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β-Amyloid of Alzheimer's Disease Induces Reactive Gliosis That Inhibits Axonal Outgrowth

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Pathological lesions in the brains of patients with Alzheimer's disease (AD) are characterized by dense deposits of the protein β-amyloid. The link between the deposition of β-amyloid in senile plaques and AD-associated pathology is, at present, controversial since there have been conflicting reports on whether the 39–43 amino acid β-amyloid sequence is toxic or trophic to neurons. In this report, we show that β-amyloid peptide when presented as an insoluble substrate which mimics its conformation *in vivo* can induce cortical glial cells *in vitro* and *in vivo* to locally deposit chondroitin sulfate containing proteoglycan. *In vitro* the proteoglycan-containing matrix deposited by glia on β-amyloid blocks the usual ability of the peptide to allow cortical neurons to adhere and grow. Chondroitin sulfate-containing proteoglycan was also found in senile plaques of human AD tissue. We suggest that an additional effect of β-amyloid in the brain, which compounds the direct effects of β-amyloid on neurons, is mediated by the stimulation of astroglia to become reactive. Once in the reactive state, glial cells deposit large amounts of growth-inhibitory molecules within the neuropil which could impair neuronal process survival and regeneration leading to neurite retraction and/or dystrophy around senile plaques in AD. © 1993 Academic Press, Inc.

INTRODUCTION

Astrogliosis is a classic hallmark of trauma and disease of the central nervous system (5, 6). It is believed that the role of gliosis in the adult is to wall off the area of injury which, in turn, prevents local regeneration of neuronal processes (25). Recently, a molecular component associated with the extracellular matrix of astroglial scars following trauma has been shown to be a chondroitin-6-sulfate-containing proteoglycan (21). *In vivo*, this proteoglycan, found in the adult cortex, colocalizes with intensely GFAP-positive astrocytes in and immediately adjacent to induced scars, but not with other GFAP-positive astrocytes distant from the injury site (21). Neurons fail to elaborate axons *in vitro* on the surface of scars removed intact from adults, unless the glycosaminoglycan chains are digested with chondroitinase, suggesting that chondroitin sulfate-containing proteoglycan deposition may be involved in limiting the growth of regenerating axons in the CNS (22). This idea is supported by the finding that certain proteoglycans found during development have been shown to be regulatory to axonal outgrowth. For instance a chondroitin sulfate/keratan sulfate-containing proteoglycan, located on astrocytes in boundary regions of the developing embryo, limits axon elongation *in vitro* (7, 32) and a chondroitinase-sensitive proteoglycan regulates the patterning of axons in the retina (4).

Apart from trauma, gliosis may be a normal consequence of senescence in the brain and especially in neurodegenerative diseases, where there is an increase in reactive glia in response to failing CNS mechanisms (9, 12, 18, 26). Accordingly, astrogliosis could play a fundamental role in the development of pathological lesions in Alzheimer's disease (AD) (10). In the AD brain, reactive astrocytes are found in high abundance around molecularly heterogenous cores of β-amyloid found within senile plaques (9, 12, 13, 23). Also, the presence of various sulfated proteoglycans in association with amyloid fibrils (29, 30, 31, 37) suggests that reactive gliosis could contribute specified extracellular matrix components to the forming β-amyloid aggregate. Indeed, the plaque-related glial interactions may be triggered by the deposition of β-amyloid (19) and might represent an attempt to wall off the β-amyloid core.

Neither the source of plaque-associated proteoglycans nor the effect these molecules have on neuronal survival or neurite dystrophy in AD are known. In this report, we have investigated the hypothesis that neurite retraction or neuronal death in the AD brain is a consequence of secondary cellular reactions in the form of a functionally reactive gliosis, triggered by insoluble 1–40 and 25–35 peptide fragments of β-amyloid variously reported to be neurotoxic (see the complete issue of *Neurobiol. Aging* **13**(5) for up-to-date review). This reactive

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gliosis, we propose, leads to the deposition of inhibitory molecules which are severely adverse to neuronal process survival.

METHODS

In Vitro Spot Assay

For preparation of substrates for the spot assays, 0.5–1 μl of a 2 m*M* solution of β-amyloid peptides (1–40 and 25–35 amino acids were obtained from Bachem California Inc., scrambled 25–35 and scrambled 1–40 kindly donated by Gliatech, Inc.) were allowed to air-dry under sterile conditions onto nitrocellulose-coated 35-mm petri dishes or Flaskette chambers (for time-lapse videomicroscopy). This produced peptide spots in the range of 0.7–1.2 mm in diameter which visibly adhered to the surface of the dish and persisted for the duration of the experiments (confirmed by ponceau S staining of peptide). Prior to seeding cells, substrates were overlayed with a 5 μg/ml solution of laminin in calcium–magnesium-free Hanks' balanced salt solution (CMF).

Culture of Cortical Neurons

Cortical neurons were obtained from Embryonic Day 16 rat embryos. Following removal of fetuses from the mother, cerebral cortices were removed into CMF and cut into small pieces and trypsinized for 10 min at 37°C. Following the addition of DMEM-F12 supplemented with 10% FCS, cells were resuspended to a determined cell density and plated onto prepared peptide spot substrates. For seeding onto conditioned spots, glial cells were first removed from the dishes after 2–14 days by extensive washes with sterile distilled water, after which cortical neurons were seeded as above.

Filter Implant Assay

For testing the *in vivo* response to β-amyloid peptides, pieces of Millipore filter either impregnated with β-amyloid peptide ($n = 10$) or saline (control; $n = 10$) were implanted into the cerebral cortex of neonatal rats. Briefly, both groups of P0 animals were anesthetized on ice. An incision was made on the dorsal surface of the head and the skin was retracted. The skull was cleaned of the periosteum and a small rectangular opening made through the skull. Next, a 2 × 2-mm piece of sterile nitrocellulose filter (Millipore Corp.) was implanted into the cerebral cortex, and the incision was closed. After 30 days animals were anesthetized with chloral hydrate and perfused with 4% paraformaldehyde. The brains were postfixed for 4 h in the same fixative and then cryoprotected with sucrose prior to cryostat sectioning and staining for the presence of chondroitin sulfate-containing proteoglycan. Sections from the brains of these animals were also stained for the presence of β-amyloid (antibody kindly provided by G. Perry) and showed that β-amyloid does not noticeably dissipate over the 30-day time period, remaining in the Millipore filters.

Culture of Glial Cells

Glial cells were obtained from P0–P2 neonatal rats by the following method. Brains were dissected into CMF and then left and right cerebral cortices were freed of surrounding tissue and cut into small (0.5 mm) pieces. Dissociation into single cells was achieved by briefly trypsinizing (0.25%) the tissue and passing it through a Nitex filter (60 μm). The cell suspension was then seeded into Falcon tissue culture flasks in DMEM-F12 supplemented with 10% FCS. After 3 days, nonadherent cells were lightly shaken off and the medium was changed. Before the cells became confluent, adherent cells were passaged into fresh flasks by trypsinization (0.25%) for 15 min at 37°C and replated in DMEM-F12 supplemented with 10% FCS. Under these conditions approximately 95% of the cells were found to be GFAP positive. At this point some cultures were incubated with a small volume (1 μl 10 ml^{-1}) of 0.06-μm Fluorespbrite carboxylate microspheres (Polysciences Inc.) which are readily phagocytosed by glial cells and allows identification of cells on phase opaque substrates with fluorescent optics. Cells were passaged an additional two times before use in the substrate assays. For seeding onto peptides in the spot assays, glial cells were removed from these cultures following a similar trypsinization protocol, resupended to a determined concentration, and plated onto prepared peptide substrates (as described above).

Timelapse Videomicroscopy

Once seeded with glia or neurons, time-lapse videomicroscopy was performed on selected cultures using an inverted Zeiss microscope at various magnifications. Time-lapsed cultures were maintained at 37°C for up to 3 days with a thermostatically controlled heated air blower and filmed using a Hamamatsu CCD camera. Films were made of cells on and off the β-amyloid spots after differing times postseeding (from Time 0 to 14 days postseeding). Under either Nomarski or phase-contrast optics, images were taken every 100–360 s using a MetelTek shutter control system operated by an Image 1 system. Digitized images were averaged, contrast-enhanced where required, and stored on a Pansonic LF 7010 optical disc recorder. Selected videoprints were made from stored images with a Sony 500-W video printer.

Immunohistochemistry

For immunocytochemistry, cultures were briefly rinsed in PBS and fixed for 12 h at 4°C with 4% formal

saline. For the filter implants, brains were perfused with the same fix and cryosectioned after which sections were treated the same as cultures. Fixed cultures and sections were washed extensively with PBS and blocked for 2 h with 0.1% BSA/4% NGS in PBS. Cultures and sections were then exposed to a mixture of antibodies: GFAP (Accurate Chemical, specific for glial acidic fibrillar protein) and CS-56, (ICN ImmunoBiologicals, which recognizes chondroitin 6-sulfate-containing proteoglycans), optimally diluted in staining buffer (0.01% BSA/0.4% NGS in PBS containing 0.01% Tween 20). After 1 h, the primary cocktail was washed away with multiple changes of staining buffer and then exposed to an optimally diluted mixture of biotinylated goat anti-mouse IgM and fluorescein-conjugated goat anti-rabbit IgG in staining buffer. After incubation with secondary cocktail, cultures were washed as before and incubated with strepavidin-conjugated Texas Red (Amersham). Cultures were rinsed and coverslipped in Citifluor (Citifluor Ltd.) and viewed on a Leitz Orthoplan 2 microscope fitted for phase-contrast, DIC, and fluorescence optics. Control staining was carried out as follows. Cultures which had either or both primaries omitted in the above protocol did not show any significant staining. Likewise, peptide spots exposed to culture medium alone for 14 days in the absence of cells stained negative with CS-56. The presence of chondroitin sulfate proteoglycan in our cultures and filter implants was confirmed with another monoclonal antibody, 3B3, which recognizes the sugar stubs of chondroitin-6-sulfate glycosaminoglycan side chains near the protein core of the proteoglycan after chondroitinase digestion.

For immunocytochemistry of AD tissue, human hippocampus and frontal cortex were obtained postmortem, fixed in Methacarn (MeOH, 60%; acetic acid, 10%; chloroform, 30%), and embedded in paraffin wax. Chondroitinase ABC, heparinase, or heparitinase were applied to the sections overnight at 20°C in Tris-buffered saline (pH 7.6). Sections were processed for immunostaining using the peroxidase–antiperoxidase method, with 3,3,5,5-diaminobenzidine tetrahydrochloride as the substrate. The presence of GFAP-positive astrocytes was visualized using polyclonal anti-GFAP with alkaline phosphatase-linked anti-rabbit IgG and Fast Blue R (biotin–avidin). The 3B3 monoclonal antibody recognizes the sugar stubs of chondroitin-6-sulfate glycosaminoglycan side chains near the protein core of the proteoglycan after chondroitinase digestion.

RESULTS

Adherence of Cortical Neurons to β-Amyloid

To examine the interaction between substrate bound β-amyloid and neurons, we first challenged neurons in culture with immobilized synthetic peptides of the 39–43 amino acid β-amyloid molecule and followed their responses using a plaque-like "spot" assay. Cortical neurons seeded onto spots of 1–40 or 25–35 β-amyloid peptides showed a marked preferential adherence to the peptide surface over the surrounding laminin substrate (Figs. 1 and 3A). By contrast, cortical neurons seeded at similar cell densities on the scrambled 1–40 or scrambled 25–35 peptides did not adhere at all. This suggested that neuronal cells of the cortex have an adhesive affinity for the 1–40 amino acid sequence of the β-amyloid molecule, and that this affinity may be mediated by the 25–35 sequence of the β-amyloid molecule.

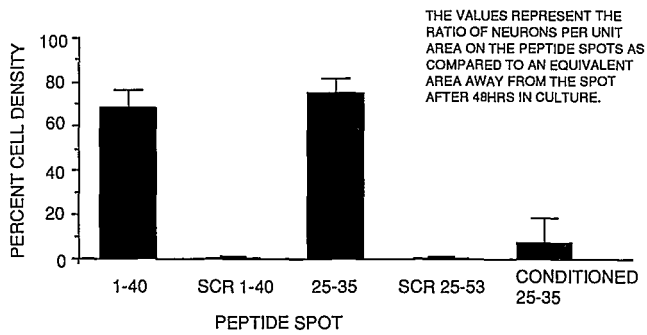


FIG. 1. Density of cortical neurons on β-amyloid peptide spots *in vitro*. The values represent the ratio of neurons per unit area on the peptide spots as compared to an equivalent area away from the spot after 48 h in culture. Neurons preferentially adhere to the 1–40 and 25–35 β-amyloid peptides over the surrounding laminin alone substrate (n = 18). With the scrambled 1–40 and 25–35 peptides, no cells were observed to adhere to the spot when this peptide was used as a substrate (n = 15). After conditioning by exposure to cortical glial cells for 14 days, the 25–35 peptide spot showed a very much diminished ability to support neuron attachment (n = 21).

Behavior of Cortical Glia on β-Amyloid Substrates

When glial cells were seeded onto the 25–35, 1–40, scrambled 25–35, or scrambled 1–40 amino acid peptides of the β-amyloid molecule, they initially attached and spread on all of the peptide surfaces showing no preference for the peptide spots over the surrounding laminin substrate. However, time-lapse videomicroscopy of the glial cultures on each of the nonscrambled amyloid components showed that glial cells exhibit different behaviors on the peptide substrates compared to cells off the peptide spots. Within a matter of hours, cells resident on the peptide spots underwent a profound change in morphology by producing many elongated processes. In addition, the motility of cells on the peptides dramatically increased relative to those seeded away from the spot. During their time on the 25–35 and 1–40 peptides some cells showed alternating periods of motility and quiescence. However, even during the stationary phase, these cells showed a pronounced ruffling of the cell surface not shown by cells off the spots. Glial cells traversed the border on and off the peptide sub-

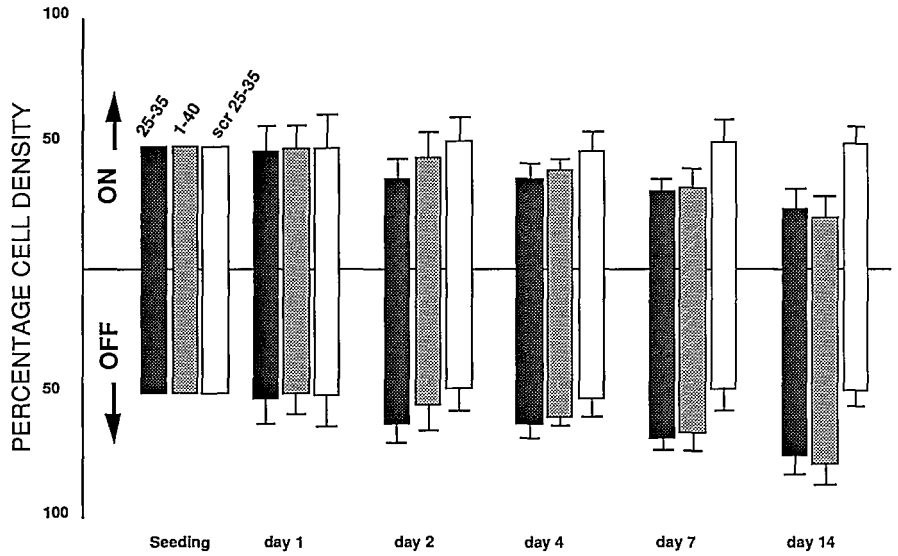


FIG. 2. Change in relative glial cell density on β-amyloid peptide spots *in vitro*. Each bar represents an equivalent number of cells. Changes in the ratio of cells on the peptide spot to regions away from the spot per unit area are shown by the intersection with the abscissa. On the 1–40 and 25–35 peptide spots, cells remain resident for many days but gradually the density on these peptides declines at comparable rates ($n = 46$). Cells on the scrambled 1–40 and 25–35 peptide spots remain at similar densities as on the surrounding substrate ($n = 12$).

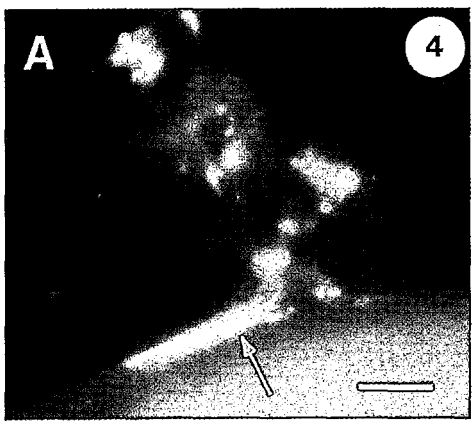
strate freely, indicating that the border of the peptide was not a physical barrier to cell movements and that the peptides were not inherently nonadhesive or repulsive to glial cells. Glial cells do not exhibit increased motility or the same changes in morphology in preparations of scrambled 25–35 or scrambled 1–40 peptides. With time, the density of glial cells on the 1–40 or 25–35 peptide substrate decreases relative to the cell density off the spot (Fig. 2). This appears to occur by cells on the peptide spots eventually wandering off the peptides without returning. Cells left on these peptides sometimes form aggregates on the peptide surface which is not seen by cells off the spots. Thus, time-lapse videomicroscopy revealed glial cells to have altered behavior, in terms of cell movements and morphologies, on the 1–40 and 25–35 peptides. This seemingly "reactive" behavior on the 1–40 and 25–35 β-amyloid peptide spots led us to examine the possibility that glial cells had initiated the deposition of gliotic scar components while in contact with the β-amyloid peptides.

Cortical Glia Condition β-Amyloid Substrates by ECM Deposition

After 14 days exposure of the 25–35 and 1–40 peptide spots, glial cells were replaced with cortical neurons. Unlike cortical neurons seeded directly onto unconditioned 25–35 and 1–40 peptides, cells now failed to show an adhesive preference for the preconditioned spot, with far fewer cells adhering to the spot than the surrounding laminin substrate (Figs. 1 and 3B). Control spots left in culture for the same time period but without exposure to glial cells still permitted preferential neuron adhesion. This indicated that the surface of the peptide spot exposed to glia had become an unfavorable substrate to the attachment and growth of neurons as a consequence of glial reactivity.

We therefore tested glial-conditioned 25–35 and 1–40 peptide spots for the presence of glycosaminoglycan-bearing proteoglycans, an indicator of functionally reactive gliotic scar formation after trauma (21). After 5 days in culture, chondroitin sulfate-containing proteoglycan deposition could be seen at points of cell contact with the peptide (Fig. 4). The deposition of chondroitin sulfate proteoglycan on the peptide spots increased with longer exposures to glial cells (Fig. 5). Over the surface of the peptide spots irregularly shaped, cell-sized blotches of chondroitin sulfate-containing proteoglycan were found (Fig. 5A) and the complete circumferences of the 25–35 and 1–40 peptides were eventually "walled off" by high amounts of chondroitin sulfate deposition after 14 days in culture (Figs. 5B and 5C). Chondroitin sulfate-containing proteoglycan was not deposited on scrambled 1–40 and scrambled 25–35 peptides over comparable time periods. Thus, we propose that glial cells become reactive when they are in contact with immobilized 1–40 and 25–35 peptides of the β-amyloid molecule and that this reactivity is manifested by the local deposition of enhanced amounts of chondroitin sulfate-bearing proteoglycan. Since analysis of time-lapse films revealed no apparent difference in the rates of mitosis between cells on the peptide spots and those off the spots, this form of reactive gliosis is associated with a large increase in cell motility but without any discernible increase in cell numbers.

FIG. 3. Video prints of cortical neurons cultured on immobilized 25–35 β-amyloid peptides. (A) Cortical neurons 48 h postseeding on a 25–35 peptide spot. Relatively more neuronal processes and cell bodies are found on the peptide surface than away from the spot. (B) Cortical neurons 48 h postseeding on a 25–35 peptide spot which had been previously exposed to mixed glia for 14 days. Unlike A, cells preferentially adhere to the surrounding substrate rather than the conditioned peptide surface. Inset at low power magnification shows conditioned peptide surface to be largely devoid of neuronal cells. Scale bars: A, 50 μm; B, 50 μm.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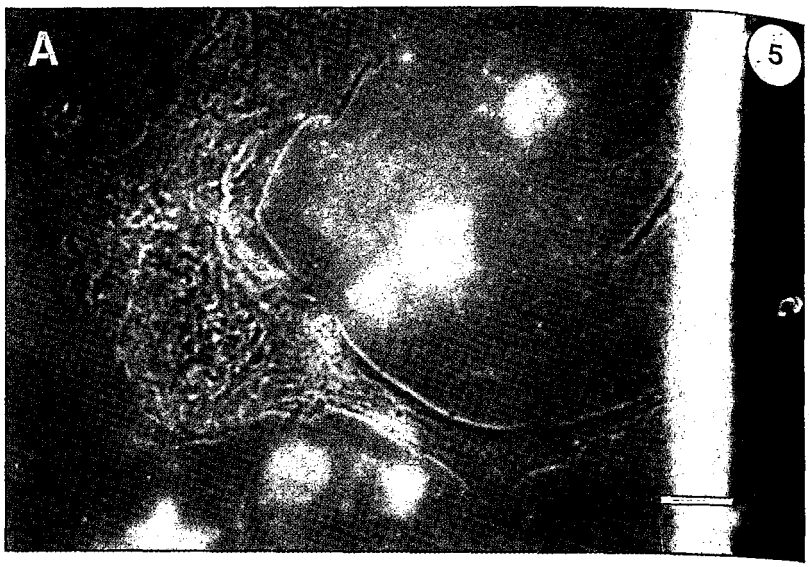
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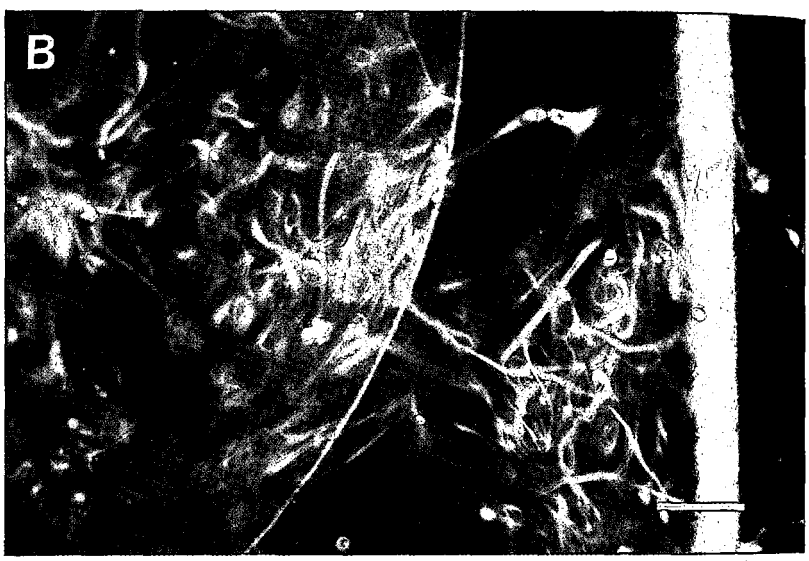
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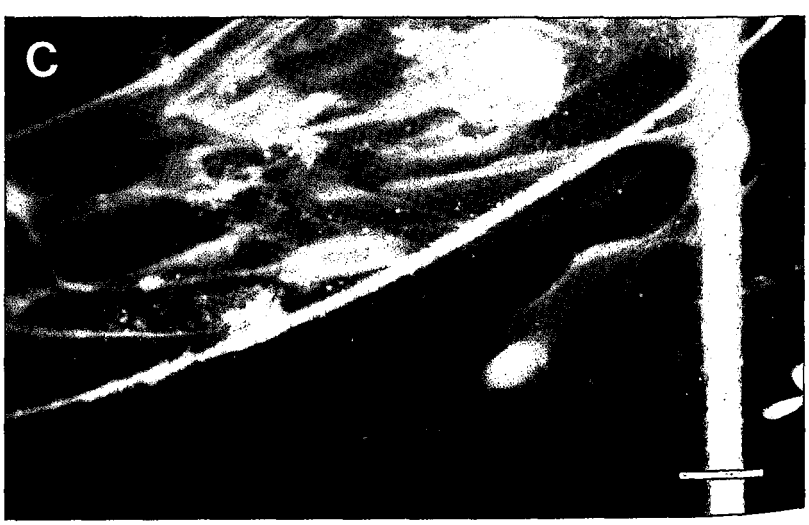
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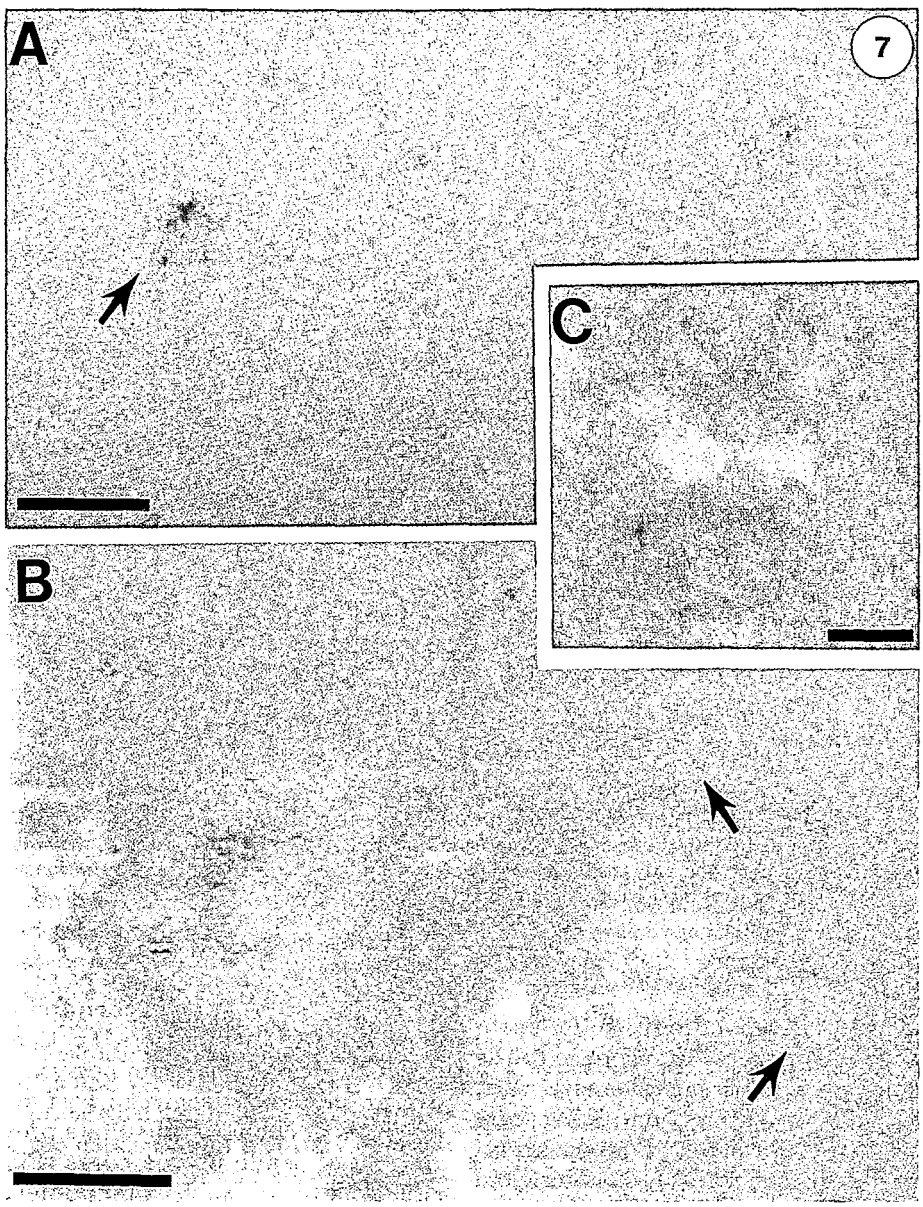

FIG. 4. Deposition of chondroitin sulfate-containing proteoglycan by cortical glial cells on β-amyloid peptide spots *in vitro*. (A) A 25–35 β-amyloid peptide, (B) 1–40 β-amyloid peptide sequence. Chondroitin sulfate positivity is shown in red and GFAP positivity is shown in yellow–green. As cells approach the peptide spot from the side, chondroitin sulfate can be seen at the points of contact of the cells with the peptide border (A, arrow). This becomes progressively more intense with time, as cells migrate along and over the edge of the peptide (B, arrow). The bright green spots in A and B are fluorescent spheres endocytosed by glial cells used for tracking during timelapse videomicroscopy. Scale bars: A, 5 μm; B, 10 μm.

FIG. 5. Deposition of chondroitin sulfate-containing proteoglycan on immobilized 25–35 β-amyloid peptide spots *in vitro* after 14 days exposure to cortical glial cells. Chondroitin sulfate positivity is shown in red (A, B, and C) and GFAP positivity is shown in yellow–green (B and C). (A) On the surface of the peptide after 14 days, localized areas of chondroitin sulfate deposition are left on the peptide surface showing that the surface has been subjected to glial reactivity. The complete circumference of the peptide spot and much of the surface is coated with chondroitin sulfate (B and C), areas which time-lapse videomicroscopy has shown to be repeatedly contacted by glial cells. The orange hue of the peptide spots is an artifact of long camera exposures during fluorescence photography. Scale bars: A, 20 μm; B, 100 μm; C, 10 μm.

FIG. 7. Immunolocalization of chondroitin sulfate proteoglycan in sections from cortex and hippocampus of patients diagnosed with Alzheimer's disease. (A) Hippocampus: Chondroitin sulfate proteoglycan (brown) is found in intimate association with numerous senile plaques (arrow). (B) Cortex: GFAP-positive astrocytes and astrocytic processes (purple–mauve stain; arrows) are located near the periphery of senile plaques (brown stain). Scale bars: A, 50 μm; B, 25 μm; C, 10 μm. (C) Hippocampus: Higher magnification of a solitary senile plaque immunostained with monoclonal 3B3 and Congo red. Birefringence of Congo red identifies the β-amyloid core as the cross-shaped structure in the center of the plaque. Chondroitin sulfate proteoglycan is located primarily around the periphery (brown).

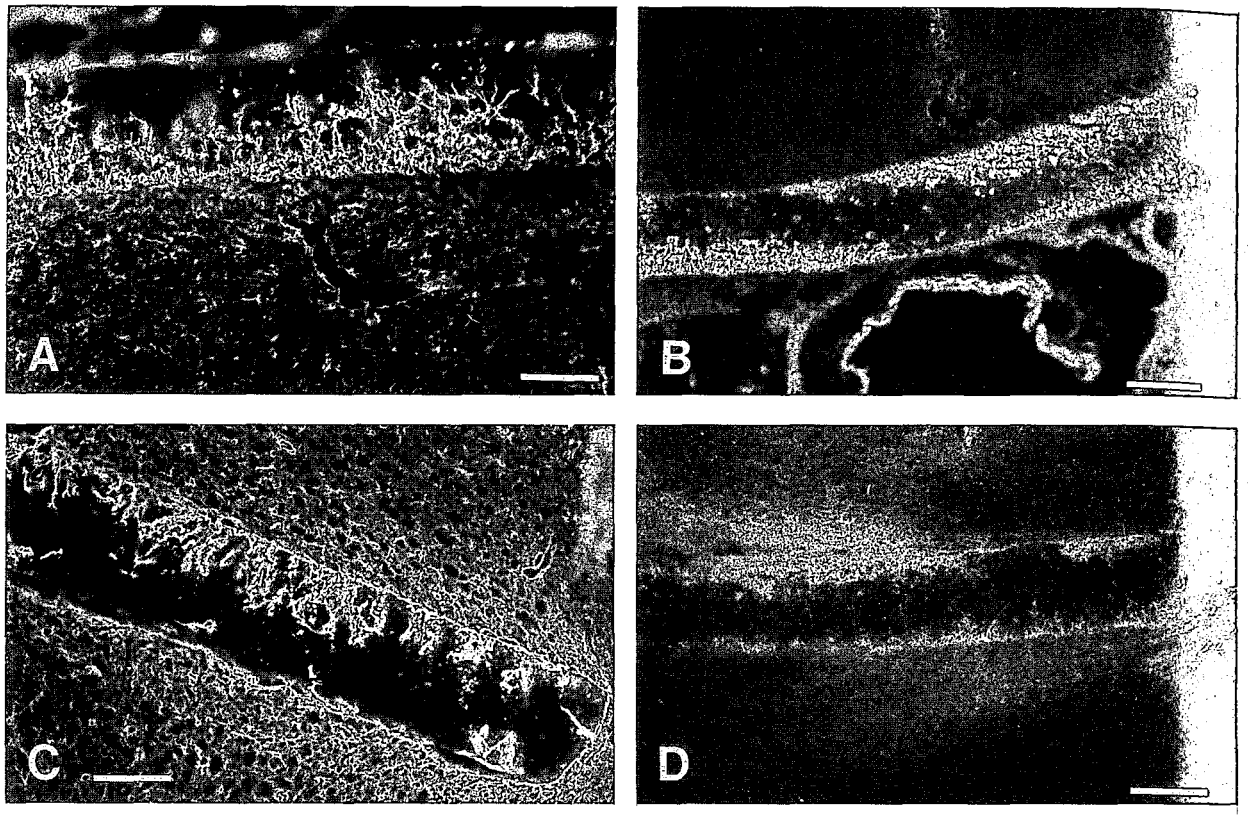

FIG. 6. Reactive gliosis to nitrocellulose filters saturated with β-amyloid peptide implanted into the cortex of neonatal rats. GFAP positivity is found at comparable levels in both the β-amyloid-impregnated filter (A) and control filter (C), showing that glial cells infiltrate both experimental and control filters. However, only the β-amyloid-containing filter elicits a significant deposition of chondroitin sulfate (B), which stains more intensely than the control filter without β-amyloid (D). Scale bars: A, 500 μm; B, 800 μm; C, 500 μm; D, 800 μm.

In Vivo Response to Immobilized β-Amyloid

We then examined whether chondroitin sulfate proteoglycan is also deposited in excess in response to the presence of β-amyloid in intact tissue. Neonatal rats, which do not form chondroitin sulfate-containing scars after lesions to the cortex (21), were implanted with nitrocellulose filters impregnated with 1–40 β-amyloid and allowed to survive 30 days (21, 22). Approximately the same number of GFAP-positive processes and cells were seen infiltrating control and β-amyloid implants (Figs. 6A and 6C). However, β-amyloid filters contained significant amounts of chondroitin sulfate-containing proteoglycan while comparable control filters revealed little or no staining (Figs. 6B and 6D). Thus, glial cells in intact living tissue react to the presence of β-amyloid by producing chondroitin sulfate-bearing proteoglycan in a manner similar to glia confronted with β-amyloid *in vitro*.

Chondroitin Sulfate Proteoglycan in Human AD Tissue

The finding that β-amyloid peptides induce enhanced deposition of chondroitin sulfate proteoglycan in the cortex of living rats, even when removed from the direct effects of trauma, prompted us to search in human tissue for the association of chondroitin sulfate proteoglycan with senile plaques in AD patients. Using antibodies against various glycosaminoglycan portions of chondroitin sulfate proteoglycan, we found greatly enhanced amounts of chondroitin sulfate proteoglycan at the periphery of senile plaque cores (Fig. 7). Bearing some similarity to our *in vivo* filter implants, the proteoglycan was found to be associated with GFAP-positive astrocytes in the near vicinity of senile plaques (Fig. 7C). Importantly, this distribution of chondroitin sulfate proteoglycan corresponds closely to the region around senile plaques which has both a decline in neuritic density and an increase of axons exhibiting AD-associated cytoskeletal damage (2). Therefore, it appears likely that the same events of reactive gliosis and deposition of chondroitin sulfate proteoglycan are taking place in the AD brain as in our *in vitro* and *in vivo* models.

DISCUSSION

Our study has focused on the possibility that neuritic retraction and/or neuronal dystrophy associated with Alzheimer-like plaques is not simply a consequence of

direct neurotoxicity by β-amyloid (as it has been suggested in solution (15, 20, 34–36) or as insoluble peptide aggregates (8, 24), but also of secondary glial cell reactions to the insoluble molecule. The β-amyloid peptides were bound to a substrate in our experimental model systems and, therefore, it may be argued that this presentation of β-amyloid peptides to cortical cells more closely mimics the situation of AD plaques *in vivo* where β-amyloid is present in fibrillar form. We have shown for the first time that β-amyloid peptide is able to induce glial cells to become functionally reactive both in culture and in the intact animal in that they specifically deposit enhanced quantities of chondroitin-6-sulfate proteoglycan, a known inhibitor of neuronal regeneration found in glial scars. We propose that the chondroitin sulfate proteoglycan present around senile plaques of patients with AD, perhaps along with other proteoglycans previously described in association with senile plaques (8, 24, 29–31), may be a link in the chain of events that ultimately leads to neuronal dystrophy and possibly neuronal loss.

The distinguishing feature by which β-amyloid is able to act as an inducer of reactive gliosis is an especially important but perplexing problem in light of the recent observation that the ubiquitiously distributed (33) amyloid precursor protein (APP), from which β-amyloid is abherrantly cleaved, may itself be a chondroitin sulfate-bearing proteoglycan core protein (27). If this were the case, then deposition of APP by a variety of injured cells of which there is some evidence (14, 28) could be a global initiator of scar formation in the CNS if the molecule were fragmented and modified extracellularly (by microglia or reactive astrocytes (1)), perhaps in response to the abnormal presence of a proteoglycan core protein. In this way, β-amyloid could be considered to be a molecular trigger of gliosis among other candidate molecules such as cytokines and growth factors (11, 17). It remains to be determined whether gliosis triggered by β-amyloid results in the deposition of a variety of scar-associated molecules including different species of proteoglycans along with other extracellular matrix molecules such as tenascin (21). In the AD brain, such a process may explain why plaque-associated axonal damage extends out and away from the dense β-amyloid core (2) into the reactive astroglial areas and why characteristic neuritic dystrophy occurs specifically in response to β-amyloid deposition (3). Furthermore, our findings that β-amyloid supports neuronal attachment, perhaps via some modification of laminin (16), unless conditioned by glia suggests that the extracellular matrix surrounding plaques could curtail neurite regeneration in the AD brain. Given the potent inhibitory nature of certain proteoglycans to neuronal cells, especially the inhibition by types of chondroitin-6-sulfate proteoglycan (32), this would suggest that glia can play important roles not only in the creation of axonal boundaries during normal development, but also in the pathology of CNS lesions and neurodegenerative disorders such as Alzheimer's disease.

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