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RESEARCH ARTICLE

Microglial Activation in Alzheimer Disease: Association with APOE Genotype

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Microglial cells are considered to play an important role in the pathogenesis of Alzheimer disease. Apart from producing the Alzheimer amyloid precursor (APP) as an acute phase protein, microglial cells seem to be involved in the deposition of its amyloidogenic cleavage product, the amyloid-β peptide (Aβ). Aβ is bound by apolipoprotein E (APOE) in an isoform-specific manner, and it has been demonstrated that inheritance of the AD susceptibility allele, APOE ε4, is associated with increased deposition of Aβ in the cerebral cortex. However, the relationship between APOE ε4 gene dose and microglial activation is unknown. Using microglial expression of major histocompatibility complex class II molecules as a marker, we have performed a quantitative genotype-phenotype analysis on microglial activation in frontal and temporal cortices of 20 APOE genotyped AD brains. The number of activated microglia and the tissue area occupied by these cells increased significantly with APOE ε4 gene dose. When a model of multiple linear regression was used to compare the relative influence of APOE genotype, sex, disease duration, age at death, diffuse and neuritic plaques as well as neurofibrillary tangles on microglial activation, only APOE genotype was found to have a significant effect. Thus, the APOE gene product represents an important determinant of microglial activity in AD. Since microglial activation by APP has been shown to be modulated by apoE in vitro, a direct role of microglia in AD pathogenesis is conceivable.

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Introduction

Microglial cells are the resident macrophages of the brain and spinal cord. It has become clear in recent years that these cells are exquisitely sensitive "sensors" of tissue pathology (28). Thus, alterations in the phenotype of microglial cells are among the earliest indications of pathological tissue change and often represent its first sign. Microglia have been suggested to play an active role in several important disease conditions including neurodegenerative disorders such as Alzheimer disease (AD) (14, 33, 38). The involvement of microglia in the pathogenesis of AD is under intense scrutiny. Activated microglia are predominantly found within and around neuritic or core plaques (33, 50) but are usually absent from diffuse amyloid deposits (20, 43). This selectivity in the distribution of activated microglia suggests that specific signals within neuritic and core plaques mediate their activation. Apart from producing the Alzheimer amyloid precursor protein (APP) (3) and apoE (41, 55, 59), microglial cells are activated by amyloid-β peptide (Aβ) (39) and are likely involved in the processing of APP (33). Microglia could thus facilitate Aβ deposition and plaque formation (31, 33). In addition, microglial cells have been shown to express receptors which are considered necessary for the clearance of compact amyloid (2, 42), and are primary candidates for the production of inflammatory mediators in the AD brain (35, 61).

Major histocompatibiltiy complex (MHC) antigens are established markers of microglial activation in both rodent and human CNS (26, 37, 56, 57). Expression of MHC class II molecules has been found in numerous neurodegenerative diseases and is prominent in AD (36). Yet, the function of reactive upregulation of MHC class II by microglia is unclear (10, 18). Although activation

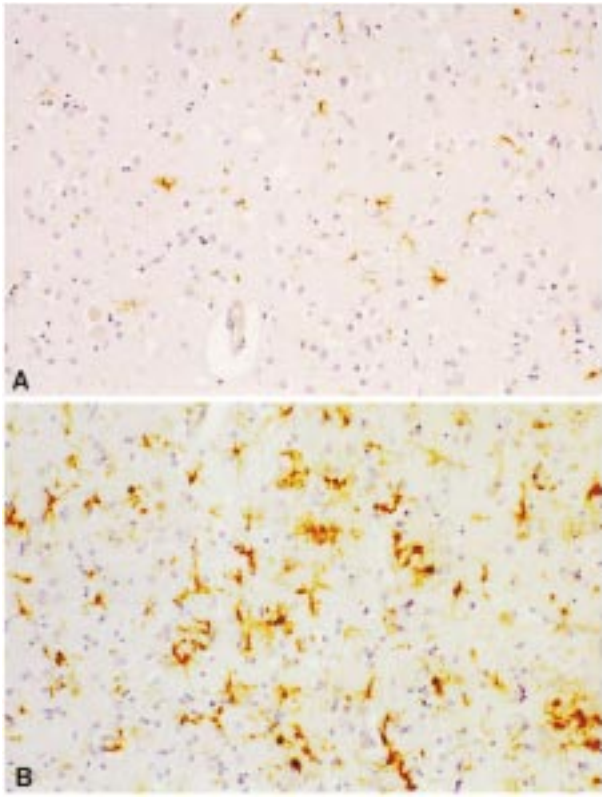

Figure 1. Comparative cortical pathology of the frontal lobe in an APOE ϵ3 (**A**) and an APOE ϵ4 (**B**) homozygote AD patient, respectively. There are significantly more activated microglial cells expressing MHC class II molecules in the APOE ϵ4/4 AD brain. CR3/43 monoclonal antibody, peroxidase/DAB. Magnification: X 190.

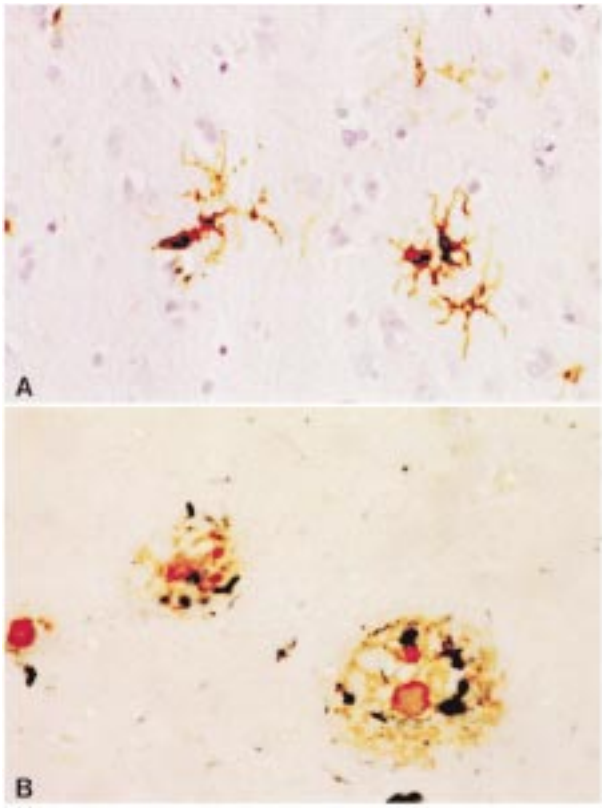

Figure 2. Numerous microglial cells in AD cortex show an activated morphology exhibiting enlarged, class II positive cell bodies and tortuous ramified cell processes. CR3/43 monoclonal antibody, peroxidase/DAB.; X 400 (**A**). Double immunostaining for βA4 and tau protein in neuritic plaques; X 400 (**B**).

of T cells may ensue following presentation of antigen in the molecular context of MHC class II molecules, a neuroprotective role of MHC class II molecules in neurodegeneration seems possible (4, 10).

The APOE ϵ4 allele (APOE4) is a strong susceptibility factor for AD (reviewed in (49)). It confers an increased risk of expressing the disease and is associated with a younger mean age of onset as well as modification of the neuropathological phenotype in a dose-dependent manner (8, 48, 52, 53, 58). Microglial activation by APP has been shown to be modulated by apoE in vitro (5). We were therefore interested in the question whether the activation of microglial cells in AD brain may be related to APOE genotype.

Materials and Methods

Subjects. Brain tissue was obtained from 20 cases with clinically and neuropathologically confirmed AD (mean age 75.9 ± 7.31 years) and 5 controls (mean age 71.6 ± 6.23 years). All cases had been collected at the Institute of Neuropathology of the University of Munich. The neuropathological diagnosis of AD was made according to established criteria (6, 40). The control group consisted of brains from cases without neurological and neuropathological abnormalities.

DNA extraction. Genomic DNA was extracted from paraffin-embedded brain tissue as described (27). In brief, tissue was scraped off from paraffin blocks using a sterile scalpel blade, deparaffinized with xylene and ethanol, and digested in 4 mg/ml (80 U) of proteinase K (Boehringer Mannheim, Germany) at 55°C for 24 h (27). DNA was further purified with phenol-chloroform-isoamylalcohol (25:24:1) and concentrated with an Amicon microcon-30 concentrator.

APOE Genotyping. APOE genotypes were determined using a previously established polymerase chain reaction (PCR) assay which allows analysis of DNA iso-

Case no.	Age at death	Age at onset	Sex	APOE genotype	No. CR3 frontal	No. CR3 temporal	Area CR3 frontal	Area CR3 temporal	No. plaques frontal	No. plaques temporal	Area plaques frontal	Area plaques temporal	No. pl. neurit. frontal	No. pl. neurit. temporal	Area pl. neurit. frontal	Area pl. neurit. temporal	NFT frontal	NFT temporal
S 001	79	74	F	44	129,05	120,83	3,14	1,93	97,20	94,60	9,41	8,81	31,01	26,50	2,90	3,03	23,96	21,99
S 002	71	65	F	44	154,74	183,40	3,38	2,68	98,10	63,90	10,95	7,83	11,10	24,18	2,68	3,48	22,34	16,67
S 003	63	54	M	33	59,60	51,04	1,04	1,14	33,35	44,55	2,91	4,40	3,13	3,35	0,50	0,66	9,61	14,43
S 004	80	76	F	33	21,53	0,00	0,23	0,00	80,55	27,75	4,85	2,54	3,47	5,90	0,54	0,93	13,08	6,94
S 005	80	75	F	44	163,31	193,10	4,37	4,73	146,00	119,00	10,95	9,44	11,10	10,76	2,58	2,13	15,39	22,92
S 006	74	67	M	33	47,33	26,70	0,69	0,54	57,50	32,20	3,18	1,85	3,47	5,21	0,38	0,51	16,55	22,45
S 007	80	75	F	33	12,85	7,99	0,16	0,10	59,85	21,50	3,91	1,50	3,82	5,21	0,50	0,71	15,63	11,92
S 008	70	67	F	34	109,49	33,90	1,71	0,60	61,70	40,50	2,68	2,15	9,49	11,34	0,99	2,11	20,37	19,09
S 009	70	68	F	33	0,23	0,35	0,04	0,04	54,95	24,45	2,23	1,44	6,59	5,60	1,20	1,03	11,92	11,34
S 033	85	77	F	34	49,42	6,48	0,71	0,08	104,85	101,45	9,65	8,48	17,01	13,43	2,03	1,81	10,07	15,86
S 034	78	71	F	34	82,41	104,70	1,54	0,91	139,40	126,60	10,00	9,49	7,75	2,43	1,99	0,48	13,42	20,60
S 035	85	79	F	33	51,74	49,88	0,63	0,68	60,15	66,15	3,88	5,19	2,43	3,70	0,39	0,66	7,99	7,41
S 036	83	75	F	34	26,97	95,25	0,63	0,99	144,40	126,20	10,89	15,70	7,29	6,71	1,03	2,24	16,55	33,45
S 037	79	68	F	34	67,82	95,49	1,19	1,05	89,40	82,35	7,26	6,26	1,04	2,31	0,14	0,38	8,92	17,01
S 039	63	60	M	34	60,53	103,00	1,11	1,83	134,00	68,15	9,11	6,56	6,01	6,94	1,15	1,10	20,60	18,51
S 040	70	61	F	34	84,03	167,80	0,61	2,84	55,65	55,35	6,75	7,64	16,32	11,23	2,63	1,75	12,27	12,73
S 042	63	56	F	34	113,43	125,23	2,20	3,91	73,20	62,85	6,89	5,69	8,45	16,55	1,08	2,00	22,57	43,41
S 043	80	69	F	34	78,70	53,13	1,43	0,69	127,15	115,00	15,60	11,48	4,98	4,40	1,11	0,91	9,26	15,40
S 044	83	76	M	34	28,01	74,88	0,38	1,08	120,00	60,65	9,86	6,03	3,13	5,90	0,40	1,23	13,08	18,52
S 046	81	65	M	33	4,40	40,74	0,09	0,91	128,40	112,65	15,96	12,04	2,08	2,20	0,69	0,63	7,64	14,01

Area, number [mm^{-2}]; age [yrs]

Table 1. Clinical, molecular genetic and histopathological data of the AD cases used in this study.

lated from paraffin-embedded brain tissue (16). The primer pairs Rup1 (5'-CTGGGCGCGGACATGGAG-3')/Rup2 (5'-GCAGGTGGGAGGCGAGGC-3'), and Rup3 (5'-GGCCAGAGCACCGAGGAG-3')/Rup4 (5'-GCCCCGGCCTGGTACACT-3') amplify 115- and 119-bp fragments, respectively, each encompassing one of the two polymorphic sites at codons 112 and 158 of the APOE gene. *Hha*I digestion of the Rup 1/2 and Rup 3/4 amplicons distinguishes between ϵ3/4 and ϵ2/3 alleles. Conditions for PCR amplification were denaturation at 94°C (5 min), followed by 43 cycles at 94°C (30 sec), 68°C (30 sec) and 72°C (1 min) on a TRIO-Thermoblock (Biometra). Final extension was carried out at 72°C for 10 min. Nucleotides (250μM) from Invitrogen were used in combination with a buffer containing 300 mM Tris-HCl, pH 9.5, 75 mM $(NH_4)_2SO_4$, and 10 mM $MgCl_2$ (Buffer J, 5x, Invitrogen) in a total volume of 50μl. PCR products were cleaved with *Hha*I and separated on a 5% intermediate melting temperature agarose gel containing 0.15 μg/ml ethidium bromide.

Immunohistochemistry. After fixation of whole brains in 3.7% formaldehyde for 5-7 days, tissue blocks were embedded into paraffin. 12 μm thick paraffin sections were dewaxed in xylene and rehydrated using an descending ethanol series. After quenching of endogenous peroxidase activity in 7.5% H_2O_2 for 10 min, tissue sections were incubated in 0.01 M phosphate buffered saline (PBS) for 15 min followed by incubation in 1% rabbit normal serum for 20 min. Incubation with the following monoclonal antibodies was carried out at 4°C overnight (dilutions in PBS are given in brackets): monoclonal anti-βA4 (DAKO; 6F/3D; M 0872; 1:100), monoclonal anti-PHF-tau (Innogenetics; AT-8; 1:2000), and polyclonal anti-tau (DAKO; A 0024; 1:8000). Detection of antibody binding was performed using biotinylated secondary antibodies (DAKO) and peroxidase-conjugated streptavidin (DAKO). Visualization was performed with DAB Chromogen (DAKO) in 0.05 M TRIS-HCl, containing 0.15 M NaCl, pH 7.6, and 3% H_2O_2.

For labeling of microglial cells, the monoclonal antibody CR3/43 (DAKO; M 0775, 1:50) was used. This antibody recognizes the β-chain of all products of the MHC class II gene subregions HLA-DR, -DQ and -DP and is a marker for activated microglia in paraffin-embedded human brain tissue (21). Sections were first immersed in 0.01 M citrate buffer (pH 6.0) and boiled in a microwave oven for 9 min (600 W). Detection and visualization of primary antibody binding was carried out as described above. Visualization of neuritic plaques was performed using two-color double-labeling immunohistochemistry with monoclonal anti-βA4 (DAKO) and polyclonal anti-tau (DAKO). First, tau-positive structures were detected using the anti-tau anti-

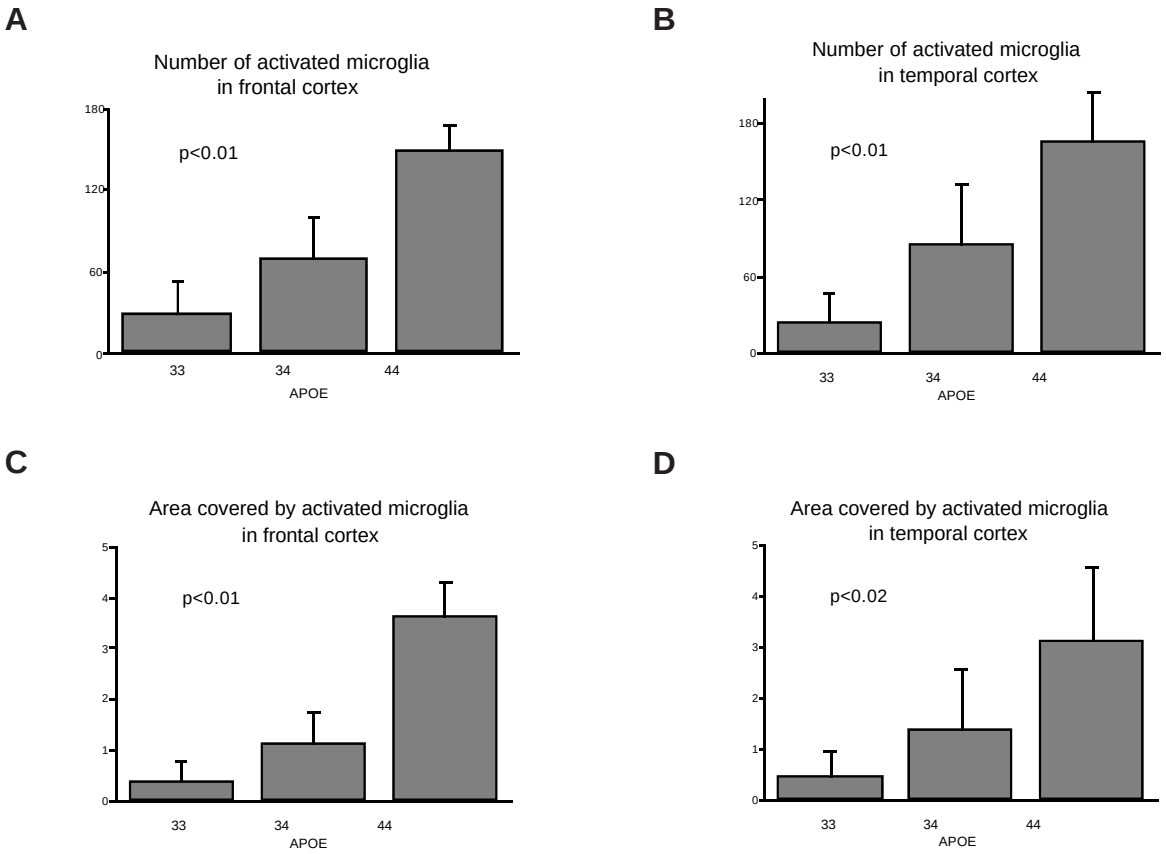

Figure 3. Relationship between APOE ϵ4 gene dose and the number of activated microglia (**A, B**) and the tissue area taken by these cells (**C, D**) in AD brain. Quantitative image analysis was performed using the computer program Optimas 5.1.

body which was visualized using a blue peroxidase substrate (Vector SG substrate Kit, Vector Laboratories). Subsequently, βA4-immunohistochemistry was performed as described above with DAB Chromogen as a peroxidase substrate giving Aβ-deposits a brownish-red color.

Image analysis. Sections from the medial frontal and the medial temporal gyrus were used. Immunophenotypes of AD brains were analyzed quantitatively by determining the number and tissue area covered by CR3/43 labeled microglial cells, cortical βA4-immunoreactive plaques and βA4/tau-positive neuritic plaques, as well as the number of neurofibrillary tangles. Slides were imaged using a Leitz Aristoplan microscope with a 10X or 6.3X objective connected to a color video camera (Sony, 3 CCD, DXC-930P) and analyzed using the computer-based image analysis system Optimas (version 5.1, Optimas Corporation, Seattle, Washington). Illumination voltage, camera setup and calibration parameters were held constant throughout all measurements. In each slide, 8 consecutive representative fields from severely affected tissue areas (53) were evaluated. Areas used for quantification of microglia, neurofibrillary tangles (NFT), and neuritic plaques were 1.08 mm^2 (0.90 x 1.20 mm) and 2.48 mm^2 (1.38 x 1.80 mm) for βA4-positive plaques, respectively. The Optimas image analysis software package allows the identification of individual immunoreactive profiles. In brief, after background correction, profiles were identified based on color-thresholds that had been empirically determined for each type of profile, i.e., microglia, plaques, neuritic plaques, NFT. Minimum object boundaries were defined with 10 pixels in the case of microglial profiles and neurofibrillary tangles, and 40 pixels for amyloid plaques. Finally, manual correction of selected fields was performed to achieve a complete match between the visual screen mask and the original microscopic fields and to prevent counting of rare artifacts. For discrimination of neuritic from diffuse plaques by means of double-

immunostaining, two independent thresholds were set for each color. Neuritic plaques were defined as βA4-positive amyloid deposits containing tau-positive profiles (Fig. 2B) (11). Dense core plaques without neurites were not assed in this analysis.

Statistical analysis. The number of MHC class II positive microglial cells and the cortical tissue area covered by them were compared between Alzheimer and control cases using the Mann-Whitney test. Significance of the differences in distribution of activated microglia between AD patients carrying one, two or no APOE4 alleles was computed using the Kruskal-Wallis test. The correlation between different immunohistochemical phenotypes was tested using the Pearson correlation coefficient. In addition, multiple linear regression analysis was performed. SPSS 6.1.3. (Statistical Package for the Social Sciences) was employed for the statistical analyses.

Results

Activated microglia in AD and controls. MHC class II immunoreactive microglia were readily detectable in the cerebral cortex of all AD cases (Fig. 1). In contrast, in the cortical grey matter of controls the number of activated microglial cells was very low. Whereas the tissue area taken by cell processes of activated microglia in grey matter was more than 50 times larger in AD cases than in controls ($p<0.005$), the average number of activated microglial cells was 16 times higher in AD patients ($p<0.005$). Cells resembling microglia were often found in the vicinity of dense plaques as already shown by Alzheimer (1). Plaque-associated activated microglia possessed a characteristic morphology with stout and sometimes tortuous cell processes (Fig. 2A). Their cell bodies were occasionally rounded and enlarged especially in the immediate vicinity of plaques, and in some instances rounded microglia showing features of phagocytosis were also present (22).

APOE ϵ4 dosage effect on microglia in AD brain. In our sample of 20 sporadic AD cases, 7 patients were APOE ϵ3/3, 10 patients were APOE ϵ3/4 and 3 patients possessed the genotype ϵ4/4. This corresponds to APOE allele frequencies of 0.00 ϵ2, 0.60 ϵ3 and 0.40 ϵ4, respectively, which is in agreement with published APOE allele frequencies in autopsy confirmed sporadic AD. Original data are shown in Table 1.

When the degree of microglial activation was analyzed in relation to the number of APOE4 alleles, an APOE4 allele-dependent increase in the number of microglia as well as in the tissue area taken by MHC class II positive microglial cellular profiles was found (Figs. 1A, 1B). This difference was statistically significant for both the frontal (Figs. 3A, 3C) and temporal cortex (Figs. 3B, 3D). When time of disease duration, age at death, and APOE genotype were analyzed in a model of multiple linear regression, only APOE genotype showed a significant influence upon microglial activation.

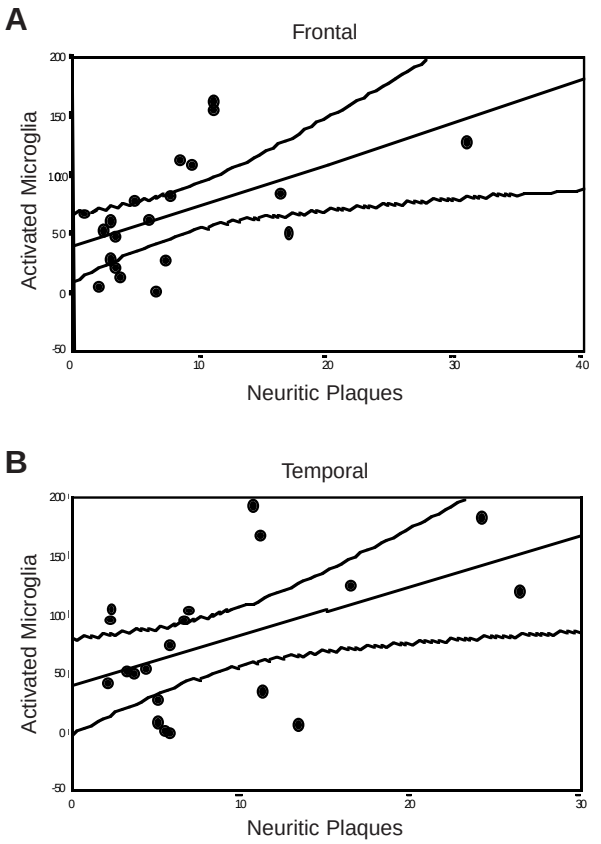

Figure 4. Linear regression analyis of the number of activated microglial cells and the number of neuritic plaques in the frontal (**A**) and temporal (**B**) cortex. The 95% confidence interval is shown.

Correlation between activated microglia, amyloid plaques and neurofibrillary tangles. Quantitative morphometric analysis of diffuse and neuritic amyloid plaques, neurofibrillary tangles and microglia demonstrated a positive correlation between the presence of activated microglia and neuritic plaques (Figs. 4A, 4B). This association was confirmed for both the number and the tissue area occupied by neuritic plaques in the

	Correlation (r)	Significance(p)
Number AM vs. NFT frontal	0.5521	0.012
Number AM vs. plaques frontal	0.1489	0.531
Number AM vs. NP frontal	0.5292	0.016
Area AM vs. plaques frontal	0.2691	0.251
Area AM vs. NP frontal	0.6607	0.002
Number AM vs. NFT temporal	0.4121	0.071
Number AM vs. plaques temporal	0.4067	0.075
Number AM vs. NP temporal	0.4990	0.025
Area AM vs. plaques temporal	0.2801	0.232
Area AM vs. NP temporal	0.5255	0.017

AM, Activated microglia; NFT, Neurofibrillary tangles; NP, Neuritic plaques

Table 2. Correlation between microglial activation and hallmarks of AD histopathology in frontal and temporal cortices

frontal and temporal cortex (Table 2). In contrast, there was no correlation between activated microglia and diffuse amyloid deposits (Table 2).

Multiple linear regression. In order to determine whether microglial activation was influenced by sex, time of disease duration, age at death, the number and distribution of plaques and tangles and APOE genotype, a model of multiple linear regression was employed where the number of MHC class II immunoreactive microglia and the cortical tissue area taken by their profiles were studied as a function of APOE genotype, sex, duration of disease, age at death, the number of neurofibrillary tangles, diffuse amyloid depositis and neuritic plaques. Only APOE genotype was found to have a significant effect on microglial activation. Both the number of MHC class II immunoreactive microglia (frontal, $p<0.001$; temporal, $p<0.02$) and the tissue area occupied by their processes (frontal, $p<0.02$; temporal, $p<0.05$) were significantly influenced.

Discussion

Our finding of an association between microglial activation and APOE genotype in AD raises the question whether microglia are directly involved in AD pathogenesis. Since microglial activation by APP has been shown to be modulated by apoE (5), the involvement of microglial cells in APP and Aβ metabolism deserves special consideration. Both genetic and molecular biological data provide support for the role of amyloid deposition as a causative factor in AD (24). Microglial cells have been shown to produce the Alzheimer amyloid precursor protein (APP) (3) as well as apoE (41, 55, 59), and they are activated by Aβ (39). In turn, activated microglia can mediate neuronal injury in response to Aβ through the release of neurotoxic factors (20) and proinflammatory cytokines such as TNF-alpha (13) which enhance the release of NO and other reactive oxygen species. Preincubation of mixed glial cultures with apoE reduces TNF-alpha secretion in a dose-dependent fashion (30). The recent finding that microglia-derived nerve growth factor causes cell death in the developing retina (19) further points to the possibility that microglia have an active role in neuronal degeneration. Interestingly, microglia show a predominant association with neuritic or core plaques (33, 50) but largely ignore diffuse deposits (20, 43). This selectivity in the distribution of activated microglia suggests that specific signals are associated with neuritic or core plaques which can activate microglial cells. Our finding of a positive correlation between neuritic plaques and activated microglia would also support the notion of a microglial involvement in the formation of amyloid plaques (33).

The association between APOE4 gene dose and amyloid deposition (53, 62) complicates the interpretation of our results because apoE4 protein and Aß may interact directly (58). Since activated microglia are predominantly found in the vicinity of dense plaques, the observed APOE4-dependent increase in the number of activated microglial cells could result from the increased number of neuritic plaques in carriers of the APOE4 allele. However, we found that the effect of APOE4 gene dose on microglial activation was stronger than on neuronal changes, i.e., the formation of neurofibrillary tangles and neuritic as well as diffuse plaques (data not shown). Therefore, microglia may participate in early stages of neuritic plaque development (23, 46). Yet, the possibility that APOE4 dose dependent microglial activation represents a secondary phenomenon cannot be ruled out completely.

There is evidence that microglia can play a role in the phagocytosis and/or processing of amyloid (54). Specifically, it has been shown that microglial cells internalize aggregates of Aβ via a scavenger receptor (17, 42) and that secreted Aβ (1-40) and Aβ (1-42) peptides are constitutively degraded by a metalloprotease released by microglial cells (44). These findings suggest a possible microglial mechanism of Aβ clearance in brain tissue. The ability of factors secreted by cells of the monocyte/macrophage lineage to reduce Aβ accumulation in smooth muscle cells supports this view (34). Furthermore, apoE has been linked to the clearance of Aβ (29), and apoE may induce microglial plasticity either by interacting directly with the microglial plasma membrane or by binding to receptors for VLDL, LDL, or low density lipoprotein receptor related protein (LRP) (30).

Consequently, Aβ processing by microglia may be modified by apoE.

The association between APOE4 genotype and microglial activation first observed for MHC class II molecules (15) is in line with the finding of an effect of APOE risk on the distribution of microglial cells in AD brain (51). These associations could be due to several factors. One possibility concerns a differential influence of apoE isoforms on microglial activation as the ability of β-APP to activate microglia can be blocked by incubation with apoE3 but not apoE4 (5). This hypothesis would be in agreement with the finding that Aβ can activate microglia through receptors for advanced glycation end-products (AGE) (60). AGEs co-localize with apoE as shown by immunohistochemical studies on AD brains (14), and apoE4 exhibits significantly greater AGE-binding activity than the apoE3 isotype (32). Thus, apoE could participate in the formation of amyloid aggregates in the AD brain by binding to AGE-modified plaque components (32). The presence of glycated substances could also attract microglia to senile plaques and subsequently activate the cells (12).

Epidemiological studies (7, 25) and the results of preliminary clinical trials (47) indicate that anti-inflammatory drugs have some therapeutic effect in AD. Yet, the underlying mechanisms have remained unclear (9). Since activated microglial cells represent a potent source of various immunological mediators (39), inflammatory mechanisms in the AD brain are likely to involve these cells. Therefore, means to control the in vivo effects of apoE on microglial activation could be of therapeutic relevance.

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