β-Amyloid Precursor Protein and β-Amyloid Peptide Immunoreactivity in the Rat Brain After Middle Cerebral Artery Occlusion

Effect of Age

A. Popa-Wagner, PhD*; E. Schröder, MS*; L.C. Walker, PhD; C. Kessler, MD

Background and Purpose—Previous studies have shown that the β-amyloid precursor protein (βAPP) is upregulated after cerebral ischemia and that the β-amyloid (Aβ) fragment may be toxic to brain cells. Although stroke in humans usually afflicts the elderly, most experimental studies on the nature of cerebral ischemia have used young animals. To test the hypothesis that the upregulation and/or persistence of amyloidogenic proteins is exacerbated in aged rats after cerebral ischemic stroke, we studied the expression of βAPP and its proteolytic product Aβ in the brains of young and old rats 7 days after temporary cerebral ischemia.

Methods—Focal cerebral ischemia was produced by reversible occlusion of the right middle cerebral artery in 3- and 20-month-old male Sprague-Dawley rats. After 1 week, brains were removed and immunostaining was performed for βAPP, Aβ, and ED1 for macrophages and glial fibrillary acidic protein (GFAP).

Results—Histological staining revealed that the degree of necrotic cavitation in the infarct core was relatively less in aged rats than in young rats, suggesting a slower pace of degenerative change and/or tissue removal in older animals. βAPP immunoreactivity was robustly increased, primarily in macrophage-like, ED1-positive cells in the infarct core and in the penumbra of both young and aged animals. Aβ immunoreactivity was evident in GFAP-positive astrocytic somata and processes, and also in clusters of small spherical structures in the penumbra. These Aβ-immunoreactive minispheres were more numerous in aged rats than in young rats.

Conclusions—The presence of βAPP and Aβ immunoreactivity in the infarct core and penumbra indicates that cerebral ischemia promotes conditions that are favorable to the focal accumulation of βAPP and its proteolytic fragments, especially in the aged brain. (Stroke. 1998;29:2196-2202.)

Key Words: stroke ■ amyloid ■ aging ■ rats

Ischemic stress increases intra-axonal β-amyloid precursor protein (βAPP) levels and promotes the deposition of β-amyloid (Aβ) in the human brain. In rodents, βAPP expression is increased in the brain after cerebral ischemia, and there is evidence for the accumulation of Aβ as well. Kalaria et al showed that at 4 and 7 days postocclusion, βAPP immunoreactivity was preferentially localized within axonal swellings, dystrophic neurites, and neuronal perikarya along the periphery of the infarct.

Substantial evidence suggests that βAPP is involved in the regulation of neuronal growth and survival. Alternative processing of βAPP can result in the production of the secreted forms of βAPP (βAPPs), which may enhance neuronal plasticity and viability, or the Aβ peptide and C-terminal segment of βAPP, which can be neurotoxic. Intrathecal administration of a 17-mer peptide of βAPP, 20 minutes before ischemia and once daily for 3 days thereafter, has been shown to significantly reduce neurological damage. Furthermore, postischemic administration of βAPPs intracerebroventricularly protects neurons in the CA1 region of rat hippocampus against ischemic injury.

On the other hand, cultured neuroblastoma cells degenerate around the infusion site of aggregated Aβ 1–40, and intracerebral injections of Aβ have been shown to be neurotoxic in aged primates. Furthermore, conditional expression of the carboxyl-terminal portion of βAPP by using a tetracycline-responsive promoter system in neuroblastoma cells results in pronounced cytotoxicity. Recent evidence derived from mice expressing the 100–amino acid carboxy-terminal fragment of βAPP indicates that this fragment may promote synaptic degeneration and neuronal death. Likewise, there is an accumulation of the cytotoxic fragment of
βAPP in the hippocampus 7 days after global forebrain ischemia. Notably, this process is accelerated with increasing age. Although the incidence of ischemic stroke increases dramatically with advancing age, relatively few studies have been conducted on aged animals, which would mimic most closely the context in which stroke occurs in humans. We hypothesized that the upregulation and/or persistence of amyloidogenic proteins would be greater in aged rats than in young rats after cerebral ischemic stroke. To test this hypothesis, we studied the expression of βAPP and its proteolytic product Aβ in the brains of 3- and 20-month-old rats 7 days after temporary occlusion of the middle cerebral artery.

Materials and Methods

The experiments reported in this study were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 93–23, revised 1985), according to the recommendations of Gärnter, and were approved by a federal animal care committee.

Eighteen hours before surgery, male Sprague-Dawley rats (n=13 for each age group) were deprived of food to minimize variability in ischemic damage that can result from varying plasma glucose levels. Water remained available at all times. In all cases, surgery was performed between 8 AM and 1 PM.

Reversible Occlusion of the Middle Cerebral Artery

Blood flow through the middle cerebral artery was temporarily interrupted using a modification of the method originally described by Brint et al., Dirnagl et al., and Zhang and Iadecola. Throughout surgery, anesthesia was maintained by spontaneous inhalation of 1% to 1.5% halothane in a mixture of 75% nitrous oxide and 25% oxygen through a specially designed mask. Body temperature was kept at 37°C by a Homeothermic Blanket System (Harvard Apparatus). The anesthetized animals were immobilized in a supine position and the tail artery catheterized with PP50 tubing (ID=50 mm). This catheter enabled the continuous measurement of blood pressure and the withdrawal of blood samples for determination of pH and blood gases (Blutgassystem IL 1620; Instrumentation Laboratory) and arterial glucose levels (Omnicare7 Balance; B. Braun, Melsungen). Under a surgical microscope, the left and right common carotid arteries were exposed and each loosely encircled with a silicon thread to facilitate the temporary closure of these vessels (see below).

The animals were placed in a prone position and the right lateral skull surface surgically exposed. Using a microdrill (Fine Science Tools), a small segment of the skull above the middle cerebral artery was removed 2- to 3-mm rostral to the juncture of the zygomatic arch and the pars squamosa of the temporal bone. The bone was thinned using a low drill speed and superfusion with physiological saline to minimize friction-induced warming. The bone flap was carefully removed with forceps and the underlying dura opened with a fine needle. The middle cerebral artery was then slowly lifted with a tungsten hook attached to a micromanipulator (Maerzhaeuser Precision Micro-manipulator Systems; Fine Science Tools) until blood flow through the artery was completely interrupted. Blood flow through the common carotid arteries was then stopped by tightening the prepositioned thread loops (see above). The surgical field was kept warm and moist with artificial cerebrospinal fluid (Liquicheck, Spinal Fluid Control, Bio-Rad) for the duration of the procedure.

Three hours later, the middle cerebral artery and the common carotid arteries were reopened, allowing full reperfusion of the brain. The surgical wounds were sutured shut, the catheter withdrawn from the tail artery and the animals returned to their cages. After a 7-day survival time, the rats were deeply anesthetized with 2.5% halothane in 75% nitrous oxide and 25% oxygen and perfused with buffered saline followed by buffered 4% freshly depolymerized paraformaldehyde. The brain was removed, postfixed in 4% buffered paraformaldehyde for 24 hours, cryoprotected in 20% sucrose prepared in 10 mmol/L phosphate-buffered saline (PBS), flash-frozen in isopentane, and stored at −70°C until sectioned.

Determination of Infarct Volume

The area and partial volume of the 20th section of the ipsilateral cerebral hemisphere and infarct site were measured stereologically. An integration of the resulting partial volumes provided the volume of the ipsilateral hemisphere along with the volume of the cortical infarct, which was then expressed as percent of the total volume of the hemisphere.

Histology

The brains were cut on a freezing-cold microtome, and 25-μm-thick coronal sections were collected in 4% paraformaldehyde in 100 mmol/L PBS, pH 7.2, postfixed for 30 minutes, immersed in polyethylene glycol, and stored at −20°C until use.

Every 20th section was stained using acid-vanadium-fuchsin to detect neuronal degeneration. In normal neuronal tissue, the nuclei are stained blue and the cytoplasm is light pink or unstained, whereas in the infarcted tissue, nuclei stain bright pink and are sometimes surrounded by a pink cytoplasm.

Immunocytochemistry

Free-floating sections were first treated with 0.3% hydrogen peroxide in PBS to inactivate endogenous peroxidase and then processed for immunocytochemistry. After blocking in 3% donkey serum/10 mmol/L PBS/0.3% Tween 20, sections were incubated overnight at 4°C with monoclonal mouse antibodies recognizing either (1) the N-terminal epitope of βAPP (clone 22C11, Boehringer Mannheim), (2) the cytoplasmic carboxyl fragment 643–695 of βAPP (clone 2.F2.19B4, Boehringer Mannheim), (3) a cytoplasmic determinant of brain macrophages (clone ED1, Camon); or (4) the astrocytic marker glial fibrillary acidic protein (GFAP) (clone G-A-5, Boehringer Mannheim), diluted 1:800 in PBS containing 3% normal donkey serum and 0.3% Tween 20. The primary antibody was detected using the ABC system (Vectastain Elite Kit, Vector, ). After extensive washing in PBS containing 0.3% Tween 20, sections were incubated overnight at 4°C with biotinylated donkey antiserum IgG (Jackson ImmunoResearch Laboratories) and diluted 1:400 in PBS containing 1% normal donkey serum and 0.3% Tween 20. After washing in PBS, sections were incubated for 2 hours at room temperature in ABC Elite reagent diluted 1:100 in PBS containing 0.3% Tween 20. The antibody complex was then visualized with 0.025% 3’,3’-diaminobenzidine and 0.005% hydrogen peroxide in 100 mmol/L Tris buffer (pH 7.5) for 5 to 10 minutes. Finally, the sections were mounted onto slides, air-dried, and placed on coverslips using a xylene-based mounting medium. For double-labeling experiments, the sections were first incubated with the primary antibody and then by secondary antibodies conjugated with alkaline phosphatase. For color development, we used the nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate system (dark blue). After brief fixation, sections were further incubated with the secondary primary antibody followed by secondary biotinylated antibodies. Color development ensued using diaminobenzidine (brown) for the second antigen.

The specificity of the antibodies was verified by Western blot analysis and by omission of the primary antibodies in immunocytochemical experiments. For Aβ immunochemistry, specificity was further assessed by preincubation of the primary antibody with an excess of the Aβ peptide (Sigma).

Results

Twenty-four hours after surgery, the clinical status of the animals was assessed using the Bederson test. At this time, all animals that had undergone cerebral ischemia for 3 hours showed obvious neurological deficits, including paresis of the contralateral extremities, particularly the hindlimbs, and rotation when lifted above the cage floor (grade 3 in the Bederson test, except for 1 young rat and 1 old rat that were...
Serum Arterial Blood Gas Level, Glucose, and BP Values

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<tr>
<th>Groups</th>
<th>pH Before</th>
<th>During</th>
<th>Po2, mm Hg Before</th>
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<th>Po2, mm Hg Before</th>
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<th>Glucose, mg/dL Before</th>
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<td>45.8±1.4</td>
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<td>3 mo (n=9)*</td>
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<td>7.35±0.01</td>
<td>42.2±3.1</td>
<td>44.5±2.5</td>
<td>205±19</td>
<td>204±27</td>
<td>96±10</td>
<td>90±7</td>
<td>3.8±0.5</td>
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<tr>
<td>20 mo (n=9)**</td>
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<td>49.8±3.6</td>
<td>186±27</td>
<td>167±40</td>
<td>122±6</td>
<td>106±13</td>
<td>3.5±0.7</td>
<td>3.2±0.8</td>
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Data were obtained 20 minutes “before” and 20 minutes “after” middle cerebral artery occlusion.

*One rat died, two rats had no infarct.
**Three rats died.

scorable as grade 2). Control animals showed no neurological deficits (grade 0). In the first 24 hours after surgery, the animals were somewhat listless, probably in part due to the aftereffects of the anesthesia. This condition improved to some extent in the ensuing days, although the animals tended to remain sensitive to external stimulation and noises. There was no evident difference in the clinical status of young and aged rats during the 7-day postsurgical survival time. Although the blood parameters showed some age-associated differences, notably in blood pressure, the differences were not statistically significant (Table). Likewise, there was no significant difference in the volume of cortical infarcts between young and aged rats (42.7±11.4% for the young rats and 43.9±11.3% for the aged rats).

Histology

Acid-vanadium-fuchsin staining revealed age-related differences in the viability of cells in and around the infarct core. Within the infarct core of young animals, necrosis of the tissue progresses to cavitation (Figure 1A), whereas in older animals the degree of removal of necrotic tissue was reduced (Figure 1B). Likewise, the intensity of acid-vanadium-fuchsin staining of necrotic cells in the penumbra was greater in young animals (Figure 1C) than in aged animals (Figure 1D). Within the infarct core of older rats, some neuronal debris, most notably degenerating axons as revealed with antibodies against neurofilament-68, could still be discerned 7 days after ischemia (not shown).

Glial Fibrillary Acidic Protein

In the region surrounding the infarct core (penumbra) of young animals, the GFAP antibody revealed numerous hypertrophic astrocytes with long, profuse processes (Figure 1E); in the corresponding region of older animals, there were many reactive astrocytes as well, but the cells had shorter and thicker processes than in the young rats (Figure 1F), suggesting a more advanced reactive phenotype (Jorgensen et al, unpublished data, 1993).

Aβ, βAPP, and ED1

In both young and aged rats, Aβ-immunoreactivity was associated with cells in the penumbra that have an astrocytic morphology (Figure 2A and B). However, the reactive phenotype of Aβ-positive astrocytes was more pronounced in old rats than in young rats (Figure 2B). A double-labeling experiment using antibodies to Aβ and GFAP confirmed that the Aβ-immunoreactive cells are astrocytes (Figure 2A, inset). In addition, small spherical objects that were immunopositive for Aβ were observed, most commonly in the penumbra of aged rats (Figure 2B, inset). Although these minispheres resembled some of the small dots seen with βAPP and ED1 antibodies (see below), the Aβ spheres were fewer in number and tended to occur in clusters. Whether the antibody to Aβ is recognizing the cleaved Aβ peptide or the exposed epitope on holo-βAPP or fragmented βAPP remains to be determined.

Using a monoclonal antibody against the cytoplasmic carboxyl fragment 643–695 of βAPP, we found that βAPP-immunostaining was associated primarily with cells that had a rounded morphology in both young and aged rats (Figure 2C, 2D, insets). In sections counterstained with hematoxylin, these immunoreactive cells resembled reactive macrophages, and some of them had the appearance of foam cells and multinucleated giant cells (not shown). In addition, numerous βAPP-positive punctate structures of various sizes were scattered among the larger immunoreactive somata. The abundant βAPP staining circumscribed the infarct core in which the necrotic tissue was still evident in the brains of many aged rats 7 days postischemia (Figure 2D). The ED1 antibody showed a staining pattern that was similar to that of βAPP; ie, ED1-immunostaining was associated with rounded cells in the vicinity of the infarct core, with microglia-like cells in the penumbra and with numerous punctate structures (Figure 2E and F and insets). Double-immunostaining showed that the cells that contained βAPP also were reactive with antibody ED1 (Figure 2E, insets). Since ED1 is a marker for macrophages, we conclude that the βAPP-positive cells are mainly macrophages derived from the brain and/or blood.25,26 We did not detect significant differences between the 2 age groups in the intensity or pattern of βAPP and ED1 immunostaining.

Discussion

Our findings confirm previous studies showing that βAPP is upregulated in the area of infarct after ischemic stroke.1–6,27,28 Immunocytochemical analysis revealed heavy staining of βAPP epitopes at both the amino and the carboxyl ends of the molecule in large round cells, most probably macrophages derived from the blood and/or brain. Aβ immunostaining was more prominent in penumbral reactive astrocytes and especially in small spherical structures of older rats as compared with young rats, suggesting that the aged brain presents relatively favorable conditions for the focal accumulation of Aβ. This is in line with other studies that have localized βAPP by immunostaining to activated microglia3 and to reactive astrocytes5,29 after ischemia in animal models.
The persistence of necrotic tissue in the infarct core of aged animals at 7 days postischemia indicates that the degenerative process and/or glial removal of damaged tissue evolves at a slower pace in the infarcted area of aged rats than in that of young rats. These findings confirm a previous study in which the disintegration of injured brain tissue is considerably reduced in the ischemic area of aged rats, apparently because of the diminished infiltration of the infarct site by macrophages. A slower rate of phagocytic removal of cellular debris in the aged brain might explain the presence of Aβ-positive minispheres in the penumbra of our aged, but not young, animals. The same phenomenon may also account for the persistence and buildup of β-amyloid deposits in the brains of older humans. Such findings also are compatible with a shift from rapid necrosis in young neurons to delayed necrosis in aged neurons. However, our data do not allow us

Figure 1. A through D, Acid-vanadium-fuchsin staining of coronal brain sections after cerebral ischemia. Note the relatively severe necrotic cavitation in the infarct core of 3-month-old rats (A) compared with the 20-month-old rats (B). Degenerating cells in the penumbra of 3-month-old rats (C, arrows) were more intensely stained than those in the penumbra of 20-month-old rats (D, arrows). E and F, Immunohistochemical staining of GFAP. Note the presence of numerous reactive astrocytes with profuse processes in the infarct penumbra of 3-month-old rats (E) versus astrocytes in 20-month-old rats, which had fewer, thicker processes (F). PE indicates penumbra; IC, infarct core. Bars: A, B =250 μm; C, D =100 μm; E, F =50 μm.
to distinguish between the delayed appearance of ischemic change in neurons that are destined to die and a delayed detrimental effect of ischemia on viable neurons. How-
however, given that ischemic neurons display enhanced acidophilia, then it follows that the neurons from the young rats are in a more advanced state of ischemic degeneration than those from the old rats.

Growing evidence suggests a synergistic and perhaps etiological link between vascular disease and Alzheimer’s disease. In persons with numerous Alzheimer-like lesions, the

Figure 2. A and B, Immunohistochemical staining of Aβ. Note the robust immunostaining associated with cells resembling astrocytes in old (B, arrows) and, to a lesser extent, in young (A) rats. (inset). Double-labeling for Aβ and GFAP was especially evident in the cell bodies (dark color, arrows) and was less evident in processes, where GFAP labeling prevailed (dark blue, arrowheads). In the penumbra of old rats, clusters of small spherical Aβ-positive structures were also apparent (B, inset, arrowheads). C and D, βAPP was intensely immunostained in rounded cells and numerous small dots in the penumbra adjacent to the infarct core of young rats (C, inset, arrowheads) and old rats (D, inset, arrowheads). E and F, Immunohistochemical staining of ED1. Note the association of ED1 immunostaining with rounded cells circumscribing the infarct core as well as with microglia-like cells and punctate structures in the penumbra of both young (E) and old (F, insets) rats. Double-immunostaining revealed that the βAPP-containing cells also were reactive with antibody ED1 (E, insets). PE indicates penumbra; IC, infarct core. Bars: A, B, C, D, and F = 50 μm; insets, A, E = 18 μm.
expression of dementia is augmented by a history of stroke. 35 One means whereby stroke might promote the pathogenesis of Alzheimer’s disease is through an upregulation of βAPP. βAPP responds to cerebral insult like an acute-phase protein 36 and therefore is likely to participate in the cellular response to brain insult. For example, head injury in humans is associated with an increased number of βAPP-immunoreactive neurons, 37–39 as are other chronic and acute disorders of the central nervous system, including infarction. 1 Blood-derived cells also could be a significant source of βAPP in ischemic brain regions. On stimulation, βAPP is expressed by the major functional types of T lymphocytes, leukocytes, and peripheral blood monocytes. 40 The pathological accumulation of βAPP after a variety of insults suggests a role for this protein in the central nervous system response to injury. 41–45 A 5-fold increase in Aβ has been reported for aged transgenic mice overexpressing the 695-amino acid isoform of human βPP containing a Lys670→Asn, Met671→Leu mutation 46; it would be interesting to determine whether overexpression of Aβ in these animals influences the response of the brain to ischemic stroke. The potential role of apolipoprotein E in the response of the brain to ischemia 28 also warrants further study, particularly in aged animals. A delayed response to damage in aged animals, in conjunction with the secretion of molecules having a deleterious effect on tissue regeneration, such as Aβ, may explain, in part, the failure of older systems to recover function after cerebral ischemia.

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References
34. Popa-Wagner et al October 1998 2201
Until recent years, the age of experimental animals has often been ignored. The use of juvenile animals to study a disease associated with aging may be a factor in the poor correlation, particularly of treatment effects, between experimental models of stroke and human patients with stroke. There are important differences in the age-related tissue responses to ischemia, emphasized by Popa-Wagner et al. Although there has been evidence that ischemic stress increases Aβ production, there is also evidence that Aβ enhances hippocampal neuronal survival in vitro. Rather than being contradictory, this may indicate that there are complex relationships between βAPP or Aβ with neurons. Evaluation of differences between model systems (in vitro versus whole-animal models), along with studies of age related differences, may help elucidate the role of βAPP and Aβ in tissue damage from ischemia. The increased accumulation of βAPP and Aβ in aged animals could suggest a detrimental role of these substances, as age is related to more severe outcome from stroke, but this possibility needs to be studied specifically.

Another interesting question is the identity of the cell producing the βAPP and Aβ. There is evidence that human neurons undergoing apoptosis generate excess Aβ. The comparative mechanisms of death (apoptosis versus necrosis) in ischemic tissue in aged animals has not been studied. Although βAPP was localized to macrophages in the present study, it is not clear that macrophages produce βAPP or whether the macrophages are removing the βAPP. In situ hybridization for βAPP mRNA will be necessary to elucidate the source of the βAPP.

Nancy Futrell, MD, Guest Editor
Intermountain Stroke Research Foundation
Salt Lake City, Utah

References
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