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Microglia and Astrocytes in Postmortem Human Brain Increase in Parallel with Aging

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Abstract

We showed previously significantly increased fractional areas (FA) of HLA- DR and GFAP immunoreactivities (microglia and astrocytes) in brains of patients with Alzheimer´s disease (AD). This prompted us to assess FA and qualitative alterations in HLA-DR, GFAP, amyloid-β-peptide (Aβ) and phospho-tau immunoreactivities during aging in human neocortex of 11 young (mean 26 years), 9 middle aged (mean 48 years) and 11 elderly (mean 82 years) healthy persons.

The mean FAs of HLA-DR and GFAP were similar in young and middle-aged persons, but significantly greater ($p < 0.05$ resp. <0.004) in young vs. elderly persons. In middle-aged vs. elderly persons only the increase in FA-GFAP reached significance ($p < 0.001$). while that in FA-HLA-DR did not. In the young microglia were located predominantly perivascularly, in the elderly widely in the neuropil. Aβ immunoreactivity did not appear until in middle- aged persons, tau-staining remained negative throughout.

In conclusion: Brain cells of innate immunity, microglia and astrocytes, increase during aging probably reflecting cumulative immunologically challenging events, including those of AD, possibly also compensating for their senescence and fading protective capacity.

***Keywords**: Microglia; Astrocytes; Inflammation; Aging; Senescence; Amyloid; β-peptide; Tau; Quantification*

Introduction

Microglia originate from precursor cells of monocyte-macrophage lineage, which populate the brain tissue during embryonic development and during postnatal period ([13,25,37]). Microglia are thought to be similar to other tissue macrophages, e.g. mediating phagocytosis, antigen presentation, production of cytokines and matrix metalloproteases. Later in adult life circulating monocytes may be recruited to the brain, if certain pathological conditions necessitate to become "auxiliary bone-marrow derived microglia" (e.g. Longo., *et al*. 2010; Beck., *et al.* 2010; [12]).

According to the present view microglia are considered to function in the innate immune system of the CNS ([18,25,42]). Under normal conditions, microglial cells are in a resting (quiescent) state with ramified appearance. Yet, this phase is not as quiescent as previously thought, but instead the ramified microglia are actively participating in monitoring the microenvironment and responding not only acute microbial invasion but also to chronic neuronal stress or damage including for example the functional status of synapses ([21]; Wake., *et al*. 2009). Thus, microglia are not, as previously thought, mainly neurotoxic effector cells [2], but according to the present view also important neuroprotective cells, directed to bring about recovery of the neurons at risk by providing growth factors and cytokines that help the neurons to recover (e.g. [21,33]; Brown and Neher, 2010; [12,28]).

If recovery is not successful and acute neuronal death ensues microglia can adopt amoeboid form and become phagocytic and the debris is cleared [12,21,28], 2010) in which activity astrocytes also participate [31]. After the process is completed microglia turns back to the resting stage [32] or they are pruned by apoptosis ([21,35]). It is suggested that if the microglial response is uncontrolled or prolonged, the proinflammatory cytokines secreted by microglia become neurotoxic and result in loss of surrounding neurons. To prevent that blood derived monocytes are needed and recruited to control local inflammation [28].

Another cell included in the innate immune system of the CNS is astrocyte, a critical mediator of the CNS homeostasis, which appears to have many of the same functions as microglia, these two cells having active cross-talk ([3,30,31]). Astrocytes appear to survive and persist as reactive cells also forming glial scars as an indicator of the repair process [15]. All these phenomina are in principle neuroprotective.

In chronic conditions of cellular stress or damage both microglia and astrocytes may be chronically activated. This may eventually lead to microglial and/or astrocytic degeneration, observed during aging of experimental animals and humans, i.e. microglia and astrocytes become senescent (Streit and Xue, 2009; ([3,12,31]).

Activated microglial cells are considered have the potential to cause neurodegeneration in Alzheimer´s disease by producing different cytotoxic agents as a reaction to β-amyloid (Aβ has ([6,11,19,27]); Leung., *et al*. 2009). However, this view has been challenged by a recent study in which microglia were not activated by deposition of Aβ (Streit., *et al*. 2009) and thus rather the dysfunction of senescent microglia and consequent fading neuroprotection is more important ([34], 2009). Furthermore, cytoprotective role of microglia is supported by the therapeutic trials using immunization with Aβ in which microglia appear to actively participate in the removal of Aβ deposited in senile plaques (SP; [22], 2006; [5]).

In our previous study [38] we observed a significant increase in the fractional area (FA) of microglial cells identified by immunoreactivity for HLA-DR in the brains of AD cases compared to controls. Besides, the FA of astrocytes in controls and possible AD cases was significantly lesser than in definite AD. This previous study prompted us to assess, how the populations of microglial and astrocytic cells develop during aging, i.e. in healthy individuals from 19 to 96 years-of-age. Although many different alterations have been reported to occur in microglia and astrocytes during aging (e.g. Sheffield and Bermann, 1998; ([3,39]), basic morphometric analysis has not been published to show the quantitative reaction of microglia and astrocytes to normal aging in human brain.

Materials and Methods

The brain tissue

Post mortem brains of the young (mean age 26 years, range 19-38, n = 11) and middle aged (mean age 48 years, range 41-59, n = 9) individuals were obtained from Maryland Medical Examiner's Office. These young and middle aged individuals were selected from among individuals who had died unexpectedly without any known neurological disease, neither were individuals with known head trauma included. Since these individuals had died unexpectedly, no clinical evaluation was available from these cases. The experimental setting is the same as in the first author´s previous study [38]. Thus, the results from these two younger cohorts are comparable with our previous results on the elderly controls (cognitively normal with no neuritic plaques in the post-mortem examination; mean age 82 years; range 69-96; n = 11) in that study. To make the comparison easier the results of the elderly controls originally published in [38] are presented also in this article (Table 1 and 2).

Neuropatho- logical marker	Statistics	Young individuals (n = 11)	Middle- aged individuals (n = 9)	Elderly individuals* (n = 11)	Definite AD* (n = 9)
GFAP fractional area	Mean	1.33	1.17	5.00	9.25
	Median	1.07	1.21	3.54	8.23
	Percent of AD median[1]	13%	15%	43%	100%
	Range	0.4 - 2.7	0.28 - 1.94	1.05 - 11.77	3.2 - 19.67
HLA-DR fractional area	Mean	0.07	0.11	0.77	1.37
	Median	0.06	0.12	0.31	1.23
	Percent of AD median[1]	5%	10%	25%	100%
	Range	0 - 0.15	0.03 - 0.27	0 - 2.78	0.07 - 2.86

Amyloid- peptide fractional area	Mean	0.00	0.19	0.22	5.14
	Median	0.00	0.03	0.18	5.15
	Percent of AD median[1]	0%	0%	3%	100%
	Range	0.00	0 - 0.78	0 - 2.9	2.23 - 9.11
Phospho-tau fractional area	Mean	0.00	0.00	0.00	1.07
	Median	0.00	0.00	0.00	1.00
	Percent of AD median[1]	0%	0%	0%	100%
	Range	0.00	0.00	0.00	0.08 - 3.5

Table 1: *Summary of results on neuropathological markers in healthy individuals and in definite AD patients.*

** Values for elderly individuals and definite AD patients from Vehmas., et al. 2003.*

[1]Percentage of the median in question out of the median in the definite AD group.

Neuropatho- logical marker	Comparison group	Middle-aged individuals (n = 9)	Elderly individuals* (n = 11)	Definite AD* (n = 9)
GFAP fractional area	Young individuals	NS	0.004	<0.001
	Middle-aged individuals	--	0.001	<0.001
	Elderly individuals	--	--	0.048
HLA-DR fractional area	Young individuals	NS	0.050	<0.001
	Middle-aged individuals	--	NS	0.005
	Elderly individuals	--	--	NS
Amyloid- peptide fractional area	Young individuals	<0.001	<0.001	<0.001
	Middle-aged individuals	--	NS	<0.001
	Elderly individuals	--	--	<0.001
Phospho-tau fractional area	Young individuals	NS	NS	<0.001
	Middle-aged individuals	--	NS	<0.001
	Elderly individuals	--	--	<0.001

Table 2: *P-values for Wilcoxon Sign Rank Test comparing fractional area of four neuropathological markers between different groups.*

** Values for elderly individuals and definite AD cases from Vehmas., et al. 2003.*

Diagnostic neuropathology

Customary 4% buffered formaldehyde was used to fix the brain tissues for at least two weeks. Tissue blocks from selected brain areas were routinely embedded in paraffin. Sections cut at 10 μm were stained with hematoxylin and eosin and Hirano silver method [41].

Immunohistochemistry

Immunohistochemical methods (IHC) have been previously described in detail [38]. Shortly, serial tissue sections from middle frontal gyri (MFG) or superior and middle temporal gyri (SMTG) were immunostained for microglia, astrocytes, Aβ peptide and hyperphosphorylated tau. Standard avidin-biotin complex (ABC) procedure or peroxidase-antiperoxidase (PAP) technique were used in immunohistochemical (IHC) stainings. Microglia were identified with monoclonal mouse anti-human HLA-DR (HLA-DR; DAKO, Carpinteria, CA, USA), astrocytes were identified with polyclonal rabbit anti- glial fibrillary acidic protein (GFAP; DAKO), Aβ peptide with mouse monoclonal antibody (10D5) to A peptide amino acids 1-28 (Elan Pharmaceuticals, San Francisco, CA, USA) and tau protein with mouse monoclonal antibody to amino acids 95-108 of hyperphosphorylated tau (Tau- 2; Sigma, St. Louis, MO, USA). All sections were pretreated with methanol/ H_2O_2 for 30 min to eliminate endogenous peroxidase activity, rinsed in deionized water for 10 min followed by microwaving for 7 minutes.

Sections for Aβ IHC were pretreated with concentrated formic acid (80%) for 3 minutes. 3 % normal goat serum was used as blocker for one hour at room temperature followed by incubation for 24 h in the appropriate primary antibody at room temperature. The concentrations for primary antibodies: Aβ 1:200, HLA-DR 1:100, GFAP 1:400, Tau 1:1000. Next the sections were incubated with appropriate secondary antibodies (ABC-kit, Vector Elite or mouse-/rabbit- IgG and mouse-/rabbit-PAP) at room temperature one hour in each step. A standard diaminobenzidine (DAB) reaction was used to visualize the bound antibodies. The sections were counterstained with hematoxylin.

Negative controls, processed in the same run with no primary antibody, were used to assess specificity.

Qualitative analyses

The qualitative analyses included observations of microglia, astrocytes, Aβ and tau immunostainings in all different groups.

Quantitative analyses

Using single immunostains, immunoreactivity for all antibodies was measured using the fractional area (FA) method [7] to estimate the percent area of interest (in MTG or SMTG) covered by immunoreactivity of each specific antibody. The study design was done as previously described [38]. Shortly, using a light microscope interfaced with a Stereo Investigator (MicroBrightfield, Inc., Williston, VT, USA), a 100 sq mm area of cortical gray matter that included all neuronal layers was outlined.

Using a random start, a systematic sample of the outlined field (i.e., divided into 1000 μm x 1000 μm squares) was selected using a computer algorithm. At each selected site a counting frame (130 μm x 100 μm) was projected and a counting grid of small equidistant crosses was superimposed. On each frame, we counted all crosses that fell on the cerebral cortex (total number of crosses) and crosses that coincided with immunoreactivity ("hits"). The sum of hits for all counting frames divided by the total number of crosses is the FA. This procedure was repeated for all antibodies using the same cortical area. The individual conducting the measurements was blinded to the categories.

Statistical analyses

Summary statistics (mean and 95 % confidence intervals) were derived for each of the neuropathological markers by the three different age groups. Statistical differences in the FA of immunoreactivity for microglia, astrocytic cells, Aβ and tau in different groups were

evaluated using the Wilcoxon Sign Test. Significance testing is based on a comparison of the sum of the rank scores derived for each group. All groups were compared to each other on each marker.

Results

The mean FA of HLA-DR immunoreactivity in the MFG or SMTG of the healthy individuals increased gradually with age: in the young individuals it was 0.07% (range: 0 - 0.15; SEM+/-0.02), in the middle aged 0.12% (range: 0.03 - 0.27; SEM+/-0.02) and in the elderly individuals 0.77% (range: 0 - 2.78; SEM+/-0.3), (Table 1 and 2). In the young individuals, microglia were located predominantly in association with blood vessels (Figure, upper). This pattern was less distinct in the middle aged individuals (Figure, middle) and even less so in the elderly individuals, where microglia were detectable widely within the neuropil (Figure, lower).

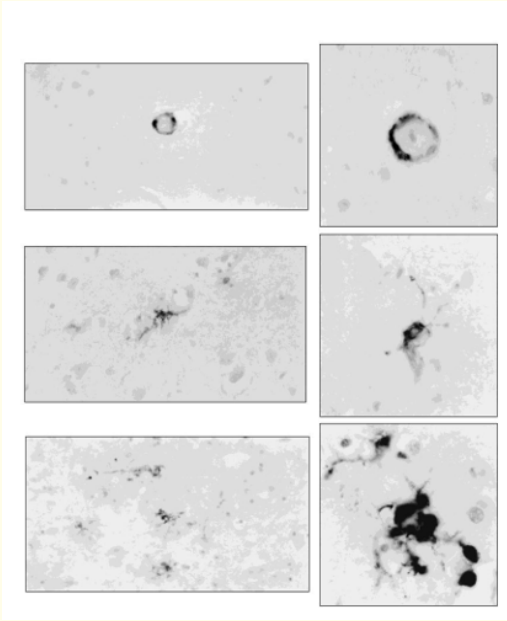

Figure 1: *HLA-DR immunostainings in human neocortex in young (upper figures), middle aged (middle figures) and elderly (lower figures) healthy persons showing the distribution of microglia from perivascular site towards neuropil.*

The mean FA value of GFAP immunoreactivity in young individuals 1.3% (range: 0.4-2.7; SEM +/- 0.3) was actually lower than that in the middle aged individuals 1.17% (range: 0.28-1.94; SEM +/-0.17), whereas the elderly individuals had significantly higher value of GFAP (5.0%; range 1.05-11.77; SEM +/-1.0) than both young and middle aged individuals (Table 1 and 2).

Aβ peptide deposits were not detected in the young individuals. Only very few diffuse deposits and no neuritic plaques were encountered in the middle aged 0.11% (range: 0-0.78; SEM +/-0.09) and elderly individuals 0.2% (range: 0-2.9; SEM +/-0.06). The difference between the middle aged and elderly group did not reach significance ($p > 0.05$). Tau 2 immunoreactivity was not detected in any of these healthy individual groups (Table 1 and 2).

Discussion and Conclusion

This study is an extension of the first author´s previous morphometric study on the association of microglial and astrocytic cells to accumulation of Aβ peptide and tau pathologies in AD [38]. In that study we showed a significantly larger fractional area (FA) of microglial cells identified by immunoreactivity for HLA-DR in the brains of possible (cognitively normal with frequent cortical neuritic plaques) and definite (demented, neuritic plaques and tau pathology) AD cases than in controls (cognitively normal, no neuritic plaques). The FAs of astrocytes in controls and possible AD cases were significantly lesser than in definite AD. This prompted us to analyse, whether these cells show during normal aging similar quantitative reaction as in the pathological aging associated with AD. The results in the previous and present study are comparable, because the same methods, compounds and technical assistance were used.

In the present study presence of possible subclinical AD cases among the healthy individuals is unlikely, because tau-immunostaining was completely negative in all three groups and only minor diffuse Aβ peptide deposits were observed in the two older groups. Lesser amounts of Aβ has been reported to occur in cognitively intact individuals at the age of our two older age groups (e.g. ([17,29]).

We could detect a definite age related increase in the mean values of the FA of microglia from young through middle aged to elderly healthy individuals. In our previous study we showed that the mean FA value even in the elderly controls (0.77%) was significantly lesser ($p < 0.05$) than in AD. The mean FA of HLA-DR in AD patients was 1.2% (SEM +/-0.3) in possible AD and 1.4% (SEM +/-0.3) in definite AD group [38], although, there was some overlapping of these values even between the middle aged healthy individuals and definite AD patients.

During aging the balanced homeostasis of the CNS cells is disposed to alterations, yet a basic morphometric analysis of the quantity of microglia in aging human brain has not been reported. In aging rats it was shown that the relative number of activated (OX-6 +) microglia increased in the white matter, whereas the total number remained the same [24]. Several qualitative alterations in microglia have been reported, e.g. microglial reactivity to fibrillar Aβ was reported to be greater in elderly than in younger monkeys [10]. Age-linked increases in microglial expression of MHC Class II antigen were seen during normal aging in non-human primates and in monocyte cytokine production of IL-6 and IL-1Ra by human peripheral blood mononuclear cells [26].

The role of microglia seems to be dual: neuroprotective and beyond certain physiological limits neurotoxic. Activated microglia have been shown to secrete neurotoxic products (e.g. Sheffield and Berman, 1998; [26]; Leung., *et al*. 2009) which could play a significant role in neurodegeneration after the physiological safe margin is exceeded. On the other hand, degeneration of microglia has been shown to occur in CNS in aging implying impaired neuroprotection ([20]; Graeber and [28,35])). In our study microglia is increasing with aging, which most likely is physiological and protective in CNS to a certain point. This raises the question, whether the increased FA of microglia reflects cumulative reaction to pathological processes affecting the brain over the years or an attempt to compensate for microglial senescence and thereby loss of their neuroprotective effect by increasing the number microglia. The latter alternative of microglial senescence is supported by our results using HLA-DR as marker for microglia, which revealed similar tendency for senescence (dystrophy) as described (e.g. [12,35]. We could observe clearly enlarged HLA-DR positive microglia in the elderly individuals, while to a lesser degree in the younger groups.

In young individuals microglia was predominantly located in association with blood vessels, but assumed progressively wider distribution in the middle aged and elderly individuals, being in the latter detectable widely in the neuropil.

The perivascular location of reactive microglia in the young individuals suggests an important role for these cells in immune mechanisms at the interface between CNS and the vascular system. [14] showed similar kind of shift of activated microglia from perivascular location towards neuropil in brain-biopsies of living patients taken from several hours to several days after brain trauma.

GFAP-FA values (obviously reflecting the number of astrocytes) increased gradually in aging (Table 1 and 2) from young to elderly cohorts. The increase was quite irregular: the mean GFAP-FA was actually lower in middle aged than in young individuals and there was a remarkable rise from these two younger cohorts to the elderly cohort, though the mean GFAP-FA in definite AD cases as reported earlier (9.3%; [38]) was still significantly higher than in any of our healthy individual groups.

Astrocytes play an important role in maintaining CNS homeostasis and in the interaction with neurons ([4,40]); Munch., *et al*. 1997; [1]; Sofroniev and Vinters, 2010). Astrocytes respond to all forms of CNS insults through increase in size and number which process is called reactive astrogliosis. This phenomenon has become a pathological hallmark of many types of CNS structural lesions including AD [31]. The recent studies have shown that astrocytes are in the CNS similarly as microglia cells of innate immunity, which have both protective and detrimental effects, for example in inflammatory processes astrocytes may secrete both pro- and anti-inflammatory molecules [31]. In AD when reactive astrocytes are stimulated by Aβ, they have been shown to produce neurotoxic chemokines and cytokines, suggesting that released substances may contribute to the cognitive decline in AD. Similarly as microglia, astrocytes can as a consequence of a variety of age- related stresses become senescent. Furthermore, brain may be especially vulnerable to aging, since astrocytes appear to be more sensitive to stress- induced senescence than many other cell types (e.g. fibroblasts; [3]). Interestingly, the mean FA of HLA-DR increased steadily from the young age onwards, while the increase in FA of GFAP occurred later.

In conclusion: We demonstrated an increase in the mean FA of the microglial marker HLA-DR with aging and in that of astrocytic marker GFAP at old age. Although these increases were significant, there was considerable overlapping of the FA values of different groups and our cohorts were still of limited size. Thus, further analyses of larger populations and preferably with more information about the participants´ genetic and medical history are warranted.

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Disclosure Statement

There are no actual or potential conflicts of interest.

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