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The Relationship of Amyloid Plaques to Cerebral Capillaries in Alzheimer's Disease

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The authors examined the hypothesis that senile plaques of Alzheimer's disease (AD) are formed by abnormal leakage of amyloidogenic precursors from brain capillaries by quantitative analysis of the spatial relationship between capillaries and amyloid plaques. Vibratome sections (40 μ) of the hippocampus, including the entorhinal cortex, obtained at autopsy from AD subjects, were immunostained with a monoclonal antibody to β-protein and counterstained with rabbit serum to either the glucose transporter protein, a cerebral endothelial marker, or collagen type IV, a basal lamina marker. The authors found that while 60% to 77% of amyloid plaques were associated with capillaries, only 8% to 13% were penetrated by a capillary, the remainder being adjacent. To test whether 1) the area occupied by amyloid plaques or 2) the border zone (10-μ rim) surrounding amyloid plaques has a statistically higher density of capillaries than 3) the remaining gray matter, similarly double-stained 6-μ sections from five AD subjects were photographed and the capillary densities in the three areas calculated. Capillary density was significantly lower in 1) than in 3) and higher in 2) than in 3), while the combined area of 1) and 2) showed the same capillary density as 3). Similar results were obtained by using either the glucose transporter or the collagen type IV antibodies. Because capillary density is low within, and high in regions that immediately surround amyloid plaques, our findings suggest that amyloid plaques exclude capillaries or lead to their degeneration, or both. The latter possibility was investigated by triple-staining tissue sections with antibodies to β-protein, glucose transporter, and collagen type IV. The proportion of glucose transporter-negative capillaries was not significantly different in areas inside or outside of the plaques. Thus, the authors found no evidence of basal lamina remnants consistent with capillary degeneration preferential to amyloid plaques. Although a small number of capillaries showed amyloid deposition just beneath the basement membrane, the authors conclude that capillaries play only a limited direct role, if any, in amyloid plaque formation, and that the apparent association of amyloid plaques and capillaries is no more than a chance contact. (Am J Pathol 1990, 137:1435–1446)

Amyloid plaques are a hallmark of the lesions of Alzheimer's disease (AD).[1] There is much debate regarding the origin and location of the extracellular amyloid plaques that are easily seen in histochemically and immunocytochemically stained sections, probably because until recently we lacked the tools to consistently identify amyloid plaques in all cases. The sequencing of the β-protein, the primary component of the amyloid deposit found in all senile plaques, made the unambiguous identification of amyloid-containing plaques possible.[2–6] The cloning of the gene for the β-amyloid precursor protein (βAPP) and subsequent production of probes specific to βAPP showed that degradation of the βAPP to form the β-protein in the amyloid plaque probably occurs *in situ*.[7] Therefore, βAPP accumulation may be an important initiating event in the genesis of the amyloid plaque.

Two main hypotheses were advanced to explain the source of βAPP in the amyloid plaque: the neuronal and vascular hypotheses. The primary evidence supporting neuronal origin is the high expression of βAPP in neurons.[8,9] The neuronal hypothesis, however, does not address what determines the location of amyloid plaques.

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The vascular hypothesis rests mainly on the deposition of amyloid in the walls of blood vessels in AD and on the intimate association of capillaries with amyloid plaques. It was reported that all the brains of AD subjects show congophilic angiopathy, although the extent of amyloid deposition is inconsistent among subjects.[10] Furthermore many investigators reported that some amyloid plaques are penetrated by or are in close proximity to capillaries, suggesting that microvessels may play an important and direct role in amyloid plaque formation.[11-17] In these reports, however, the association between amyloid plaques and microvessels was not consistent, varying from 36% to 100%,[14,17-19] and rigorous quantitative analysis was not performed.

To understand the role played by the blood vessels in amyloid plaque pathogenesis, we addressed the following three issues in this study: 1) Are amyloid plaques topographically associated with capillaries? We found that 60% to 77% of amyloid plaques are associated with blood vessels; however, 8% to 13% are penetrated by capillaries. The rest have capillaries at their periphery. 2) Is the capillary density greater within the amyloid plaque than in the rest of the cortical gray matter? This question is important to rule out chance association between these two structures. We found that the capillary density in plaques is significantly lower, but that capillary density is higher in the border zone of plaques. 3) Is the low capillary density in amyloid plaques due to disappearance or degeneration of endothelial cells? To answer this question, we compared the numbers of capillaries visualized by the two microvessel markers: antibodies to the glucose transporter, a marker of cerebral vascular endothelial cells, and antiserum to collagen type IV, a marker of the basement membrane. Our hypothesis was that glucose transporter immunoreactivity is lost by the degeneration or dysfunction of endothelial cells, while collagen immunoreactivity is retained for some time after the disappearance of endothelial cells. Our results do not demonstrate a definite discrepancy in immunostaining with these two microvessel markers, either inside or outside the plaque.

In keeping with terminology used in previous studies, the term *capillary* is used interchangeably with *microvessel*, although the latter term may be more appropriate. A vessel size of less than 10 μ in diameter was the criterion that we used to identify capillaries. Vessels with apparent smooth muscle layer in their walls were excluded. Despite these exclusions, a small number of venules and arterioles were possibly included in our study.

Materials and Methods

Tissues

A 0.5- to 1-cm-thick coronal block of the hippocampal gyrus, including subiculum and entorhinal cortex, was obtained at autopsy from eight subjects with the clinical diagnosis of AD (Table 1). The diagnosis was confirmed by histopathologic examination. None had intracerebral hemorrhage or infarction. Tissues were immersed in Bouin's fixative and then processed in two ways for immunocytochemistry. In the first, the tissue was dehydrated after 48 to 72 hours, embedded in paraffin, cut at 6 μ, and rehydrated (seven cases). In the second procedure, unembedded wet tissue was sectioned at 40 μ using an Oxford Vibratome Model G (Technical Products International Inc, Earth City, MO) and rinsed in TRIS-buffered saline, pH 7.6 until the washing solution was colorless (four cases).

Antibodies

We used three brain microvessel markers. One was a rabbit antiserum raised to the basement membrane component, collagen type IV (gift of Dr. Steven Ledbetter). Collagen type IV has been established as a capillary marker.[20] The other two were antibodies to the glucose transporter; a rabbit antiserum (gift of Dr. Ora Rosen) and a mouse monoclonal antibody (MAb) (gift of Drs. L. Andersson and P. Lundahl) were used as endothelial cell markers.[21,22] Both antibodies were raised against synthetic peptides homologous to the carboxyl-terminal of the human erythrocyte glucose transporter[23,24] and recognized identical structures by double immunostaining (data not shown). Amyloid plaques were stained using a mouse MAb raised to a synthetic peptide corresponding to residues 1 through 10 of the β-protein (2A1/10B10) (gift of Dupont and Dr. George Glenner).

Immunostaining

Double Staining

The localization of blood vessel markers (collagen type IV or glucose transporter) was detected by the peroxidase-anti-peroxidase (PAP) method, and that of the β-protein by the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method. Immunoreactivity of the amyloid plaque was increased by pretreatment of the sections with 70%

Table 1. *Alzheimer Disease Subjects*

Case	Age	Sex	Duration of dementia (years)	Postmortem time (hours)
1	85	M	5	2
2	83	M	3-5	2
3	64	M	14	—
4	72	M	5	—
5	71	F	5	5.5
6	80	M	5	4
7	63	M	8	3
8	92	F	10	3

formic acid for 5 minutes.[25] Because we noted that this procedure destroyed the antigenicity of the glucose transporter, we stained the glucose transporter before β-protein. For PAP staining, the sections were pretreated with 3% hydrogen peroxide to inactivate the endogenous peroxidase activity. The primary antibodies were applied to tissue sections and incubated at 4°C overnight at the following dilutions: the antiserum to collagen IV, 1:100; the antiserum to glucose transporter, 1:750; the MAb to glucose transporter of the hybridoma culture supernatant at 1:1, the MAb to β-protein, at 30 μg/ml. The secondary antibodies (affinity-purified goat anti-rabbit and anti-mouse immunoglobulins heavy and light chain; Organon Technika, Durham, NC) were diluted at 1:50 and incubated at room temperature for 30 minutes. The tertiary antibodies, peroxidase anti-peroxidase produced in rabbit (Organon Technika) and alkaline-phosphatase anti-alkaline-phosphatase raised in mouse (Vector Laboratories, Burlingame, CA) were diluted at 1:250 and 1:20, respectively, and incubated at room temperature for 1 hour. The alkaline phosphatase activity was demonstrated using naphthol AS-MX phosphate (Sigma, St. Louis, MO) and fast red TR (Sigma) as chromogens according to Cordell et al.[26] The peroxidase was developed by 3,3′-diaminobenzidine (DAB) (Sigma). Sections were mounted with an aqueous base medium, Aquamount (Learner Laboratories, New Haven, CT) or Crystal/Mount (Biomeda Corp., Foster City, CA).

Triple Staining

To investigate any possible discrepancy between the endothelial and basement membrane markers of microvessels in the amyloid plaques, we triple stained sections by using mouse glucose transporter, rabbit collagen type IV, and mouse β-protein antibodies. The MAb to glucose transporter raised in mouse and the rabbit antiserum to collagen IV were first applied simultaneously and visualized by the APAAP and PAP methods, respectively, using fast blue BB (Sigma)—naphthol AS-MX and DAB as chromogens. After formic acid treatment, the third antigen, β-protein, was stained by using the APAAP method and fast red TR — naphthol AS-MX as a chromogen. Although in some cases a partial development of the first mouse antibody (MAb to the glucose transporter) was noted when the second mouse antibody (MAb to β-protein) was used, this faint cross-staining did not prevent the discrimination between the endothelium and amyloid plaques, because amyloid plaques showed a pure red color and the two structures are morphologically distinct.

Morphometry

Quantitation was performed to determine whether the density of microvessels within amyloid plaques or in the border region is different from that in the rest of the gray matter. We photographed (35-mm, Technical Pan, Eastman Kodak, Rochester, NY) the entire depth of the gray matter of the entorhinal cortex from 6-μ sections double stained by the β-protein MAb and one of the blood vessel markers (anti-collagen type IV in five cases and anti-glucose transporter in three cases) by using a Zeiss Axiophot microscope with a 10× plan apochromat objective (Optivar = 1.25) (Carl Zeiss, Thornwood, NY). The negatives were printed to a final magnification of 110× and assembled to represent the entire region. Each amyloid plaque was delineated on the print as the region stained by the MAb to β-protein. Most of the amyloid plaques were smaller than 35 μ in diameter. The border of each plaque was confirmed by direct observation in the microscope. The border zone was arbitrarily defined as the rim area extending 10 μ outside of the amyloid plaque. In these 6-μ sections, most of the capillaries appeared as circles or ellipses; however, a small number of the vessels followed the plane of section for some distance. In all cases, the position of the vessel was represented by its geometric center. Whenever uncertain on the photographic print, capillaries associated with plaques were confirmed by direct observation in the microscope. The total areas of the three categories, ie, the exterior, the border zone, and the interior of the plaques, were measured using a Bioquant image analysis system (R & M Biometrics, Nashville, TN). The total number of the vessels in each area was counted and the capillary density was calculated as capillaries per square millimeter.

Statistical Analysis

To evaluate differences in the capillary density of two regions, the null-hypothesis χ^2 test was used.

$$\chi_0^2 = \frac{(n_1 - \alpha n_2)^2}{\alpha(n_1 + n_2)}$$

where n_1, n_2 are the number of blood vessels in regions 1, 2, respectively, and α = area 1/area 2 (See Appendix).

Results

Topographic Relationship Between Amyloid Plaques and Capillaries (Double-stained 40-μ Sections)

As shown in Figure 1, although the majority of amyloid plaques were in close proximity to capillaries, most of the capillaries were adjacent to the plaques, and only a minority of them penetrated the plaques. Some vessels followed the border of amyloid plaques. Amyloid plaques

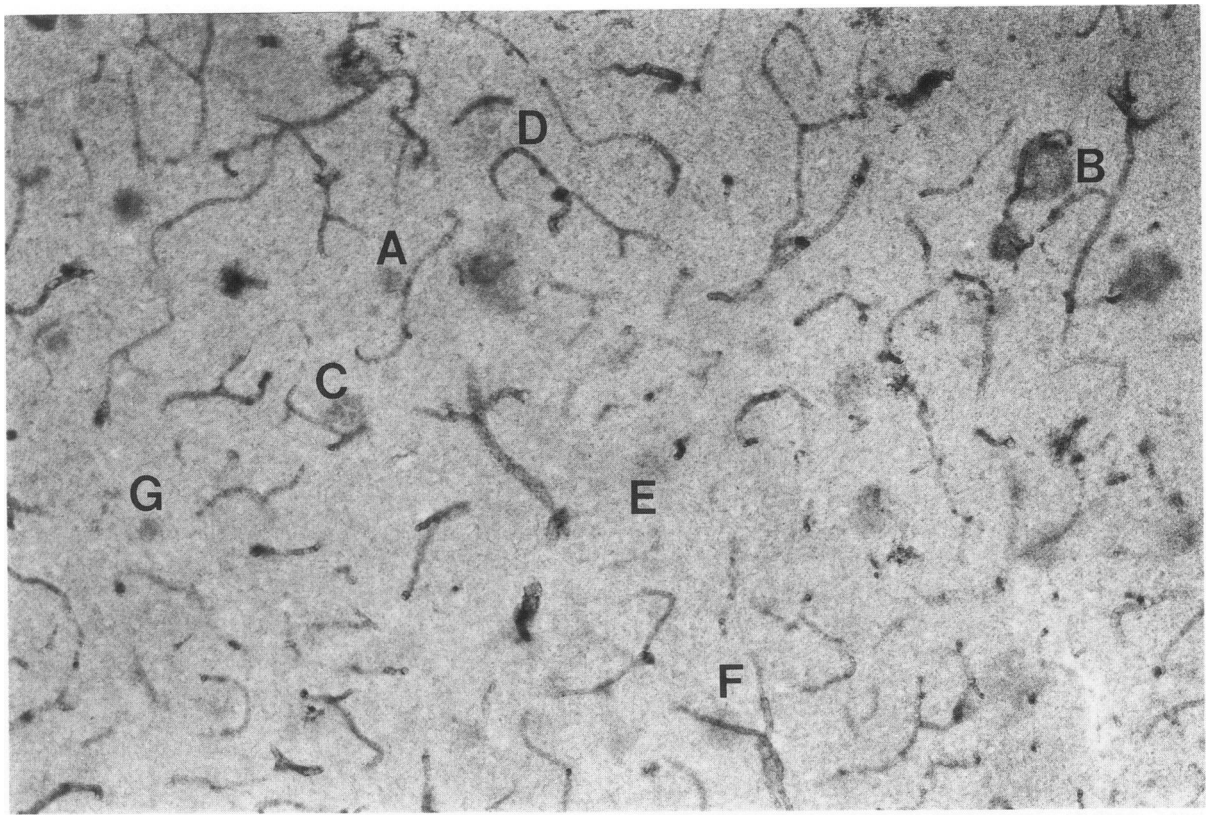

Figure 1. *Forty-micronmeter-thick Vibratome section from case 3, double stained with monoclonal antibody to β-amyloid protein and antiserum against collagen type IV (110×). The majority of amyloid plaques are associated with, attached to, or penetrated by a capilary; however some are independent. A–G are the examples of the relationship between amyloid plaques and capillaries shown in Figure 2.*

were found occasionally at capillary branch points. Some plaques were associated with two or more blood vessels.

These findings were quantified by classifying amyloid plaques in the entorhinal cortex according to the scheme shown in Figure 2. This subclassification of the relation plaques of a capillary or capillaries, although arbitrary, is important to understand the incidence of the abovementioned findings. Based on this, we found that 60% to 77% of amyloid plaques were related to capillaries, but only 8% to 13% were penetrated by blood vessels (Table 2). This result suggested that the blood vessel density is lower within amyloid plaques than in the surrounding area, or more specifically border area.

Capillary Density in the Amyloid Plaque, Its Border Zone, and in Other Areas of the Cerebral Cortex

As discussed in the previous paragraph, the majority of plaques were related in one way or another to capillaries. We were uncertain, however, whether this topographic association was statistically significant in the face of numerous amyloid plaques and dense capillary network. The high density of capillaries at the boundaries of plaques compared with their paucity within these plaques suggested three possibilities: first capillary density is low within amyloid plaques; second capillary density is high in the bordering areas; or third a mixture of the first two possibilities. To clarify these issues, we determined the capillary density of the following three compartments: the exterior, the border zone area, and the interior of the plaques, in

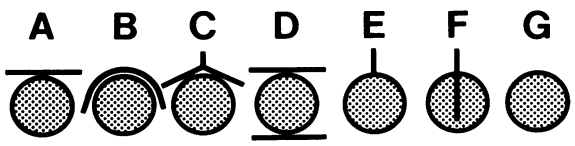

Figure 2. *Classification of the relationship between amyloid plaques and capillaries.* **A:** *Plaque tangentially attached to blood vessel.* **B:** *Plaque encircled by the vessel.* **C:** *Plaque associated with the branch point of the vessel.* **D:** *Plaque associated with multiple vessels.* **E:** *Plaque not tangentially attached to the blood vessel.* **F:** *Plaque penetrated by a vessel.* **G:** *Plaque not associated with any vessel. From A to E are the subclassifications of the plaques in contact with blood vessels. Plaque E could be penetrated by the blood vessel in an adjacent cortex.*

Table 2. *Topographic Relationship Between Amyloid Plaques and Capillaries*

Case	Number of plaques examined	A	B	C	D	E	A–E	F	G
1	152	23.7	9.2	4.6	3.9	11.2	52.6	7.9	39.5
4	181	33.1	6.1	4.4	6.6	5.5	55.8	8.3	35.9
5	155	40.6	7.1	3.9	5.8	10.3	67.7	5.1	27.1
6	91	33.0	7.6	8.8	7.7	6.6	63.7	13.2	23.1

The results in columns A to G denote percentage of amyloid plaques demonstrating the relationships to capillaries as defined in Figure 2.

6-μ-thick sections. Table 3A shows area of the three compartments of the entorhinal cortex we analyzed. Area of the interior or the border zone was not more than 3.1% of that of the exterior in all cases analyzed. In three cases (cases 2, 3, and 4), capillary density was determined for both collagen type IV (Figure 3A) and glucose transporter (Figure 3B). In two cases (cases 5 and 6), only collagen type IV was used as a capillary marker (Table 3A).

Tables 3B and 3C show the vessel density for the three compartments. In all cases analyzed, the capillary density within the amyloid plaque was significantly lower than that of gray matter outside plaques ($P \leq 0.01$). In contrast, the capillary density in the boundary area was greater than in the rest of the gray matter in all cases and was statistically significant ($P \leq 0.01$) in all except case 4 when stained by collagen type IV. These results showed clearly that vessel density was low within plaques and high in the periphery of plaques. The next question we addressed was whether the whole plaque-related area, ie, the area of the senile plaque plus the border zone, is more or less vascular than the rest of the gray matter. If plaques are more vascular, it suggests either that the plaques are generated around capillaries or that plaques attract blood vessels. If less vascular, the senile plaque may lead to capillary degeneration. If both areas are equally vascular, the exclusion hypothesis is most likely, ie, amyloid plaques displace capillaries to their periphery. When the interior plus the border zone areas of amyloid plaques were combined, the capillary density was not found to be significantly different from the rest of the gray matter ($P < 0.05$), with the exception of case 3, where the density of capillaries in areas in and around the plaques was significantly lower.

Glucose Transporter Negative Capillaries

To investigate the possibility that capillaries within senile plaques degenerate, we compared the immunoreactivity of the vessels with antibodies to the glucose transporter and collagen type IV. Our hypothesis is that, in the course of capillary degeneration, basal lamina structures stained by the anti-collagen type IV remain, as suggested by Miyakawa et al,[17] even after the disappearance of the endothelial cells recognized by the glucose transporter antibody. Thus, if collagen-positive and glucose transporter-negative capillaries are more common within than outside the amyloid plaque, this suggests capillary degeneration within amyloid plaques.

The densities of capillaries identified by the two blood vessel markers were compared. None of the three cases thus analyzed showed significant differences ($P > 0.05$) in capillary density determined by either antibody, except in the area outside amyloid plaques in case 3 (Table 3C).

Discrepancy within amyloid plaques was analyzed in triple-stained 6-μ-thick sections from four cases of AD with the two vessel markers (glucose transporter and collagen type IV) and the β-protein MAb. The antibody to the glucose transporter stained endothelial cells, which typically were surrounded by the basement membrane recognized by the collagen type IV antiserum. Additionally, the basement membrane of smooth muscles in small arteries was stained by the collagen type IV antibody. Infrequently, glucose transporter-negative vessels were observed not only in close association with amyloid plaques, but also in the remainder of the gray matter (Figure 3C).

The lack of immunoreactivity to the glucose transporter in these few microvessels can be interpreted as follows:

Table 3A. *Area of Inside, Border, and Outside of Amyloid Plaques of the Entorhinal Cortex Region Studied to Determine Capillary Density (see text) Expressed in square millimeters*

	Glucose transporter			Collagen type IV		
Case	Inside	Border	Outside	Inside	Border	Outside
2	0.547	0.509	17.929	0.575	0.520	20.071
3	0.434	0.392	26.355	0.524	0.431	25.919
4	0.271	0.320	12.778	0.171	0.173	13.116
5				0.379	0.349	22.905
6				0.250	0.192	20.718

Table 3B. *The Capillary Density Expressed as Number of Capillaries per square millimeters Inside, Outside, and Bordering Amyloid Plaques Determined by Using Either the Glucose Transporter or Collagen Type IV Antibodies as Vascular Markers*

	Glucose transporter				Collagen type IV			
Case	(1) Inside	(2) Border	(3) Inside & border	(4) Outside	(1') Inside	(2') Border	(3') Inside & border	(4') Outside
2	32.9	202.4	114.6	131.5	36.5	250.0	137.9	127.6
3	18.4	216.8	112.6	153.9	17.2	234.3	115.2	161.0
4	14.8	168.8	113.3	117.7	35.1	167.6	101.7	123.1
5					18.5	186.3	98.9	115.4
6					24.0	177.0	90.5	111.9

1) As a normal finding (eg, very thin endothelial cells may occasionally be difficult to visualize by anti-glucose transporter antibody; or if the capillary was sectioned longitudinally, only the basement membrane might be included in the section).

2) As a postmortem change.

3) As a pathologic process of AD.

Thus any discrepancy between the two blood vessel markers can be interpreted in the context of the pathologic processes of amyloid plaque formation of AD only if the frequency of glucose transporter-negative capillaries was significantly higher in amyloid plaques than in the rest of the gray matter. Therefore, the relative frequency of the glucose transporter-negative capillaries in each of the three compartments described above was determined. All vessels related to plaques in the entorhinal cortex section were counted, while 1000 capillaries unrelated to plaques were sampled from the whole depth of gray matter of entorhinal cortex. The longitudinally sectioned capillaries were excluded.

The results summarized in Table 4 indicate no significant difference ($P > 0.05$) in the density of glucose transporter-negative capillaries between the interior and the exterior, nor between the border zone and the exterior of amyloid plaques. These results indicated that even if degenerating capillaries were found in the amyloid plaque, there was no evidence that it is related to amyloid deposition. Consequently, the distribution of capillaries relative to amyloid plaques is most reasonably explained by the hypothesis that the amyloid plaque excludes capillaries.

Other Findings

Although our quantitative analysis does not support an amyloid plaque–vascular causal relationship, we did note amyloid plaques in close proximity to vessels. These amyloid plaques were not only penetrated by a vessel, but also showed a special relationship with the amyloid deposit; amyloid was demonstrated along and just beneath the capillary wall (Figure 3D). This observation led us to speculate that this type of plaque was generated in association with the blood vessel. These vessel-related plaques frequently took an elongate form along the vessel. On very rare occasions, an amyloid deposit was observed just beneath the capillary basement membrane without forming a plaque. Although not common, vessel-related deposits were observed in many but not all the sections; however, the number was very small (at most three per section), not allowing us to statistically evaluate their incidence.

Discussion

Several lines of evidence were advanced to support the hypothesis that the senile plaque amyloid originates from the leakage of hematogenous amyloidogenic proteins through the blood–brain barrier (BBB).

First the observation that most cases of AD show cerebrovascular amyloidosis. Amyloid deposition is confined to the wall of the leptomeningeal and large cortical vessels (congophilic angiopathy).[27] In contrast, in small cortical vessels and capillaries, amyloid deposits infiltrate surrounding neuropil *(drüsige Entartung der Hirnarterien und*

Table 3C. χ_0^2 *Values for Statistical Comparisons of Significance of Data Shown in Table 3B*

Case	1–4	2–4	3–4	1'–4'	2'–4'	3'–4'	1–1'	2–2'	3–3'	4–4'
2	34.95*	18.93*	2.17	36.76*	58.85*	0.93	0.10	2.58	2.30	1.42
3	51.79*	9.77*	8.62*	70.20*	11.62*	14.84*	0.02	0.28	0.00	6.86*
4	24.32*	6.90*	1.83	10.72*	2.74	1.24	1.92	0.00	0.03	1.52
5				30.64*	15.99*	1.67				
6				17.22*	8.77*	1.74				

* $P \leq 0.01$.

-capillaren and l'angiopathie dyshorique).[11,12] The latter is associated with dystrophic axons immunostained with antibodies to paired helical filament or microtubule-associated protein τ and indistinguishable from senile plaques.[28,29]

Second light microscopy studies using serial sections demonstrated that most senile plaques are topographically closely associated with capillaries. Ishii[14] showed 65% to 91% of amyloid plaques were penetrated by or attached to capillaries in serial sections stained by PAS. Recently Arai et al[30] addressed the same issue immunohistochemically using β-protein antiserum, and obtained a similar result; however no quantitative analysis was performed. At the ultrastructural level, Miyakawa et al[17] observed at least one degenerating capillary in each amyloid plaque by examining serial sections. These authors suggested that amyloid fibrils are formed at the basement membrane of abnormal capillaries and then project to the surrounding area.

Third many serum proteins, including γ-globulins, albumin, fibrinogen, and complement C_3, have been demonstrated in the area of the amyloid core.[31,32] This observation is consistent with the abnormal permeability of the BBB to macromolecules within senile plaques. The role of increased vascular permeability was proposed by Glenner,[33] who suggested that the leakage of an unknown toxic serum substance from congophilic microvessels causes neuronal degeneration and amyloid formation.

Although it is tempting to speculate that the amyloid in the senile plaque is formed from a serum precursor protein coming through a leaky microvessel on the basis of the observations mentioned above, some objections can be raised against this hypothesis. First several quantitative or semiquantitative studies yielded conflicting data concerning the relation between cerebrovascular amyloidosis and amyloid plaque formation. In 1974, Mandybur[34] studied the incidence of amyloid angiopathy in 15 AD subjects using Congo red staining and pointed out a correlation between the presence of amyloid-rich plaques and cerebral amyloid angiopathy, although two cases showed no amyloid vascular degeneration. No quantitative analysis was performed in that study. In 1982, Mountjoy et al[35] semiquantitatively investigated the possible relationship between the amyloid in senile plaques and blood vessels. They analyzed 15 demented and 30 nondemented subjects using Congo red as a marker of senile plaque and blood vessel amyloid. In the demented group, a statistically significant correlation between the two structures was obtained only in the temporal lobe, but not in other regions. Three recent studies quantitatively addressed this issue with conflicting results. Yamada et al[36] examined 123 autopsy brains from aged subjects and demonstrated that the presence and severity of amyloid angiopathy significantly correlated with the density of senile plaques (probably neuritic plaques stained by silver impregnation). Bergeron et al[37] studied 30 AD brains and found no significant correlation between amyloid angiopathy and the severity of neuritic plaques stained by silver impregnation. But vascular amyloid and plaque amyloid were significantly but weakly correlated ($r = 0.507$) in their study, although remarkable discrepancy was observed in some cases. Contrary to these studies, Rosenblum and Haider[38] reported a negative correlation ($r = -0.48$) between the number of congophilic plaques and congophilic vessels. Thus, although the results of these studies are conflicting, none of them indicated a high correlation between vascular and senile plaque amyloid and all of them mentioned several cases with remarkable discrepancy.

Second if all amyloid plaques are formed around capillaries, then capillary density should determine the density of amyloid plaques. Many studies have indicated discrepancy between capillary and amyloid plaque distribution. These include 1) although cerebral white matter is less vascular than gray matter, the difference in the density of capillary network does not explain the extreme scarcity of amyloid plaques in white matter (see Figure 1 of Wisniewski et al[39]). 2) The laminar distribution of senile plaques and capillary density was studied by many researchers.[19,40–45] In the striate cortex, lamina IV is the most heavily vascularized,[19,40] but senile plaques in AD subjects are most densely distributed in the lamina II–III,[19] with some clustering at the border of layer IVc and V.[44,45]

Finally, with regard to the percentage of plaques in close association with microvessels, some investigators reported much lower figures than those found in this study (36% by Friede and Magee,[18] 55% by Bell and Ball[19]). The actual percentage may not be important, as it probably depends on the methods and criteria used to define the association. The most important question is whether the association is different from that due to chance. Mountjoy et al[35] observed that 'a plaque would be seen in close proximity to an affected vessel, but this appeared to be no more than a reflection of random distribution of plaques,' although no prior study addressed this question quantitatively.

The advantages of our study include the fact that we used immunocytochemistry for identification of amyloid plaques and blood vessels. Antibodies to β-protein allow more sensitive visualization of amyloid deposition than conventional Congo red staining[46–48] and reveal a similar number of plaques to the Bielschowsky method.[48] By using two capillary markers, we could address the question of capillary degeneration in the plaque at the light microscopic level.

The present study demonstrated several important findings:

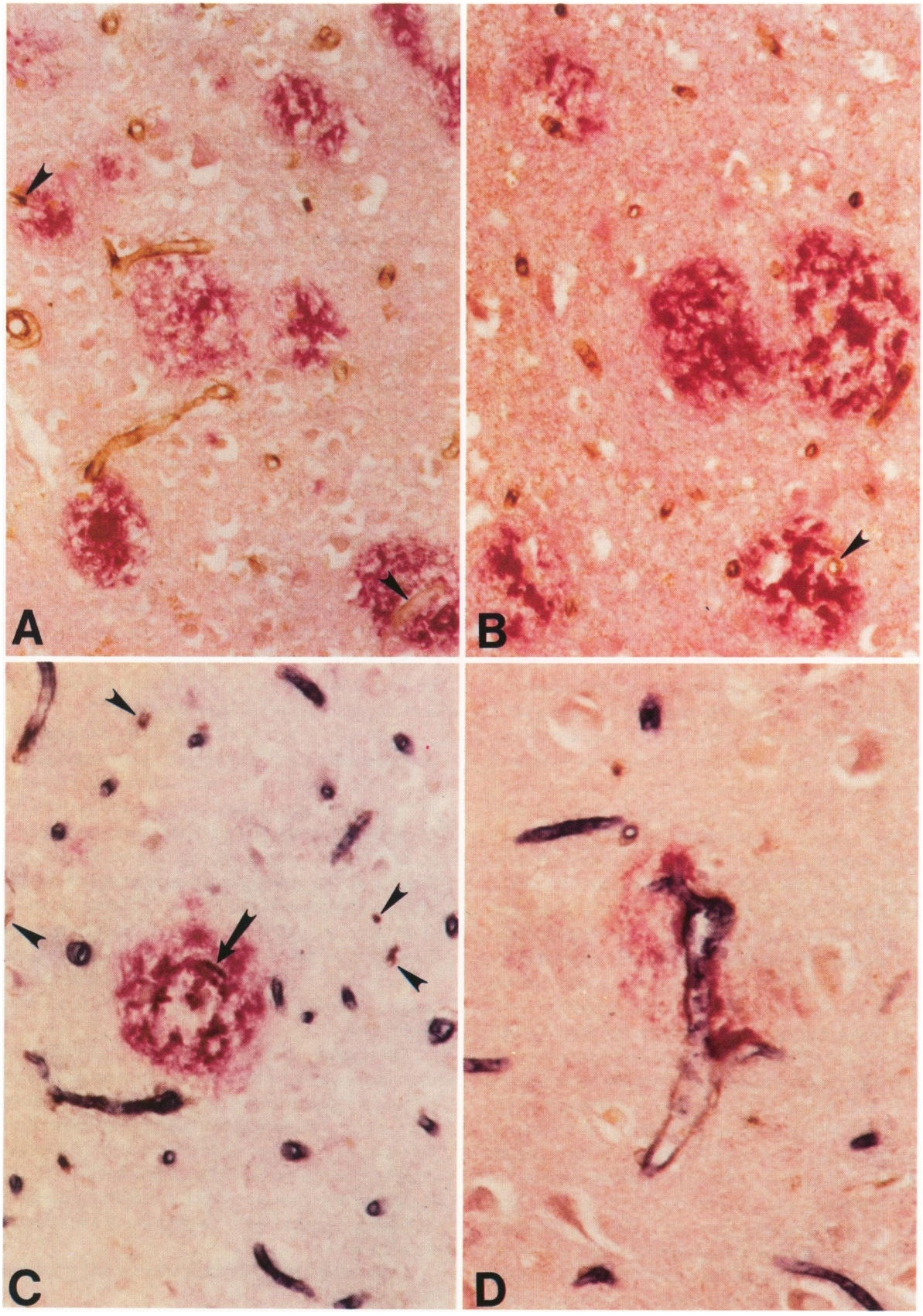
A
B
C
D

Figure 3. A, B: *Double-immunocytochemical staining of entorbinal cortex from case 3 with monoclonal antibody to β-amyloid protein (red amyloid plaques) and antiserum against collagen type IV (brown)* (A) *and antiserum against glucose transporter protein (brown)* (B), *indicate the association of vessels to amyloid plaques. While most of the amyloid plaques are associated with capillaries (brown) at their periphery, some are penetrated by a capillary (arrowheads).* C, D: *Triple immunocytochemical staining of entorbinal cortex from case 3* (C) *and case 8* (D) *with monoclonal antibodies against β-amyloid protein (red amyloid plaques), glucose transporter protein (blue capillary staining), and antiserum to collagen type IV (brown capillary staining). In* C, *glucose transporter protein negative capillaries or capillary remnants are not only observed within the amyloid plaque (arrow) but also outside the plaque (arrowheads). In* D, *an amyloid plaque associated with a blood vessel is shown, clearly β-protein deposition just beneath the basement membrane of the blood vessel* (A–D ×400).

First 60% to 77% of amyloid plaques were in contact with or penetrated by capillaries. This percentage was very close, however slightly lower than the figures reported by Ishii (65% to 91%).[14] We have undertaken a three-dimensional reconstruction study using serial sections and the same immunocytochemical techniques to address the issue of vessel and plaque association. Preliminary data showed two thirds of amyloid core containing plaques are associated with capillaries (Kawai et al, manuscript in preparation).

Second a small number of capillaries showed deposition of amyloid just beneath the basement membrane, suggesting a role for capillaries in the generation of amyloid plaques. Although this finding, presumably identical to *drüsige Entartung der Hirnarterien und -capillaren* (Scholz)[11] or *l'angiopathie dyshorique* (Morel),[12] was very impressive, the incidence was low and inconstant among the cases, suggesting that it cannot play an important role in forming the majority of amyloid plaques.

Third capillary density was not elevated in the plaque area compared with the remainder of the gray matter. This finding indicates that the apparent association of amyloid plaques and capillaries is not more than a chance contact.

Fourth there was no evidence of capillary degeneration exclusive or preferential to amyloid plaques. This result, although not precluding the capillary degeneration hypothesis, made it much less probable.

Fifth amyloid plaques were heavily vascularized at the periphery, whereas the capillary density was very low in the interior of the plaques. Our analysis indicates that this finding is most reasonably explained by the exclusion of vessels from amyloid plaques. Occasionally we did observe capillaries following the border of amyloid plaques. These capillaries probably correspond to the 'capillary loop encircling the periphery of the plaque' described by Friede and Magee,[18] and were consistent with this hypothesis.

In the light of these results, it is presumed that the capillaries play only a limited direct role in the formation of amyloid plaques. Our findings indicate that congophilic angiopathy and amyloid plaques are two distinct modes of amyloid deposition in Alzheimer disease. Although these two pathologic processes are observed simultaneously in a single case, they are not always dependent on each other, suggesting that they are independent processes that probably share some pathogenetic mechanisms. Differences in the C-terminal sequence, N-terminal blockage, and solubility of the β-protein in the two loci support this idea.[49,50] Conversely, because the majority of the plaques bear no direct topographic relationship to vessels, the question arises of what produces the amyloid precursor protein and what determines the site of amyloid deposition. At the present time available information cannot resolve the issue. Our results do not preclude the possibility that the amyloid precursor protein leaks from serum into the brain parenchyma through functionally abnormal blood vessels; however recent studies using *in situ* hybridization and immunohistochemistry have shown that almost all the cell components in the brain, ie, neurons, astrocytes, microglia, endothelial, and choroid plexus cells, express the βAPP gene,[51,52] although neurons and their projections show particularly high concentrations of the precursor.[53] Understanding of the chronologic sequence leading to senile plaque pathology, particularly the very early stages, will therefore elucidate how amyloid plaques form at discrete locations.

Table 4. *The Prevalence of Glucose Transporter (an Endothelial Marker) Negative Capillaries Was Found to Be Unrelated to the Association of the Capillary with Amyloid Plaques*

Case	Inside (1)	Border (2)	Outside (3)	χ_0^2 1–3	χ_0^2 2–3
3	0/7	12/116	127/1000	1.017	0.527
6	1/17	8/101	87/1000	0.168	0.071
7	0/2	1/27	76/1000	0.165	0.576
8	1/18	8/97	83/1000	0.176	0.000

None of the χ^2 test showed a significant difference at $P = 0.05$.

Values shown of glucose transporter negative vessels compared to total vessels for each compartment. χ_0^2 values indicate a lack of significance in the incidence of glucose transporter negative vessels and their proximity to amyloid plaques.

Appendix

Chi-square analysis was used to evaluate the significance of differences in vessel density between two regions. Suppose the blood vessels to be randomly scattered in both region 1 and region 2. Let the region 1 and region 2 be divided into N_1, N_2 unit areas, so that each unit area contains at most one blood vessel and $\frac{n_1 + n_2}{N_1 + N_2}$ is negligibly small.

$$\text{Then } \frac{N_1}{N_2} = \frac{\text{area 1}}{\text{area 2}} = \alpha$$

The problem was now replaced by the comparison of the proportion of the units with blood vessel in the following 2×2 table.

	Units with blood vessel	Units without blood vessel	Total
Region 1	n_1	$N_1 - n_1$	N_1
Region 2	n_2	$N_2 - n_2$	N_2
Total	$n_1 + n_2$	$N_1 + N_2 - (n_1 + n_2)$	$N_1 + N_2$

The null hypothesis is that the two regions have the same proportion of the units with blood vessels. Then the expected number of units for each cell is shown as follows:

	With blood vessel	Without blood vessel
Region 1	$N_1 \frac{n_1 + n_2}{N_1 + N_2}$	$N_1\left(1 - \frac{n_1 + n_2}{N_1 + N_2}\right)$
Region 2	$N_2 \frac{n_1 + n_2}{N_1 + N_2}$	$N_2\left(1 - \frac{n_1 + n_2}{N_1 + N_2}\right)$

$$\chi_0^2 = \frac{\left(n_1 - N_1 \frac{n_1 + n_2}{N_1 + N_2}\right)^2}{N_1 \frac{n_1 + n_2}{N_1 + N_2}} + \frac{\left(n_2 - N_2 \frac{n_1 + n_2}{N_1 + N_2}\right)^2}{N_2 \frac{n_1 + n_2}{N_1 + N_2}}$$

$$+ \frac{\left\{(N_1 - n_1) - N_1\left(1 - \frac{n_1 + n_2}{N_1 + N_2}\right)\right\}^2}{N_1\left(1 - \frac{n_1 + n_2}{N_1 + N_2}\right)}$$

$$+ \frac{\left\{(N_2 - n_2) - N_2\left(1 - \frac{n_1 + n_2}{N_1 + N_2}\right)\right\}^2}{N_2\left(1 - \frac{n_1 + n_2}{N_1 + N_2}\right)}$$

$$\chi_0^2 = \frac{(n_1 - \alpha n_2)^2}{\alpha}\left(\frac{1}{n_1 + n_2} + \frac{1}{N_1 + N_2 - n_1 - n_2}\right)$$

Because $\frac{n_1 + n_2}{N_1 + N_2}$ is negligibly small, $\chi_0^2 = \frac{(n_1 - \alpha n_2)^2}{\alpha(n_1 + n_2)}$

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