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21. We thank S. Holbrook for help in calculating the molecular volumes used herein. Supported by NIH grant GM35393. M.D.T. was supported in part by NIH training grant GM07232.

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Amyloid β Protein Enhances the Survival of Hippocampal Neurons in Vitro

JANET S. WHITSON, DENNIS J. SELKOE, CARL W. COTMAN*

The β-amyloid protein is progressively deposited in Alzheimer's disease as vascular amyloid and as the amyloid cores of neuritic plaques. Contrary to its metabolically inert appearance, this peptide may have biological activity. To evaluate this possibility, a peptide ligand homologous to the first 28 residues of the β-amyloid protein (β1-28) was tested in cultures of hippocampal pyramidal neurons for neurotrophic or neurotoxic effects. The β1-28 appeared to have neurotrophic activity because it enhanced neuronal survival under the culture conditions examined. This finding may help elucidate the sequence of events leading to plaque formation and neuronal damage in Alzheimer's disease.

IN ALZHEIMER'S DISEASE (AD), INSOLUBLE fibrils of β-amyloid (A4) protein accumulate in cerebral blood vessels (*1*) and in neuritic plaques (*2, 3*). Within the plaques, found in various regions of the cerebral cortex and in the hippocampus (*4, 5*), a central core containing primarily β-amyloid is surrounded by a spherical cluster of dystrophic neurites and glial cells (*5*). Although the brains of normal elderly adults exhibit neuritic plaques with their associated amyloid deposits, the density of the lesions is much greater in AD and, indeed, is a pathological marker by which diagnosis is confirmed (*5*). Thus, β-amyloid deposition could be thought of as a primary event in the pathological cascade of AD. It is also possible, however, that β-amyloid itself may constitute part of a reactive, albeit aberrant, plasticity response to the neuronal loss that accompanies the disorder. The possibility that the AD brain is capable of compensatory responses has been suggested from studies in which rats given lesions of the entorhinal cortex (to mimic the pathology observed in this region in AD) exhibited sprouting of neurites positive for acetylcholinesterase (AChE) in the outer molecular layer of the dentate gyrus. Detailed characterization of human hippocampal pathology in AD has revealed a similar sprouting response and has shown that AChE-positive neuritic plaques also accumulate in that dentate layer (*6*). Thus, Geddes and others have indicated the possible preferential location of neuritic plaques in regions of active sprouting and have proposed that an initial stage of neuritic plaque formation may be an aberrant sprouting response caused by local injury-induced increases in neurotrophic activities (*6*). As the direct effects of β-amyloid protein on brain cells have not been determined, questions of the trophic or toxic properties of β-amyloid remain unresolved.

In the present study we examined the effect of a synthetic β-amyloid peptide (β1-28) on cell survival and neuritic outgrowth in primary cultures of hippocampal neurons. The β1-28 peptide (*7*) consists of the first 28 residues of the β-amyloid protein (estimated to be 40 to 42 residues in all) and is thought to represent a portion of the extracellular domain of the amyloid precursor (*8, 9*).

In initial experiments, β1-28 was added to the medium in which embryonic rat hippocampal cells were plated and grown for 2 days. In these low-density cultures, the numbers of surviving cells was quantitated on day 2 as a percentage of the number of live cells counted 1 hour after plating. Cultures were treated with β1-28 (0, 10, and 100 μg/ml) in four separate preparations and triplicate wells were used for each concentration (Fig. 1). In the absence of β-amyloid peptide, neuronal survival at the end of 2 days was only 36% (35.6 ± 6.4). When β1-28 was included in the medium at 10 and 100 μg/ml, these values increased to 60% and 90%, respectively (60.2 ± 9.6, 90.1 ± 12.2: $P = 0.0016$, $n = 12$ ± SEM). To assess the time course of this survival effect we prepared cultures and determined cell survival after 1, 3, 5, or 9 days (Fig. 2). The percentage of neurons sustained by β1-28 at day 1 was nearly 100% (compared to about 25% without β1-28), but dropped sharply between days 3 and 5. By days 7 and

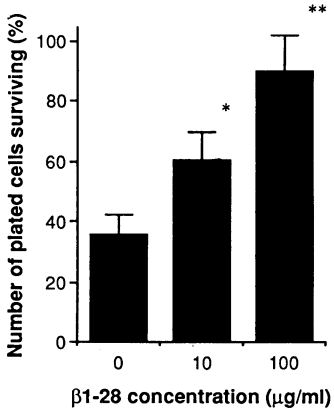

Fig. 1. Survival of hippocampal neurons as a function of β1-28 concentration. Hippocampal cells from embryonic day 18 Sprague-Dawley rats were plated in polylysine-coated 24-well culture plates (*10*). β1-28 was included in Brewer's B18 serum-free culture media (*10*) when cells were plated. After 2 days, cells were counted and survival calculated as a percentage of day 0 plating counts [$^{**}P < 0.01$, $^{*}P < 0.09$, Fisher probable least-squares difference (PLSD)]. Values are mean ± SEM; $n = 12$. Analysis of variance (ANOVA): $F(2,35) = 7.854$, $P < 0.002$.

J. S. Whitson and C. W. Cotman, Department of Psychobiology, University of California at Irvine, Irvine, CA 92717.
D. J. Selkoe, Department of Neurology (Neuroscience), Harvard Medical School, Boston, MA 02115 and Center for Neurologic Diseases, Brigham and Women's Hospital, Boston, MA 02115.

*To whom correspondence should be addressed.

9, the percentage of surviving cells in cultures given β1-28 had decreased to the level found in cultures grown without added peptide.

It is unlikely that increased neuronal survival was a nonspecific response to the addition of small amounts of protein, because the medium used in these studies contained 0.25% bovine serum albumin (*10*). Nevertheless, as a further control experiment, a peptide of similar length to β1-28, the gastrointestinal peptide secretin, was tested in hippocampal cultures at concentrations identical to those used previously for β1-28. After 2 days in vitro, the percentages of surviving neurons in the secretin-treated cultures were no different from those in control cultures (42.4 ± 13.8%, 39.0 ± 14.8%, and 26.3 ± 6.6% for 0, 10, and 100 μg secretin per milliliter, respectively; $n = 9$ ± SEM). This indicates that the increased neuronal survival observed in cultures treated with β1-28 was caused by that peptide or some portion of its sequence.

A peptide differing from β1-28 by only one amino acid was used to characterize further the specificity of the β-amyloid peptide's trophic action. Previous studies had shown that the substitution of alanine for lysine at position 16 of β1-28, producing β1-28 (Ala^{16}), was sufficient to change the tertiary structure of the peptide and to alter its aggregation properties (*7*). When tested in our hippocampal cultures, β1-28 (Ala^{16}) and β1-28 produced identical effects throughout the time course of the experiment (Fig. 3). This finding suggests that the trophic properties of β1-28 are not dependant specifically on its β-pleated sheet characteristics or its form of aggregation. Thus, the trophic activity of β1-28 appears to reside in a discrete sequence of the peptide. In vivo, the secondary structure of this region is likely to be influenced by the remainder of the β-amyloid protein sequence in ways that could diminish or enhance the trophic activity we observed.

Next, the possible effect of β1-28 on neuronal attachment was checked by plating the neurons on culture dishes that had regions to which β1-28 had been adsorbed (*11*). No difference was observed in either attachment density or neurite extension when we compared the neurons grown on the polylysine or polylysine plus β1-28 regions. To further investigate the possible neurite-promoting effects of β1-28, we quantitated neurite extension in cultures containing peptide concentrations of 0, 10, or 100 μg/ml (Fig. 4). The inclusion of β1-28 in the culture medium did not alter the number of neurites originating from the cell soma. Although 100 μg of β1-28 per milliliter did increase neuritic sprouting as measured by the number of branch points, these changes were not statistically significant (Fig. 4). Similarly, β1-28 treatment led to an increase in cumulative neurite length, but this effect did not reach significance under the conditions of our experiment.

Given the presence of β-amyloid in the cores of neuritic plaques and our demonstration of the peptide's ability to enhance neuronal survival, it might be expected that β1-28 should also affect neurite outgrowth. The inability to detect subtle changes in neurites may be attributable to (i) culture conditions in which 98% of living control neurons elaborated neurites, thereby masking the β1-28 effect; (ii) a selective effect of β1-28, which may induce sprouting in only a small subset of the hippocampal neuronal population; or (iii) insufficient sensitivity of the methods used to quantitate higher order branch points. It is more likely, however, that sprouting such as that seen in neuritic plaques in human cerebral cortex requires the interaction of several components. These might include high local concentrations of trophic substances (including β-amyloid protein), special substrates, protease inhibitors, or a combination of these features.

The trophic activity of β1-28 reported here should be considered in relation to the physiological role of the β-amyloid precursor protein (βAPP) as well as the part played by β-amyloid in AD. βAPP is the product of a gene on human chromosome 21 (*12, 13*) which, probably through alternative mRNA splicing, forms at least three varieties of βAPP mRNA (*14*). These all encode the β-amyloid protein, but one variety lacks an insert encoding a Kunitz-type serine-protease inhibitor (function unknown), whereas

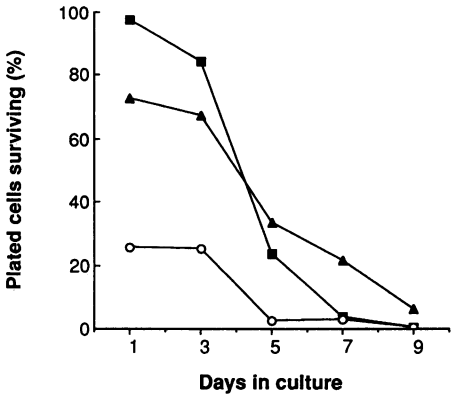

Fig. 2. Time course of neuronal survival enhancement by β1-28. Values are the mean of triplicate wells from a representative preparation counted after the indicated number of days in vitro. ■, 100 μg/ml; ▲, 10 μg/ml; and ○, 0 μg/ml.

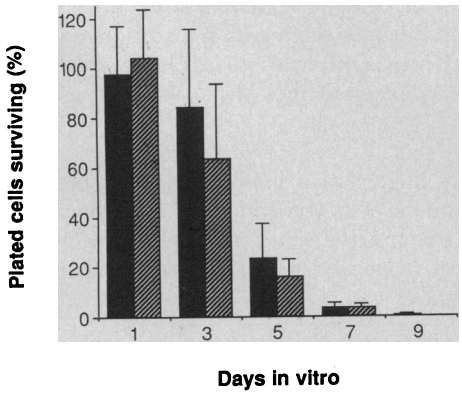

Fig. 3. Comparison of neuronal survival in the presence of β1-28 or β1-28 (Ala^{16}) counted after the indicated number of days in vitro. Values shown are for peptide concentrations of 100 μg/ml added at time of plating (mean ± SEM; $n = 3$). Solid bars, β1-28; hatched bars, β1-28 (Ala^{16}).

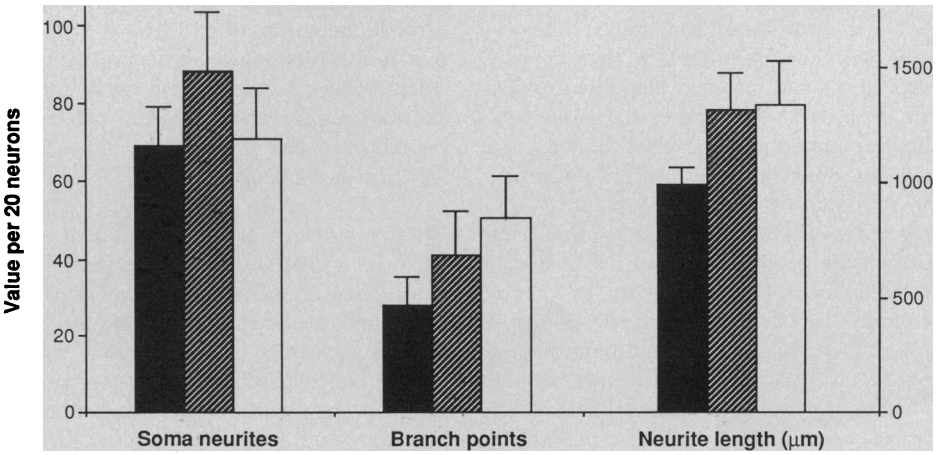

Fig. 4. The effect of β1-28 on the number of neurites projecting from the soma, the number of branch points present, and the total neuritic length. Cultures were fixed at day 2 in 2 to 4% paraformaldehyde and silver-stained (*17*). The first 20 neurons with processes seen in each well were photographed. The number of neurites and branch points were counted from tracings of projected negatives. Neurite length was measured from the same tracings with the use of an Apple IIe graphics tablet. Values are mean ± SEM; differences are not significant; $P > 0.2$ in all cases. Solid bar, 0 μg/ml; hatched bars, 10 μg/ml; and blank bar, 100 μg/ml.

the other two varieties include the Kunitz-insert at nucleotide 865. βAPP is now known to occur physiologically as a group of 110- to 135-kD membrane-associated proteins in brain and other tissues (*3*). Prediction of its amino acid sequence based on cDNA clones indicates the molecule possesses a large extracellular domain, a single membrane-spanning region, and a small cytoplasmic moiety (*8*). βAPP is not homologous to any known protein and its post-translational modifications or processing, if any, as well as its function in vivo are unknown (*14*).

On the basis of immunohistochemical elucidation of binding sites for a portion of β-amyloid and on structural similarities between βAPP and the epidermal growth factor precursor, it has been suggested that the βAPP may be processed to release an active peptide ligand (*15*) having regulatory properties, that is, a neurotrophic factor or hormone. According to this model, abnormal processing of βAPP may result in the formation of amyloid fibrils or in the production of an altered inactive ligand. Our results suggest a possible physiological trophic function for the βAPP ligand in, but not necessarily limited to (*15*), the central nervous system. The concentrations of β1-28 that produced neurotrophic effects here are higher than those required for well-characterized trophic factors such as nerve growth factor, which is typically added to cultured neurons at low concentrations (nanograms per milliliter). However, because β1-28 may be expected to represent only a fragment of any active physiological ligand formed from βAPP, it may lack both primary sequences and tertiary structures that enhance binding. It is also possible that high local concentrations of the physiological ligand occur normally in brain tissue, thus showing similarity to the in vitro situation described here.

A working hypothesis is that amyloid deposition and neuritic plaque formation are linked to a regenerative response of the brain mounted in an attempt to ameliorate the denervation or some cellular abnormality associated with AD. Evidence for such a regenerative response is found in the recent demonstration that AD brain tissue extract exhibits more trophic activity in cortical cultures than normal brain extract (*16*). Specifically, we suggest that the physiological product of βAPP may perform a trophic function supportive of normal brain plasticity and, when altered by aberrant processing, may be precipitated as amyloid in an inactivated or inaccessible form. If the βAPP product is normally retrogradely transported from target cells to the cell bodies of neurons specifically dependent on it (as is the case with nerve growth factor), aberrant processing could result in the transport of a product lacking full trophic activity. Thus, cells dependent on βAPP product for trophic support would die. Alternatively, β-amyloid and the proteins known to be associated with it in AD (for example, α1-antichymotrypsin and others) could serve as the initial nucleus for a neuritic trophic response, yielding plaques. The demonstration of biological trophic activity for the β1-28 portion of β-amyloid points to new avenues of research and may open the door to new therapeutic approaches.

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Control of Angiogenesis with Synthetic Heparin Substitutes

JUDAH FOLKMAN,* PAUL B. WEISZ, MADELEINE M. JOULLIÉ, WILLIAM W. LI, WILLIAM R. EWING

Many diseases are dominated by persistent growth of capillary blood vessels. Tumor growth is also angiogenesis-dependent. Safe and effective angiogenesis inhibitors are needed to determine whether control of angiogenesis would be therapeutic. Heparin and certain steroids, administered together, can inhibit angiogenesis in a synergistic manner. This "pair" effect suggested that specific hydrophilic cycloamyloses may be suitable heparin substitutes. β-Cyclodextrin tetradecasulfate administered with a steroid inhibits angiogenesis at 100 to 1000 times the effectiveness of heparin in the chick embryo bioassay. This cyclic oligosaccharide also augments the anti-angiogenic effect of angiostatic steroids against corneal neovascularization in rabbits when β-cyclodextrin tetradecasulfate and a steroid are inserted into the cornea or applied topically as eyedrops.

PERSISTENT CAPILLARY BLOOD VESSEL growth is often associated with disease, such as diabetic retinopathy, neovascular glaucoma, rheumatoid arthritis, and hemangiomas (*1*). Progressive tumor growth and metastases also appear to depend on angiogenesis (*2, 3*). In contrast, angiogenesis is not usually active in the normal adult except during wound repair, ovulation, menstruation, and the formation of the placenta. Therefore, the potential therapeutic benefit of controlling pathologic angiogenesis has led to a search for reliable and effective angiogenesis inhibitors.

Angiogenesis is inhibited when heparin, or one of its non-anticoagulant fragments, is administered simultaneously with a steroid of specific structure (for example, an angio-

J. Folkman, Departments of Surgery and Anatomy and Cellular Biology, Harvard Medical School and Children's Hospital, Boston, MA 02115.
P. B. Weisz, Departments of Chemical and Bio-Engineering, University of Pennsylvania, Philadelphia, PA 19100.
M. M. Joullié and W. R. Ewing, Department of Chemistry, University of Pennsylvania, Philadelphia, PA 19100.
W. W. Li, Department of Surgery, Children's Hospital, Boston, MA 02115.

*To whom correspondence should be addressed.