5-4-1994

Intercellular Interactions in PC12 Cells Overexpressing Beta/A4 Amyloid

Gladys E. Maestre
Harvard Medical School

Barbara A. Tate
Brown University

Ronald E. Majocha
Brown University

John Maguire
McMaster University, Ontario

Charles A. Marotta
Brown University

Utah State University
MERRILL-CAZIER LIBRARY

INTERCELLULAR INTERACTIONS IN PC12 CELLS OVEREXPRESSING BETA/A4 AMYLOID

Gladys E. Maestre[1,*], Barbara A. Tate[2], Ronald E. Majocha[2,4], John Maguire[3], and Charles A. Marotta[2,4]

[1]Department of Psychiatry and Neuroscience Program, Harvard Medical School, Boston, MA 02115
[2]Dept. Psychiatry and Human Behavior, and [4]Dept. Neuroscience, Brown University, Providence, RI 02912
[3]Dept. Pathology, Hamilton General Hospital, and McMaster Univ. Medical Centre, Ontario Canada

Abstract

The amyloid precursor protein (APP) is an integral membrane component of eukaryotic cells. A variety of research approaches have addressed the contribution of the β amyloid peptide region of the APP to neuritic plaque structure and formation in the Alzheimer disease brain as well as the relationship between β amyloid accumulation and the occurrence of dementia. However, there is limited information available concerning the cellular consequences of amyloid deposition. The present studies were undertaken to investigate the relationship between β amyloid and intercellular junctions. Transfected PC12 cell lines, that overexpress the β amyloid peptide, exhibit structural and functional alterations at the cell surface and tend to form aggregates more readily than normal control cells. Intermediate junctions were the most common intercellular interactions of both normal and transfected cells. However, the control and transfected cells differed since areas of continuous and extensive junctions were readily seen in transfected cells and infrequently seen in control cells. The data suggest that excess accumulation of β amyloid is associated with the junctional apparatus and may be related to increased intercellular adhesion.

*Present address and address for correspondence:
Gladys E. Maestre,
Center for Neurobiology and Behavior,
Department of Pathology, 15-402,
College of Physicians and Surgeons,
630 W. 168th Street,
Columbia University,
New York, NY 10032

Telephone number: 212-305-2017
FAX number: 212-305-5498

Introduction

Alzheimer's disease (AD) is the most common of the dementing illnesses leading to impaired intellectual, occupational and social performance (Katzman, 1986). It is the most common form of adult-onset dementia and is the fourth leading cause of death in the United States (Hay and Ernst, 1987). Among the neuropathological changes found in the brain, high concentrations of senile plaques containing β amyloid are observed in cerebral cortex, subcortical structures, and cerebellum. β amyloid is composed of fibrils of 4-8 nm diameter. The protein forms the cores of plaques and is found in congophilic angiopathy of Alzheimer brain (Glenner et al., 1981; Masters et al., 1985).

Beta amyloid is a peptide of approximately 4 kDa derived from a much larger glycoprotein, the amyloid precursor protein (APP) (Dyrks et al., 1988; Esch et al., 1990; Kang et al., 1987). The latter exists in multiple transmembrane forms (APP_{695}, APP_{751}, APP_{770}) that result from alternative splicing of primary transcripts of a single precursor gene (Kitaguchi et al., 1988). Fragments of APP containing the β amyloid peptide have been found in normal brains (Ghiso et al., 1992) and cerebrospinal fluid (Palmert et al., 1990), suggesting that β amyloid has a normal but unknown role. Determination of the function of the β amyloid peptide may help to clarify the mechanism of formation and the consequences of amyloid deposition that occurs specifically in the AD brain.

There is evidence to suggest that APP plays a role in cell-cell and cell-matrix interactions (Breen et al., 1991; Chen and Yankner 1991; de Sauvage and Octave, 1989; Dyrks et al., 1988; Klier et al., 1990; Shivers et al., 1988; Ueda et al., 1989). This putative function appears to be due, at least in part, to an amino acid sequence located within the β amyloid peptide (Chen and Yankner, 1991; Ghiso et al., 1992). However, it is not known how β amyloid is related to the organization of cellular and membranous molecules that are responsible for maintenance of the cellular matrix and intercellular junctions.

Interactions between cells may influence their viability and functional roles within tissues (Bissell and Barcellos-Hoff, 1978; Farmer *et al.*, 1978; Singer, 1992; Vasiliev, 1985). Changes in inter-neuronal associations, the relationships between neurons and other cell types, and their relationship to the microenvironment are implicated in brain aging (Vernadakis, 1985). Modifications in membranous structures including synaptic and non-synaptic junctions (Terry *et al.*, 1991), which may require cell adhesion molecules, may be prerequisites for the acquisition of memory (Bailey and Kandel, 1993). The role, if any, of the transmembrane amyloid precursor protein and the β amyloid peptide in modulating cellular interactions is unknown.

To begin to explore the relationship between β amyloid and cell-cell interactions, we examined the morphological consequences of overexpressing the human β amyloid-C-terminal peptide in rat pheochromocytoma (PC12) cells (Maestre *et al.*, 1992a,b, 1993; Majocha *et al.*, 1993; Tate *et al.*, 1991). PC12 cells were permanently transfected with human amyloid precursor protein (APP) DNA that codes for 97 amino acids. These represent 40 β amyloid residues followed by the remainder of the C-terminal region of the APP (Marotta *et al.*, 1989).

In the present work, the cells were characterized by light (LM) and transmission electron microscopy (TEM) with immuno-staining. By these approaches, we determined whether or not contacts established by cells that overexpress β amyloid were uniquely different from those formed by control cells.

Materials and Methods

Preparation of transfected cells

The initial cloning vehicle was an SV40-based vector, Pko+RI/ML, composed of PML_2, a derivative of PBR322 (lacking certain prokaryotic sequences poisonous for eukaryotic cell replication), the Lac UV_5 promotor of *E. coli*, and SV40 sequences covering the enhancer, origin of replication, early promotor, t/T antigen splice sites and polyadenylation sites. Modification of the initial vector was carried out to produce three variants, Min +1, +2 and +3, with three different translational reading frames utilizing the ATG codon of the T/t antigen (Marotta *et al.*, 1989). The starting vector or modified forms were used for experimentation. The precursor to the Min series contained a unique PvuII site (enhancer start) and a Bam HI site (poly A addition site), both of which were modified to XbaI sites by standard techniques.

From an AD brain complementary DNA (cDNA) expression vector library prepared with bacteriophage lambda, we obtained an insert, referred to as amy37,

that included the A4 sequence and the flanking regions (Zain *et al.*, 1988). The Min vector constructs were used for insertion of the EcoRI digested amy37 cDNA fragment into the three translational reading frames. Vectors were digested with EcoRI restriction endonuclease, to cleave at the unique EcoRI site, and with alkaline phosphatase. The lambda gt11-amy37 chimera (Zain *et al.*, 1988) was digested with EcoRI enzyme, and the 1.1 kb long fragment, containing the A4 site, was isolated. The 1.1 kb fragment was ligated into the Min vectors by established techniques. The amy37-1.1 kb chimeric plasmids generated separately in the three reading frames were propagated; the DNA was isolated, purified, and used for transfection experiments (see below). Sequence analyses were carried out as described elsewhere (Zain *et al.*, 1988). Earlier transfections were applied to COS-1, CV-1, HS683, SK-N-SH and A172 cell lines. The cells used in the present studies were PC12 cells, derived from rat adrenal pheochromocytoma obtained from Dr. Itzak Fisher (Medical College of Pennsylvania, Philadelphia).

Permanent transfection experiments were conducted using the general procedures of van der Eb and Graham (1980). Integration of the 1.1 kb amyloid cDNA insert was carried out as described above except that the transfection medium contained 10 μg of vectors with amy37-1.1 inserts. Controls consisted of vector DNA without an amyloid cDNA insert. Five μg PSV_2CAT DNA (the chloramphenicol acetyltransferase gene cloned into an SV_{40} based plasmid), and 5 μg of Pko+Neo plasmid DNA (in an SV_{40} based plasmid) was used. The latter carried the gene for Neomycin resistance that was sensitive to Geneticin. CAT (chloramphenicol acetyl transferase) assays to assess transfection efficiency were carried out according to Gorman *et al.* (1982). The various transfectants were selected for survival in the presence of Geneticin (G418, GIBCO) at a concentration of 0.4 gm/l for 6 days, and then at 0.3 gm/l for 3 days; the cells were subsequently maintained at 0.2 gm/l. The cells shown in accompanying figures had undergone at least 20 cell divisions. Integration and expression of the DNA insert was confirmed by southern, northern, and western blot analyses in addition to immunocytochemical staining with anti-β amyloid monoclonal antibodies (Mabs) (Maestre *et al.*, 1992a; Tate *et al.*, 1992).

Methodology for cell preparation and fixation

Cells were grown in 35 mm plastic plates. For Coomassie blue staining, cells were prefixed in Hanks buffered saline with 5 mM EGTA/well and 200 μl 2% glutaraldehyde was added to fix the cells overnight. The cells were subsequently rinsed with 10% methanol (MeOH) for two minutes, 20% MeOH for two minutes,
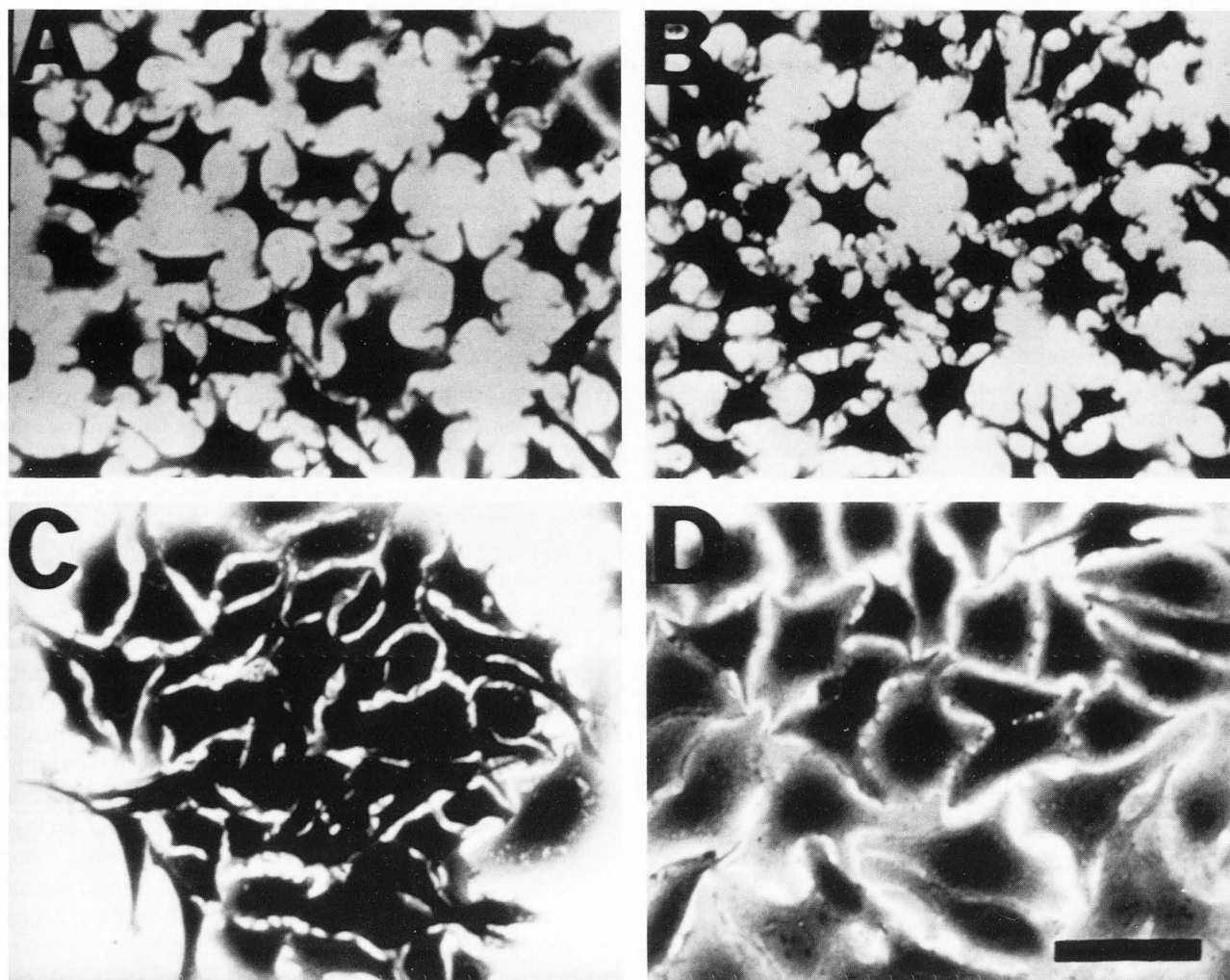
Figure 1. Light microscopy of control and β amyloid positive PC12 cells stained with Coomassie brilliant blue demonstrating the tendency to form aggregates. (**A**) Normal control (NN) cells. (**B**) Vector transfected control (V120) cells lacking a β amyloid insert. (**C**) β amyloid positive transfected AC126 cells. (**D**) β amyloid positive transfected AC127 cells. Bar = 50 μm.

and then stained with 0.1% Coomassie blue in 20% MeOH for two minutes and 1% acetic acid for 20 minutes. For destaining, the cells were rinsed with 20% MeOH plus 1% acetic acid for two minutes. The cells were finally rinsed twice in 1% acetic acid.

Cells were fixed with 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 30 minutes, postfixed with OsO_4 for 30 minutes, dehydrated through graded ethanols, and embedded in Epon on cover-slipped glass slides. The plates were rinsed briefly with Hank's salt solution and fixed with fresh 4% paraformaldehyde in 0.13 M NaCl, 0.02 M phosphate buffer, pH 7.4 (phosphate buffered saline, PBS) for 30 minutes at room temperature. They were rinsed 3 times

with PBS for 10 minutes each time. Thin sections (60 nm) were cut using an ultramicrotome and placed on grids which were then examined with a JEOL 1200 EX transmission electron microscope.

Transmission electron microscopy immunocytochemical study

After the cells reached a confluence of approximately 75%, the plates were rinsed with Hank's salt solution and fixed with 4% paraformaldehyde in 0.13 M NaCl, 0.02 M phosphate buffer, pH 7.4 (PBS) for 30 minutes at room temperature. They were rinsed 3 times with PBS. Cells were immunostained with Mabs (IgG) prepared against a synthetic polypeptide with the β amyloid sequence as reported by Masters *et al.* (1985). As

327

shown elsewhere (Majocha *et al.*, 1988), the Mabs were highly specific for β amyloid. Primary antibody supernatants were diluted 1:5 in ANP buffer (2% bovine serum albumin, 0.3 M NaCl, 0.02 M phosphate, pH 7.2) containing 0.01% Triton X-100. Incubation was overnight at 4°C. The following day, cells were washed 3 times (for 10 minutes each) with buffer containing 0.3 M NaCl and 20 mM Tris, followed by 2 hour incubation with 5 μg/ml of biotinylated goat anti-mouse IgG [Jackson Immunoresearch in buffer (without detergent)]. Cells were washed as before. They were then incubated with strepavidin-horseradish-peroxidase conjugate (Sigma) at 0.25 μg/ml in ANP buffer (without detergent) for 2 hours and washed as above. The chromogen used was diaminobenzidine (Sigma) 0.5 mg/ml, imidazole (Sigma) 1 mg/ml, in 100 mM Tris, pH 7.0. Hydrogen peroxide was added just before use at 0.015% (1 μl of 30% H_2O_2/2 ml). The reaction was allowed to proceed for 2 minutes at room temperature followed by two rinses with distilled water. The cells were then postfixed in 2% glutaraldehyde. Identification of cellular junctions was according to established procedures (Ghadially, 1988; Quinonez and Simon, 1988).

Staining of actin

Control and β amyloid-positive transfected cell lines were grown as above for 48 hours on glass slides coated with poly(L-lysine) and fixed with 4% paraformaldehyde for 5 minutes. The cells were incubated at room temperature for 30 minutes in Hank's solution containing 1 μM rhodamine-phalloidin. The cells were then postfixed with 4% paraformaldehyde for 10 minutes; the slides were rinsed and coverslipped. Photographs were taken using an inverted microscope (Bio Star) equipped with a 35 mm camera and epifluorescence optics.

Results

Light microscopy

The β amyloid positive transfectants (AC126 and AC127), when compared to normal control cells (NN) cells that were transformed by the transfecting vector minus the β amyloid DNA (V120) plated at the same concentration, tended to form aggregates (Figure 1). The current experiment confirmed an earlier observation (Tate *et al.*, 1992). The AC126 and AC127 transfectants displayed a more cohesive arrangement in culture and the points of intercellular contact were therefore more extensive and numerous than in control cells (Figure 1).

Transmission electron microscopy (TEM)

TEM was performed to determine whether or not contacts established by cells that overexpress β amyloid

were uniquely different from those formed by control cells. At the ultrastructural level, the transfected AC126 and AC127 cells demonstrated more extensive areas of intercellular contact than in the non-transfected control cells. The diverse intercellular junctions were analyzed and subclassified into the following categories:

Intermediate junctions. The most commonly observed junctions were of the intermediate type (Figs. 2C and 2D). They displayed some variability in appearance: in extended areas of intercellular contact, extensive intermediate type junctions were observed. This type of junction was more commonly observed in transfected cells as compared to control cells. The majority were characterized by paired subplasmalemmal densities that were up to 30 nm thick. The inter-membrane thickness was approximately 25 nm. Occasional subplasmalemmal densities were associated with an increased concentration of adjacent cytoplasmic intermediate filaments. The filaments were frequently organized in a parallel plane to the plasma membrane without a well developed perpendicular arrangement. Shorter less extensive intermediate type junctions were also identified. This type of intermediate junction appeared to be more readily observable in control cells than in transfected cells. Occasionally, this junctional type formed the only points of contact between control cells. Infrequent intermediate junctions demonstrated a better developed perpendicular arrangement of intermediate filaments and, therefore, more closely resembled desmosomes. Well formed desmosomes were not identified in either control (Figs. 2A and 2B) or transfected cells (Figs. 2C and 2D).

Hemi-subplasmalemmal junctions. Very occasional hemi-cell junctions were observed in PC12 cells in the absence of cell contact (Fig. 3A). These were characterized by areas of increased subplasmalemmal density without an associated perpendicular arrangement of intermediate filaments to the cell membranes. In some instances, an intercellular space between cells displaying these junctions contained a fine granular material of unknown nature. The intercellular distances at junctions were wide and measured approximately 100 nm. At these points, subplasmalemmal densities were observed. In the present study, the hemi-subplasmalemma junctions were not observed in amyloid-positive transfectants, however, they may be present to a minor extent relative to control levels.

Tight junctions. Both transfected and non-transfected control cells showed occasional points of very close intercellular approximation of cell membranes. They appeared as a series of focal connections between membranes of adjacent cells. These structures were interpreted as resembling tight junctions (Fig. 3B). On the material examined, no gap junctions were identified.

Figure 2. Transmission electron micrographs demonstrating intermediate junctions.
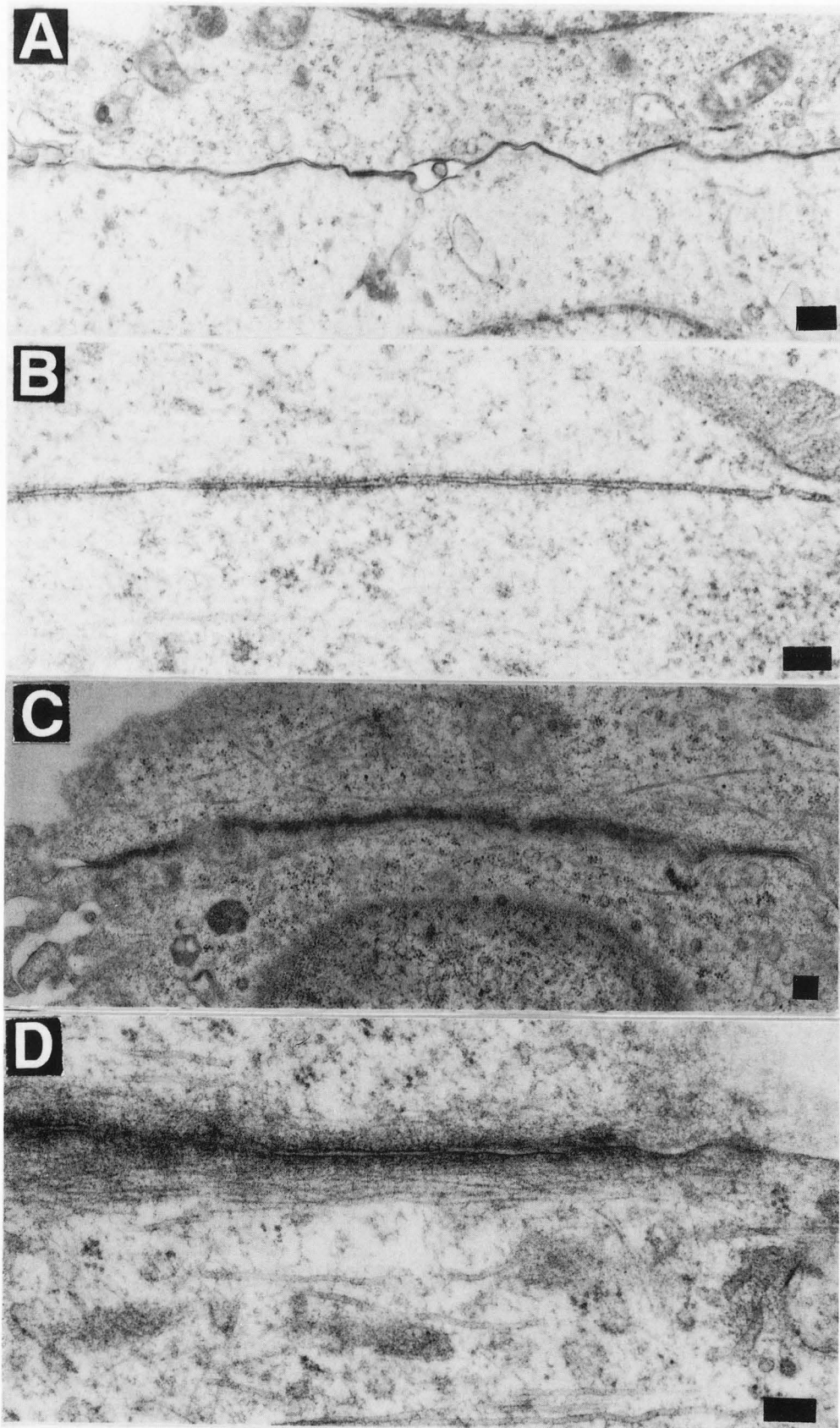
(A) Normal control (NN) cells.

(B) Vector transfected control (V120) cells lacking a β amyloid insert.

(C) β amyloid positive transfected AC127 cells.

(D) β amyloid positive transfected AC127 cells.

In areas of extended intercellular contact, intermediate type junctions were well formed. There is a corresponding organized arrangement of microfilaments, intermediate filaments, and microtubules in the underlying cellular cytoplasm. The intermediate filaments and microtubules are oriented parallel to the cellular membrane.
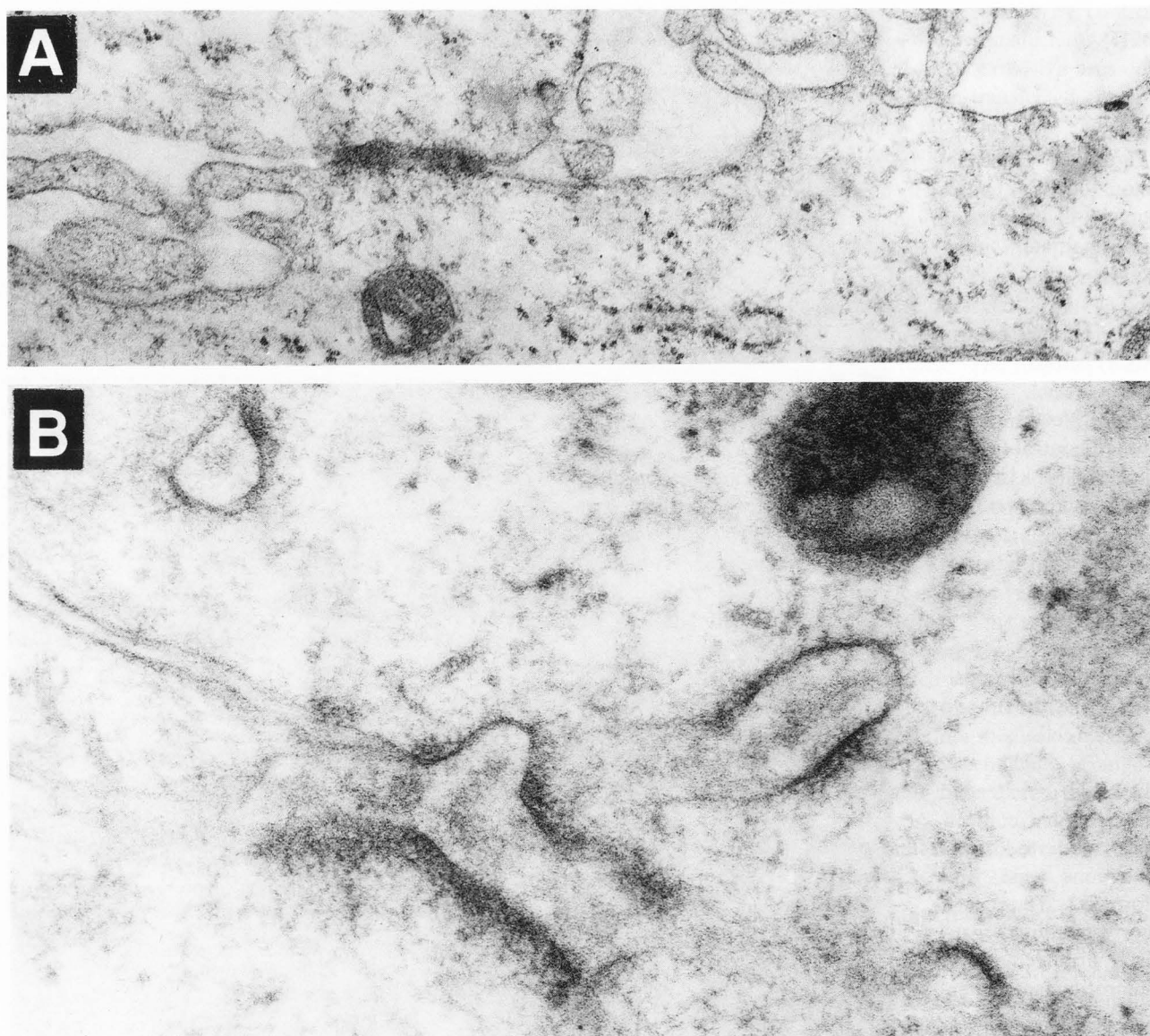
Bars = 200 nm.

Figure 3. Transmission electron micrographs demonstrating a tight junction and hemi-subplasmalemmal junction. (**A**) β amyloid positive transfected AC127 cells showing a tight junction. (**B**) NN normal control PC12 cells showing hemi-subplasmalemmal densities. Bars = 100 nm.

β amyloid epitope at contact sites

The possible relationship between the accumulation of the β amyloid peptide and intercellular contact sites was assessed by immunocytochemistry and studied with TEM in both β amyloid-positive transfectants (Figs. 4B and 4C) and in control cells (Fig. 4A). The areas of immunostaining for the β amyloid epitope corresponded to the areas of extended intercellular contact (Figs. 4B and 4C). This pattern of immunostaining was not observed in control cells (Fig. 4A). A corresponding parallel arrangement of intermediate filaments in the subplasmalemmal region was occasionally associated with membranous immunostaining for β amyloid.

To demonstrate that the immunostain label was the result of specific β amyloid accumulation, an unrelated control MAB, anti-ribonuclease inhibitor protein (RIP) (Majocha *et al.*, 1987), was used for comparative analysis. Even though identical procedures were employed, the anti-RIP MAB did not deposit on membranes or intercellular spaces (Maestre *et al.*, 1992a).
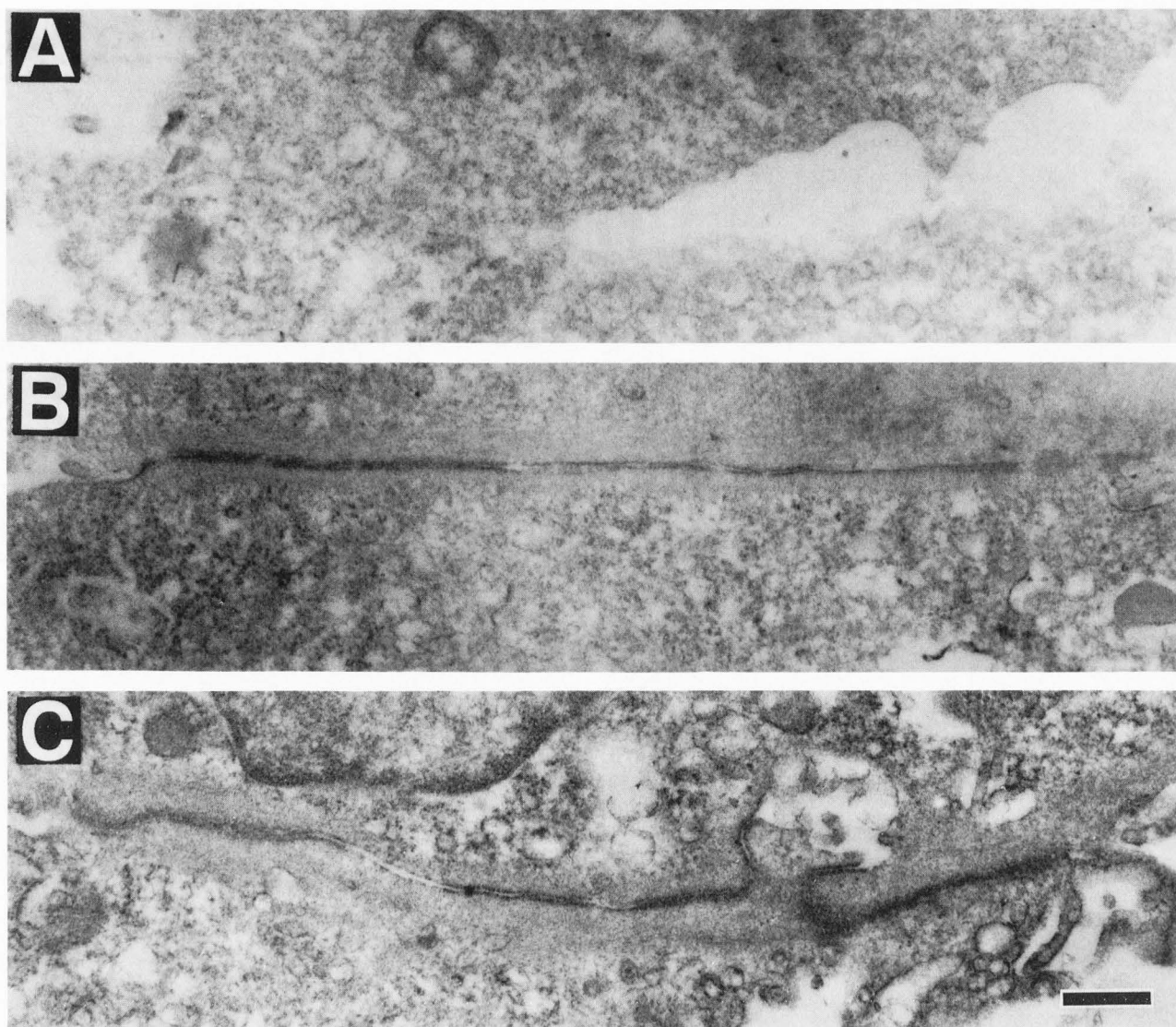
Figure 4. Transmission electron micrographs of immuno-stained control and β amyloid positive transfected PC12 cells using monoclonal antibodies to the β peptide region of the APP. (**A**) Normal control (NN) cells. (**B**) β amyloid positive transfected AC126 cells. (**C**) β amyloid positive transfected AC127 cells. In B and C, immuno-positive material is present in appreciable quantities at the intercellular junctions. Bar = 640 nm.

Distribution of actin. Cells were stained with phalloidin-rhodamine and studied at the LM level. As suggested by the TEM observations, actin accumulated subapically in linear bundles that coursed parallel to the edge of the β amyloid-positive cell types (Figs. 5C and 5D). By contrast, in control cells actin was more concentrated at the tip of neurites (Figs. 5A and 5B). When two adjacent control cells were observed, little or no accumulation of actin was observable (Figs. 5A and 5B).

Discussion

Transfected PC12 cell lines that overexpress the β amyloid peptide exhibit structural and functional alterations at the cellular surface. We previously reported that these cells display increased numbers of the membrane elaborations, including microvilli and blebs (Maestre *et al.*, 1992a), enhanced ruffling activity (Maestre *et al.*, 1993), and modified cell-substrate adhesion properties (Maestre *et al.*, 1992b). Here, we report
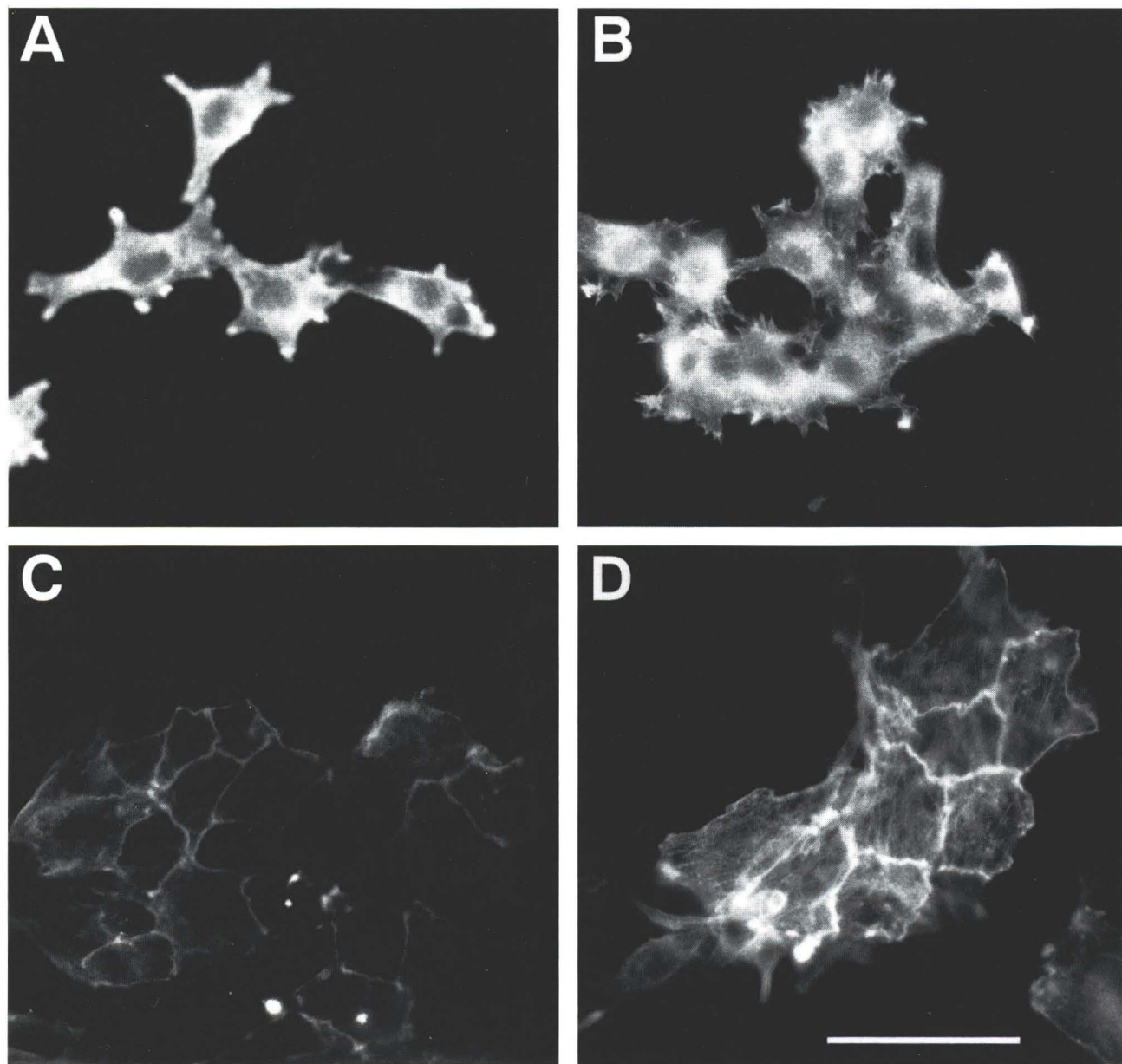
Figure 5. Fluorescence microscopy of control and β amyloid positive PC12 cells stained with phalloidin-rhodamine to demonstrate the redistribution of immunofluorescent actin fibers after transfection with β amyloid DNA. (**A**) Normal control (NN) cells. (**B**) Vector transfected control (V120) cells lacking a β amyloid insert. (**C**) β amyloid positive transfected AC127 cells photographed to emphasize the intercellular junctions. (**D**) β amyloid positive transfected AC127 cells photographed to emphasize the intracellular distribution of actin fibers. Bar = 70 μm.

that intercellular contact is also affected in β amyloid positive cells. The cells have a greater tendency to form aggregates than do normal control PC12 cells or PC12 cells transfected with a vector lacking the β amyloid-C-terminal insert. Increased electron density was found at the junctional membrane sites of transfected cells over-expressing β amyloid.

The more commonly observed type of junction that mediates clustering in the β amyloid positive aggregates is the intermediate junction. Although intermediate type junctions were also seen in control cells, the occurrence of continuous and extensive intermediate type junctions was common in transfected cells that overexpressed amyloid. However, tight junctions and hemi-subplasma-

lemmal densities were also noted. Intercellular structures resembling tight junctions, but not gap junctions, were found in control and β amyloid-positive transfected cells. Freeze etching techniques could be employed to make a more definitive identification of the tight junctions and to conclusively exclude the occurrence of gap junctions.

There are multiple determinants that influence the type of junction that can form. These include environmental cues and tissue distribution (Chiu *et al.*, 1992); regeneration (Gluck *et al.*, 1992); and the stage of development (Duband and Thiery, 1990). Junctions are also influenced by rearrangements of cytoskeletal components such as the accumulation of actin bundles at the cytoplasmic surface of the plasma membrane (Volberg *et al.*, 1986). Current investigations are aimed at identifying neuronal markers, including cytoskeletal constituents, that may be perturbed by overaccumulation of the β amyloid peptide. In a concurrent study, we observed that β amyloid-positive transfectants exhibited reduced levels of neurofilaments and synaptophysin (Majocha *et al.*, 1992).

Neither the role of the β amyloid peptide in the formation of specific junctional types, nor the consequences of this process for intercellular communication, if any, are currently understood. However, certain relevant observations can be cited. In the transfected PC12 cells, the β amyloid-C terminal region of the APP that is overexpressed includes the transmembrane domain (Marotta *et al.*, 1989). The β amyloid antigen colocalizes with the membrane (Maestre *et al.*, 1993); and from *in vitro* studies, it is known that the β peptide has a strong tendency to self-aggregate (Gorevic *et al.*, 1987; Halverson *et al.*, 1990; Honda and Marotta, 1992; Kirschner *et al.*, 1987). Based on these observations, we can hypothesize that overexpression of the β amyloid peptide enhances the ability of the cell to form symmetrical junctions directly or indirectly. A direct role may include homotypic binding among β amyloid peptides at the surface of two cells; or, heterotypic binding may occur which would include the interaction between β amyloid amino acids and a receptor or ligand on another cell. It is possible that the observed effects are due to a non-β-amyloid region of the overexpressed protein. Indeed, although β amyloid peptide has been shown to be toxic in some circumstances, it has also been reported that other regions of the c-terminal fragment not involving the amyloid region can also be toxic to PC12 cells (Kozlowski *et al.*, 1992). Therefore, additional studies are necessary to confirm that the observations reported here are due to β amyloid peptide alone.

Structural changes that underlie the acquisition of memory support the notion that synaptic growth occurs at the expense of non-synaptic junctions (for review, see Bailey and Kandel, 1993). Deficits in memory are a clinical hallmark of AD, and this impairment may be directly related to the compromised integrity of interneuronal interactions. We provided evidence that excess accumulation of β amyloid C-terminal peptide is related to increased intercellular adhesion and that the peptide is associated with the intercellular junctional apparatus. The β amyloid peptide may compromise membrane integrity (Arispe *et al.*, 1993; Nitch *et al.*, 1992) and mediate membrane turnover at these sites. This suggested process would interfere with dynamic neuronal connectivity and synaptic growth. To some extent this proposed mechanism may be reflected by the differences in actin distribution we observed in β amyloid positive cells compared with controls.

Thus, among the consequences of the suggested processes may be memory impairment. Alterations in intercellular interactions in the AD brain have not been directly explored to a notable extent. Genetically engineered PC12 cells transfected with the C-terminal region of APP DNA provide important opportunities to analyze intercellular alterations that are directly associated with the accumulation of the β amyloid peptide. These studies will extend our understanding of cellular mechanisms associated with β amyloid that contribute to the dementia of AD.

Acknowledgements

References

Arispe N, Rojas E, Pollard HB (1993). Alzheimer disease amyloid ß protein forms calcium channels in bilayer membranes: blockade by tromethamine and aluminum. Proc. Natl. Acad. Sci. USA **90**: 567-571.

Bailey CH, Kandel ER (1993). Structural changes accompanying memory storage. Annu. Rev. Physiol. **55**: 397-426.

Bissell MJ, Barcellos-Hoff MH (1978). The influence of extracellular matrix on gene expression: is structure the message? Cell **15**: 627-637.

Breen KC, Bruce M, Anderton BH (1991). Beta amyloid precursor protein mediates neuronal cell-cell and cell-surface adhesion. J. Neurosci. Res. **28**: 90-100.

Chen M, Yankner BA (1991). An antibody to beta amyloid and the amyloid precursor protein inhibits cell-substratum adhesion in many mammalian cell types. Neurosci. Lett. **125**: 223-226.

Chiu ML, Jones JC, O'Keefe EJ (1992). Restricted tissue distribution of a 37-Kd possible adherens junction protein. J. Cell Biol. **119**: 1686-1700.

de Sauvage F, Octave JN (1989). A novel mRNA of the A4 amyloid precursor gene coding for a possibly secreted protein. Science **245**: 651-653.

Duband JL, Thiery JP (1990). Spatio-temporal distribution of the adherens junction-associated molecules vinculin and talin in the early avian embryo. Cell Differ. Dev. **30**: 55-76.

Dyrks JD, Weidemann A, Multhaup G, Salbaum JM, Lamaire HG, Kang J, Muller-Hill B, Masters CL, Beyreuther K. (1988). Identification transmembrane orientation and biogenesis of the amyloid A4 precursor of Alzheimer's Disease. EMBO J. **7**: 949-957.

Esch FS, Keim PS, Beattie EC, Blacher RW, Culwell AR, Oltersdorf T, McClure D, Ward PJ (1990). Cleavage of amyloid ß peptide during constitutive processing of its precursor. Science **248**: 1122-1124.

Farmer SR, BenZe'ev A, Benecke BJ, Penman S (1978). Altered translatability of messenger RNA from suspended anchorage-dependent fibroblasts: reversal upon cell attachment to a surface. J Cell Sci Supl. **8**: 327-343.

Ghadially FN (1988). Ultrastructural Pathology of the Cell Matrix, 3rd Edition. Vol. 2. Butterworths, pp. 1099-1126.

Ghiso J, Rostagno A, Gardella JE, Liem L, Gorevic PD, Frangione B (1992). A 109 amino acid C-terminal fragment of Alzheimer's disease amyloid precursor protein contains a sequence, -RHDS-, that promotes cell adhesion. Biochem. J. **288**: 1053-1059.

Glenner GG, Henry JH, Fujihara S (1981). Congophilic angiopathy in the pathogenesis of Alzheimer's degeneration. Ann. Pathol. **1**: 120-129.

Gluck U, Rodriguez Fernandez JL, Pankov R, Ben-Ze'ev A (1992). Regulation of adherens junction protein expression in growth activated 3T3 cells and in regenerating liver. Exp. Cell. Res. **202**: 477-486.

Gorevic PD, Castano EM, Sarma R, Frangione B (1987). Ten to Fourteen residue peptides of Alzheimer's disease protein are sufficient for amyloid fibril formation and its characteristic x-ray diffraction pattern. Biochem. Biophys. Res. Commun. **147**: 854-862.

Gorman CM, Moffat LF, Howard BH (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Molec. Cell. Biol. **2**: 1044-1051.

Halverson K, Fraser PE, Kirschner DA, Lansbury Jr. PT (1990). Molecular determinants of amyloid deposition in Alzheimer's disease: Conformational studies of synthetic ß-protein fragments. Biochemistry **29**: 2639-2644.

Hay JW, Ernst RL (1987). The economic costs of Alzheimer's disease. Am. J. Pub. Health **77**: 1169-1175.

Honda T, Marotta CA (1992). Arginine specific endopeptidases modify the aggregation properties of a synthetic peptide derived from Alzheimer ß/A4 amyloid. Neurochem. Res. **17**: 367-374.

Kang J, Lemaire JG, Unterbeck A, Salbaum JM, Masters CL, Grzeschik KH, Multhaup G, Beyreuther K, Muller-Hill B (1987). The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. Nature **325**: 733-736.

Katzman R (1986). Alzheimer's Disease. N. Engl. J. Med **314**: 964-973.

Kirschner DA, Inoue H, Duffy LK, Sinclair A, Lind M, Selkoe DJ (1987). Synthetic peptide homologous to ß protein from Alzheimer disease forms amyloid-like fibrils *in vitro*. Proc. Natl. Acad. Sci. USA **84**: 6953-6957.

Kitaguchi N, Takahashi Y, Tokushima Y, Shiojiri S, Ito H (1988). Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity. Nature **331**: 531-534.

Klier FG, Cole G, Stallcup W, Schubert D (1990). Amyloid beta protein precursor is associated with extracellular matrix. Brain Res. **515**: 336-342.

Kozlowski MR, Spanoyannis A, Manly SP, Fidel SA, Neve RL (1992). The neurotoxic carboxy-terminal fragment of the Alzheimer amyloid precursor binds specifically to a neuronal cell surface molecule: pH dependence of the neurotoxicity and binding. J. Neurosci. **12**: 1679-1687.

Maestre GE, Tate BA, Majocha RE, Marotta CA (1992a). Cell surface extensions associated with overexpression of Alzheimer ß/A4 amyloid. Brain Res. **599**: 64-72.

Maestre GE, Tate BA, Majocha RE, Marotta CA (1992b). Adhesive properties of cells overexpressing ß/A4 amyloid DNA. Society for Neuroscience Abstracts **18**: 1437 (abstract 60.1).

Maestre GE, Tate BA, Majocha RE, Marotta CA (1993). Membrane surface ruffling in cells that overexpress β/A4 amyloid. Brain Res. **621**: 145-149.

Majocha RE, Sajdel-Sulkowska EM, Rodenrys A, Ventosa-Michelman M, Marotta CA (1987). Loss of alkaline ribonuclease inhibitor protein associated with Alzheimer neuronal degeneration. Society for Neuroscience Abstracts **13**: 819 (abstract 230.8).

Majocha RE, Benes FM, Reifel RL, Rodenrys AM,

Marotta CA (1988). Laminar-specific distribution and infrastructural detail of amyloid in the Alzheimer cortex visualized by computer-enhanced imaging of unique epitopes. Proc. Natl. Acad. Sci. USA 85: 6182-6186.

Majocha RE, Lai E, Marotta CA (1992). Reduced expression of neuronal proteins in PC12 cells transfected with the A4-C-terminal portion of amyloid precursor protein (APP). Society for Neuroscience Abstracts 18: 729 (abstract 304.9).

Majocha RE, Tate-Ostroff B, Marotta CA (1993). PC12 cells release stimulatory factors after transfection with ß/A4-C-terminal DNA of the Alzheimer amyloid precursor protein. Molec. Chem. Neuropathol. 18, 99-113.

Marotta CA, Chou WG, Majocha RE, Watkins R, Zain SB (1989). Overexpression of amyloid precursor protein A4 (ß-amyloid) immunoreactivity in genetically transformed cells. Implications for a cellular model of Alzheimer amyloidosis. Proc. Natl. Acad. Sci. USA 86: 337-341.

Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K (1985). Amyloid plaque core protein in Alzheimer's disease and Down syndrome. Proc. Natl. Acad. Sci. USA 82: 4245-4249.

Nitch RM, Blusztajn JK, Pittas AG, Slack BE, Growdon JH, Wurtman RJ (1992). Evidence for a membrane defect in Alzheimer disease brain. Proc. Natl. Acad. Sci. USA 89: 1671-1675.

Palmert MR, Usiak M, Mayeux R, Raskind M, Tourtellotte WW, Younkin SG (1990). Soluble derivatives of the beta amyloid protein precursor in cerebrospinal fluid: alterations in normal aging and in Alzheimer's disease. Neurology 40: 1028-1034.

Quinonez G, Simon GT (1988). Cellular junctions in a spectrum of human malignant tumors. Ultrastruct. Pathol. 12: 389-405.

Shivers B, Hilbich C, Multhaup G, Salbaum JM, Beyreuther K, Seeburg PH (1988). Alzheimer's disease amyloidogenic glycoprotein: expression pattern in rat brain suggests a role in cell contact. EMBO J. 7: 1365-70.

Singer SJ (1992). Intercellular communication and cell-cell adhesion. Science 255: 1671-1677.

Tate BA, Majocha RE, Maestre GE, Marotta CA (1991). Biological and behavioral alterations induced by ß/A4-amyloid. Bull. Clin. Neurosci. 56: 131-139.

Tate B, Aboody-Guterman KS, Morris AM, Walcott EC, Majocha RE, Marotta CA (1992). Disruption of circadian regulation by brain grafts that overexpress Alzheimer ß/A4 amyloid. Proc. Natl. Acad. Sci. USA 89: 7090-7094.

Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa, R Hill, Hansen LA, Katzman R (1991). Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. Ann Neurol 30: 572-580.

Ueda K, Cole G, Sundsmo M, Katzman R, Saitoh T (1989). Decreased adhesiveness of Alzheimer disease fibroblasts: is amyloid A protein precursor involved? Ann Neurol. 25: 246-251.

van der Eb AJ, Graham FL (1980). Assay of transforming activity of tumor virus DNA. Methods Enzymol. 65: 826-839.

Vasiliev JM (1985). Spreading of non-transformed and transformed cells. Biochim. Biophys. Acta 780: 21-65.

Vernadakis A (1985). The aging brain. Clin. Geriatr. Med. 1: 61-94.

Volberg T, Geiber B, Kartenback J, Franke WW (1986). Changes in membrane-microfilament interaction in intercellular adherens junctions upon removal of extracellular Ca^{2+} ions. J. Cell Biol. 1023: 1832-1842.

Zain SB, Salim M, Chou W-G, Sajdel-Sulkowska EM, Majocha RE, Marotta CA (1988). Molecular cloning of amyloid cDNA derived from mRNA of the Alzheimer brain. Coding and non-coding regions of the fetal precursor mRNA are expressed in the Alzheimer cortex. Proc. Natl. Acad. Sci. USA 85: 929-933.

Discussion with Reviewers

F.N. Low: The author's interpretations of cell junctions, particularly in the transmission electron micrographs, would not be approved by critical cell biologists (although not necessarily wrong). For example, in Figure 3A, the tight junction does not show pentalaminar organization. This may be because it is cut diagonally in a section too thick for satisfactory identification.

Authors: Prof. Low justifiably addresses the difficulties in confirming tight junctions in this material. For the purpose of clarification, a tight junction was defined as follows: "A tight junction consists of a series of punctuate contacts in which the dense outer leaflets of adjoining membranes converge and fuse to form a single line. Often, the outer dense line of the outer membrane is interrupted at the sites of membrane fusion. The width of diffused membranes is slightly less than the combined width of the two unit membranes" (Quinonez and Simon, 1988). Prof. Low's comments concerning the thickness of the section are appreciated. In fact, as mentioned in text, freeze-fracturing techniques could be employed to better study gap junctions and tight junctions; these techniques were not utilized in this study.

Based upon the limitations of the techniques that we used in our study, the example of tight junction presented in this paper should still be acceptable considering the above cited definition for this type of junction.

G.D. Miner: What is the relative importance of β amyloid to the basic causes of Alzheimer's Disease considering the chromosome 14AD gene, the chromosome 19 (APO-E4) gene, the chromosome 21 ("FAD") gene, and the yet to be discovered chromosome location of the Volga-German early onset FAD?

Authors: The relative importance of ß amyloid in the pathogenesis of AD is unknown. The current view is that AD is a heterogeneous syndrome, from both a clinical and a genetic point of view. The fact that mutations in the APP gene (chromosome 21) are sufficient to cause AD has been the major argument in favor of amyloid as an active participant in the events that lead to degeneration in the brains of AD victims. In addition, even though linkage of AD and chromosome 14 and 19 have been reported, the mechanisms for which these loci could be pathogenic are uncertain. In the case of the Apoliproprotein E gene (ApoE) on chromosome 19, precise definition as an AD susceptibility gene or as a gene in linkage disequilibrium with the "true AD gene" is necessary. However, in families with APP mutations, the e-4 genotypes predispose to earlier onset, which suggest that ApoE could be a "modifier" instead of a determinant factor in the pathogenic pathway leading to AD [Alzheimer's Disease Collaborative Group (1993). Apolipoprotein E genotype and Alzheimer's disease. Lancet **342**: 737-738; and St. George-Hyslop P, Crapper McLachlan D, Tuda T, Rogaev E, Karlinsky H, Lippa CF, Pollen D (1994). Alzheimer's disease and possible gene interaction. Science **263**: 537].

G.D. Miner: Are there any specific and differing (i.e., variant) functions or purposes of the three transmembrane forms of APP (e.g., APP_{695}, APP_{751} and APP_{770}) known? Are there any important reasons for these multiple forms, or is it a redundancy built into the biochemical genetic system?

Authors: The expression of the amyloid precursor protein (APP) gene is highest (all forms) during neurological development. The dominant form in the brain is the 695 isoform, whose expression has previously been found to be largely restricted to the central nervous system. During neonatal development in the rat basal forebrain, the ratio between isoform 695 and other Kunitz containing APP's is the highest. The precise function of APP during development, or at any other time for that matter, is not known [Sherman CA, Higgins GA (1992). Regulated splicing of the amyloid precursor protein gene during postnatal development of the rat basal forebrain. Brain Res Dev Brain Res **6**: 63-69].