ deposition.

Increasing studies support the hypothesis that deficiency of melatonin may be critical for the development of AD. Melatonin is a pineal hormone participating in biologic modulation of mood, sleep, sexual behavior, reproductive alterations, and circadian rhythms. It is involved in aging and age-related diseases[10]. A selective impairment of the nocturnal melatonin secretion has been observed in elder subjects, being significantly related either to the age or to the severity of dementia[15]. In AD patients, a dramatic decrease of melatonin level was found, and sleep disruption, nightly restlessness, sun downing, and other circadian disturbances were also frequently seen[11]. Results from initial therapeutic trials of melatonin in AD patients have demonstrated a significant slowing of pathological progression[10]. We have also found recently that melatonin partially inhibits wortmannin-induced tau hyperphosphorylation in rat brain, and in neuroblastoma (SH-SY5Y) cell system the hyperphosphorylation/accumulation of neurofilament-(NF-) H/M subunits and the disruption of microtubules, induced by OA, were significantly inhibited by Mel[17,18]. Therefore, we chose melatonin in the present study to investigate its protective effect, if any, on Aβ-induced NF abnormality. Our data showed that melatonin with 10 µmol/L concentration (chosen by MTT assay) partially restored the immunoreactivity of both phosphorylated and non-phosphorylated NF. Early studies suggested that the neuroprotective property of melatonin probably related to its direct and/or indirect antioxidant activity[13,16]. The potential benefit mechanism of melatonin probably also stems from its function of antiamyloidogenesis besides its scavenger and antioxidant properties[10,11]. The present study suggests that melatonin may play a neuroprotective role through regulating the production of Aβ. Therefore, melatonin might be a potential therapeutic agent in AD.

REFERENCES