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Toxic effect of a β-amyloid peptide (β22–35) on the hippocampal neuron and its prevention

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A synthetic truncated β-amyloid peptide, β22–35, was shown to have a cytotoxic effect on cultured neurons from the rat hippocampus in serum-free medium. The peptide formed aggregates and typical amyloid fibrils resembling those of the β-amyloid protein (AP) in neutral buffer solution and showed characteristic staining with Congo red and thioflavin-S. The neurotoxicity of β22–35 was suppressed by addition of calf serum, dibutyryl cAMP or insulin to culture medium, but not by addition of NGF or substance P. β22–35 had no effect on the glial cells. These results suggest that the AP can induce neurotoxicity in the hippocampal cells in vitro and the toxicity may involve a disorder in the intracellular signal transduction.

Deposition of the β-amyloid protein (AP) in the core of the senile plaque is a major hallmark of Alzheimer's disease brain [5, 10, 18]. And overproduction [9, 22] and mutation [2, 4] of the AP precursor (APP) in the brain cells are suggested as the etiology to cause the pathogenic deposition of the AP. Therefore elucidation of the processes of the AP production from the large APP molecules and the toxic effect of the AP on neuronal cells is considered to be very important for understanding the pathology of AD.

Several authors have reported the biological [19, 20] and toxicological [14–16, 21, 22] activities of the AP or its amyloidogenic fragments in the culture of brain cells or cell lines transfected with portions of the APP gene. However, the results of experiments on the toxic effect on neuronal cells of the synthetic AP and shorter peptides are not consistent [7, 11]. We adopted in the present work β-amyloid peptides, β22–35 and its C-terminal amide, to examine the neurotoxicity of them in vitro. The newly synthesized peptide, β22–35, was expected to have appropriate solubility and hydrophobicity that are mainly ascribed to polar and non-polar amino acid chains at the N-terminus and C-terminal region, respectively, and maintain neurotoxicity [21].

Primary hippocampal cultures were obtained from 17- to 19-day-old embryonic rat cerebra as described previously [13]. Dissociated (with trypsin) hippocampal cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) precolostrum newborn calf serum in poly-D-lysine-coated 24-well culture plates (Falcon) at 37°C under a gas mixture of 95% air/5% CO_2. After 6 days of culture, non-neuronal cell division was halted by 24 h exposure to 10^{-5} cytosine arabinoside. After washing cell layers with DMEM, cells were cultured with 1 ml of DMEM per well in the absence of serum for 5 days before experiment. Cells were treated for 3 days with β22–35 or its amidated (C-terminus) derivative by addition of 20 μl of stock solution (2 mg/ml of either in betaine buffer, pH 8.5, consisting of 0.7% betaine and 20 mM NH_4HCO_3) to 1.0 ml medium, unless otherwise specified. Control cells were treated with the betaine buffer alone. Morphological change of cells was checked throughout the course of experiment with a phase-contrast microscope. Dead cells were checked by trypan blue staining. Cell injury was assessed by measuring lactate dehydrogenase (LDH) activity released into medium during 3 days of treatment of neurons with peptides. The enzyme activity in the culture medium (0.5 ml) was determined by the rate of decrease of absorption at 340 nm (ΔA/min) with NADH and pyruvate as substrates.

The peptides, β22–35 (NH_2-EDVGSNKGAIIGLM-COOH, the same amino acid sequence from 22 to 35 of the AP) and β22–35-NH_2 (C-terminal amidated derivative of β22–35), were synthesized by the solid-phase method starting from Boc-Met-Merrifield resin and benzhydrylamine, respectively, using a peptide synthe-

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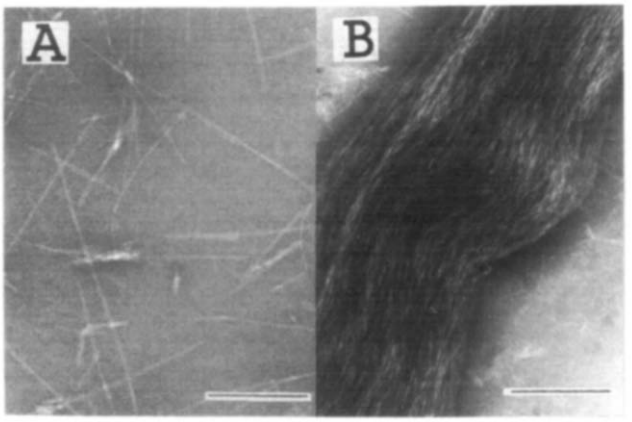

Fig. 1. Electron micrographs of the β22–35 peptide assemblies. The fibrous aggregates from betaine buffer were stained negatively with 4% (w/v) uranyl acetate or 2% (w/v) phosphotungstic acid after adsorption onto 180 mesh carbon-coated formvar grid and subjected to electron microscopic observation using JEM12EX2 at 100 kV. A: fibrils of β22–35. B: fibrous bundles. Bars = (A) 200 nm; (B) 500 nm.

sizer (Beckman Instruments, Ltd., model 990c). After HF treatment and deprotection, the crude product was purified by preparative reversed-phase HPLC connected to a column of μ-Bondasphere 5μ C_8-100A. Purity of the peptides was confirmed by amino acid analysis, analytical reversed-phase HPLC on the YMC-Pack ODS-AM column and high performance TLC of their acid hydrolysates. The products were stored at 2 mg/ml in the betaine buffer, seemingly partly solubilized. The stock mixture of β22–35 and betaine buffer exhibited aggregates forming very fine floating particles (solubilized 80% or less according to amino acid assay of the ultracentrifugation supernatant).

Electron microscopy of the aggregates revealed that β22–35 can assemble in straight fibrils with the diameter of approximately 5–10 nm and variable lengths (Fig. 1A). A part of these fibrils made large bundles resembling those of the senile plaque cores (Fig. 1B). The bundled fibrils showed green fluorescence on binding thioflavin-S and green birefringence on staining with Congo red under excitation (IMT2-DMB, Olympus) and polarized light, respectively (data not shown), as described for the senile plaque. β22–35-NH_2 also showed aggregates in the betaine buffer. The precipitates had the characteristic fluorescence after thioflavin-S treatment, but a different image of optical microscopy from that of β22–35; aggregates of entangled fibrils. We eliminated calf serum from culture medium to examine cytotoxic effect of β22–35 on the hippocampal neurons 6 days after plating the cells and for additional 5 days before experiment, because β22–35 had no effect on the cells in the presence of serum in medium for 14 days of culture.

The addition of β22–35 (40 μg/ml) exhibited a remarkable cytotoxicity without serum when the neurons were observed every day by the phase-contrast microscopy and stained with trypan blue in 3 days of culture (Fig. 2). Fragmentation of the neurites and shrinkage of both the cell body and nucleus were observed within 3 days of culture with β22–35. The neurotoxicity of the peptide was confirmed by measurement of proportion of the release of lactate dehydrogenase from the cells. β22–35 increased the release of the enzyme in a dose-dependent manner (10–40 μg/ml), whereas its amidated derivative was significantly injurious on the cells only at the highest concentration in the same range of concentration (Fig. 3). The neurotoxicity induced by 40 μg/ml β22–35 was attenuated or cancelled by the presence of dialyzed-calf serum (1%, v/v), insulin (0.2 mg/ml) or dibutyryl cAMP (1 mM) (Fig. 4). Dibutyryl cAMP plus forskolin (10 μM) was more effective than dibutyryl cAMP only. On the other hand, NGF (0.1 μg/ml), substance P (0.1 mM) and glutamate antagonists (10 μM MK-801 and 20 μM CNQX) did not prevent the toxicity induced by β22–35.

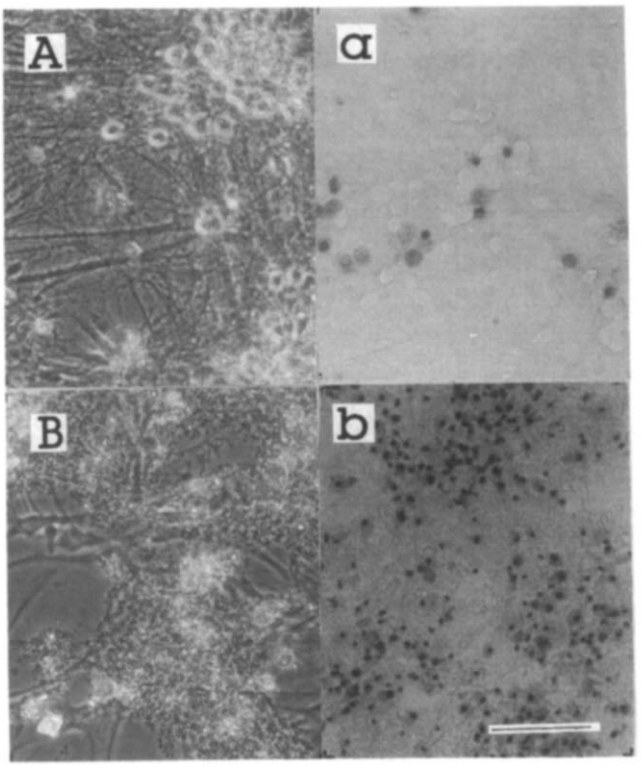

Fig. 2. Morphological change and death of the rat hippocampal neurons after β22–35 treatment. Cells (B and b) were treated for 3 days with β22–35 by addition of 20 μl of the stock mixture (2 mg/ml of β22–35 in betaine buffer (0.7% betaine and 20 mM NH_4HCO_3) pH 8.5) to 1 ml medium. Control cells (A and a) were treated with the betaine buffer alone. They were incubated at 37°C under a gas mixture of 95% air/5% CO_2. Morphological change of cells was checked throughout the course of experiment on phase-contrast microscopy (A and B). Cell viability was finally tested by Trypan blue staining (a and b). Specimens were all at 3 days of treatment. Bar = 100 μm.

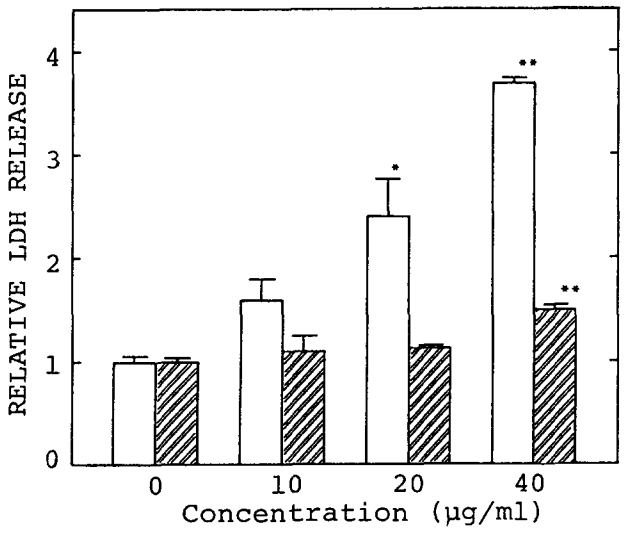

Fig. 3. Concentration dependence of the neurotoxicity of β22–35 and β22–35-NH_2. Cell injury was assessed by measuring of lactate dehydrogenase (LDH) activity released into the medium 3 days after treatment with varying concentrations of β22–35 (blank bar) or β22–35-NH_2 (hatched bar) up to 40 μg/ml. Each value represents the mean±S.E.M. of 3 sister cultures. $^{*}P < 0.05$, $^{**}P < 0.01$ (vs. no addition).

β22–35 seemed not to elicit any toxic effect on the glial cells as examined morphologically and in the LDH release (data not shown).

Yankner et al. [21] first reported that β1–38, β1–40 and β25–35 had cytotoxicity to the cultured hippocampal neuron at 4 days after cell plating (mature neurons) and it was antagonized by substance P (SP), whereas several authors demonstrated that the AP did not interact with the SP receptor [8, 12]. We could not show the inhibition of the neurotoxicity of β22–35 by SP (Fig. 4). Pike et al. [14] demonstrated the neurotoxicity of the aggregated β1–42 peptide to the hippocampal cells 1 day after culture.

Two synthetic β-amyloid peptides, β1–28 and β12–28, were shown to form fibrils (8–10 nm wide) and their bundles had a similar electron-microscopic appearance in distilled water or in saline as the extracted amyloid core proteins [3]. It has been claimed on the basis of the neurodegeneration experiment performed with aged (incubated for 7 days at 37°C in stock solution) and new peptides (β1–42, β25–35, β1–28, etc.) that the aggregated state of the peptide plays a key role in expressing neurotoxic effect of them [15]. β25–35 formed large sheet-like precipitates (far less soluble than β22–35) in the betaine buffer and culture medium as observed under light microscopy and actually showed a considerable neurotoxicity in the hippocampal neurons in our experimental condition (data not shown). Both β22–35 and β22–35-NH_2 were partially soluble in buffer solution in our preparations, but they were different in morphology of their precipitated aggregates with different activity on the hippocampal neurons in vitro.

How the AP and related peptides interact with neuronal cells and affect them is not so far explainable. The peptides may exert their neurotoxic effect through interaction with a receptor such as the serpin-enzyme complex receptor [6] or by forming calcium channels in the plasma membrane [1]. Our experimental results suggest that the β-amyloid peptide interacts with a group of specific cell membrane sites that is related to metabotrophic signals capable of being regulated by insulin and cAMP, and accelerates the death of neurons. It is a very interesting finding in this regard that the apoptotic cell death of PC12 cell is induced by depletion of serum from culture medium and can be rescued by stimulation of the multiple pathways including cAMP, insulin and basic fibroblast growth factor [17]. It is also plausible that the AP interrupts in cells a trophic signal transduction which is essential to survival of neurons in cooperation with other apoptotic factor(s).

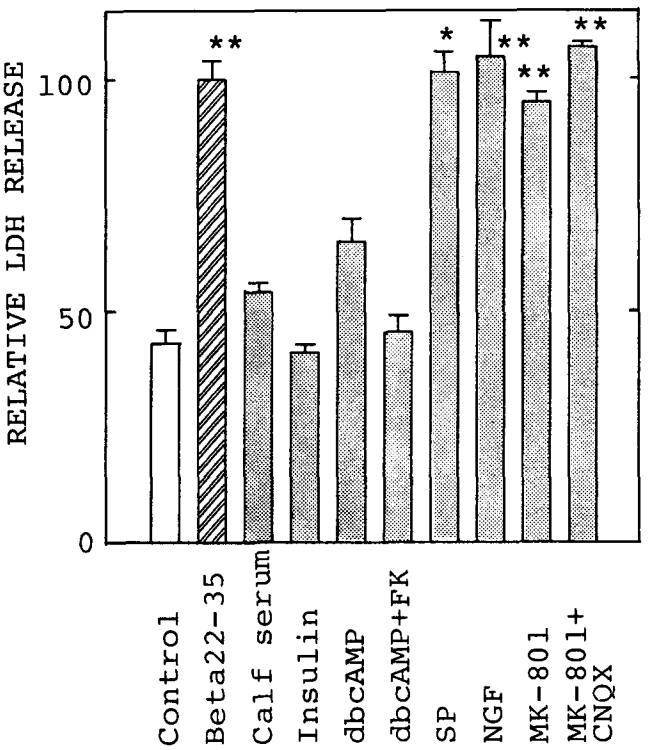

Fig. 4. Effects of various agents on the neurotoxicity induced by β22–35. Hippocampal neurons were cultured with 40 μg/ml of β22–35 alone or in the presence of 1% calf serum, 0.2 mg/ml insulin, 1 mM dibutyryl cAMP, 1 mM dibutyryl cAMP+10 μM forskolin (FK), 0.1 mM substance P, 100 ng/ml NGF, 10 μM MK-801 or 10 μM MK-801+20 μM CNQX as described in the legend to Fig. 2. Control was with betaine buffer only. Cell injury was evaluated by LDH release. Each value represents the mean ± S.E.M. of 3 sister cultures. $^{*}P < 0.05$, $^{**}P < 0.01$ (vs. control).

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1 Arispe, N., Rojas, E. and Pollard, H.B., Alzheimer disease amyloid β protein forms calcium channels in bilayer membranes: Blockade by tromethamine and aluminum, Proc. Natl. Acad. Sci. USA, 90 (1993) 567–571.

2 Cai, X.-D., Golde, T.E. and Younkin, S.G., Release of excess amyloid β protein from a mutant amyloid β protein precursor, Science, 259 (1993) 514–516.

3 Castano, E.M., Ghiso, J., Prelli, F., Gorevic, P.D., Migheli, A. and Frngione, B., In vitro formation of amyloid fibrils from two synthetic peptides of different lengths homologous to Alzheimer's disease β-protein, Biochem. Biophys. Res. Commun., 141 (1986) 782–789.

4 Citron, M., Oltersdorf, T., Haass, C., McConlogue, L., Hung, A.Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I. and Selkoe, D.J., Mutation of the β-amyloid precursor protein in familial Alzheimer's disease increases β-protein production, Nature, 360 (1992) 672–674.

5 Glenner, G.G. and Wong, C.W., Alzheimer's disease: Initial report of the purification and characterization of a novel cerebrovascular amyloid protein, Biochem. Biophys. Res. Commun., 120 (1984) 885–890.

6 Joslin, G., Krause, J.E., Hershey, A.D., Adams, S.P., Fallon, R.J. and Perlmutter, D.H., Amyloid-β peptide, substance P and bombesin bind to the serpin-enzyme complex receptor, J. Biol. Chem., 266 (1991) 21897–21902.

7 Koh, J.-Y., Yang, L.L. and Cotman, C.W., β-Amyloid protein increases the vulnerability of cultured cortical neurons to excitotoxic damage, Brain Res., 533 (1990) 315–320.

8 Kozlowski, M.R., Spanoyannis, A., Manly, S.P., Fidel, S.A. and Neve, R.L., The neurotoxic carboxy-terminal fragment of the Alzheimer amyloid precursor binds specifically to a neuronal cell surface molecule: pH dependence of the neurotoxicity and the binding, J. Neurosci., 12 (1992) 1679–1687.

9 Maruyama, K., Terakado, K., Usami, M. and Yoshikawa, K., Formation of amyloid-like fibrils in COS cells overexpressing part of the Alzheimer amyloid protein precursor, Nature, 347 (1990) 566–569.

10 Masters, C.L., Multhaup, G., Simms, G., Pottgiesser, J. Martins, R.N. and Beyreuther, K., Amyloid plaque core protein in Alzheimer disease and Down syndrome, Proc. Natl. Acad. Sci. USA, 82 (1985) 4245–4249.

11 Mattson, M.P., Cheng, B., Davis, D., Bryant, K., Leieberburg, I. and Rydel, R., β-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity, J. Neurosci., 12 (1992) 376–389.

12 Mitsuhashi, M., Akitaya, T., Turk, C.W. and Payan, D.G., Amyloid β protein substituent peptides do not interact with the substance P receptor expressed in cultured cells, Mol. Brain Res., 11 (1991) 177–180.

13 Ohkubo, T., Mitsumoto, Y. and Mohri, T., Characterization of the uptake of adenosine by cultured rat hippocampal cells and inhibition of the uptake by xanthine derivatives, Neurosci. Lett., 133 (1991) 275–278.

14 Pike, C.J., Walencewicz, A.J., Glabe, C.G. and Cotman, C.W., In vitro aging of β-amyloid protein causes peptide aggregation and neurotoxicity, Brain Res., 563 (1991) 311–314.

15 Pike, C.J., Burdick, D., Walencewicz, A.J., Glabe, C.G. and Cotman, C.W., Neurodegeneration induced by β-amyloid peptide in vitro: the role of peptide assembly state, J. Neurosci., 13 (1993) 1676–1687.

16 Roher, A.E., Ball, M.J., Bhave, S.V. and Wakade, A.R., β-Amyloid from Alzheimer disease brains inhibits sprouting and survival of sympathetic neurons, Biochem. Biophys. Res. Commun., 174 (1991) 572–579.

17 Rukenstein, A., Rydel, R.E. and Greene, L.A., Multiple agents rescue PC12 cells from serum-free cell death by translation- and transcription-independent mechanisms, J. Neurosci., 11 (1991) 2552–2563.

18 Selkoe, D.J., Abraham, C.R., Podlisny, M.B. and Duffy, L.D., Isolation of low-molecular-weight proteins from amyloid plaque fibers in Alzheimer's disease, J. Neurochem., 46 (1986) 1820–1834.

19 Whitson, J.S., Selkoe, D.J. and Cotman, C.W., Amyloid β protein enhances the survival of hippocampal neurons in vitro, Science, 243 (1989) 1488–1490.

20 Whitson, J.S., Glabe, C.G., Shintani, E., Abcar, A. and Cotman, C.W., β-Amyloid protein promotes neuritic branching in hippocampal cultures, Neurosci. Lett., 110 (1990) 319–324.

21 Yankner, B.A., Duffy, L.K. and Kirschner, D.A., Neurotrophic and neurotoxic effects of amyloid β protein: reversal by tachykinin neuropeptides, Science, 250 (1990) 279–282.

22 Yoshikawa, K., Aizawa, T. and Hayashi, Y., Degeneration in vitro of post-mitotic neurons overexpressing the Alzheimer amyloid protein precursor, Nature, 359 (1992) 64–67.