Neuronophagia in the motor cortex in amyotrophic lateral sclerosis

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This study was designed to identify which phagocytic cells in the cerebral cortex of amyotrophic lateral sclerosis (ALS) patients are involved in the process of neuronophagia. For this purpose a number of single and double immunocytochemical stains were carried out on five ALS cases which were selected on the basis of the presence of degenerative and phagocytic phenomena in the cerebral cortex. The cortical degenerative process is mainly present in the third and fifth layers and is not restricted to the fifth layer which contains the cell bodies of the Betz cells. The present study indicates that a number of cells are involved

in the process of phagocytosis in ALS. Resident macrophages (from microglial or perivascular origin) and astrocytes seem to play an immunologically-mediated role in the disappearance of neurons. Some of the cells involved in the degenerative process, i.e. rounded macrophages and microglia, expressed major histocompatibility class II antigen. The phagocytic cells in neuronophagia were phenotypically identical to perivascular macrophages and not to microglia. Therefore, the process of phagocytosis of neurons appears to be primarily the task of the perivascularly located macrophage.

Keywords: amyotrophic lateral sclerosis, immunocytochemistry, monoclonal antibodies, macrophages, microglia

Introduction

The basic mechanism of neuronal destruction in amyotrophic lateral sclerosis (ALS) is unknown [27]. In the cerebral cortex, cell loss is sometimes accompanied by mild gliotic scarring and neuronophagia [25, 27].

Neuronophagia is the active process by which severely degenerate or dead neurons are surrounded, destroyed and ingested by phagocytic cells [22]; the triggering mechanisms, however, are not known [2, 6]. Generally, microglial cells are held responsible for the phagocytosis in neuronophagia [6, 17]. Neuronophagia is not a process restricted to ALS. This phenomenon has been shown to occur in, e.g. viral, paraneoplastic and neurodegenerative

processes. Two macrophage precursor cell populations have been identified in the central nervous system in animal experiments: microglia and perivascular (synonym for 'adventitial' or 'perithelial') cells [3, 15, 29, 34]. Both cell types are candidates for an active role in neuronophagia. In an earlier study, microglial proliferation and activation of microglia and astrocytes have been demonstrated in the cortex, subcortical white matter and spinal cord of ALS patients [17, 37–39]. However, the possible involvement of astrocytes, resident and non-resident macrophages has not been clarified. The selective neuronal degeneration in the motor cortex in ALS prompted us to study this phenomenon in relation to the microglial reaction and the enhanced expression of MHC class II antigens on different cell types in the ALS motor cortex.

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Table 1. Patients with amyotrophic lateral sclerosis (ALS): clinical details

Case number	Diagnosis	Sex	Age at death	Months duration
1	B.ALS	F	35	15
2	L.ALS	M	56	18
3	A.ALS	M	59	8
4	L.ALS	F	62	25
5	B.ALS	F	68	18

F, Female. M, Male. B.ALS, ALS with initial bulbar symptoms. A.ALS, ALS with initial symptoms in the arm. L.ALS, ALS with initial symptoms in the leg.

Material and methods

Five ALS patients were selected from the material of the Netherlands ALS tissue bank (Table 1). These cases exhibited the general pathological criteria of ALS [27], but also showed clear-cut evidence of neuronophagia in the cerebral motor cortex. This selection was needed because neuronophagia and motor cortex damage is not always present in ALS [25]. The average age of onset of the disease was 54 years (range 33–66 years of age). The median survival time after the onset of the disease was 17 months (range 8–25 months). Five patients, age matched, who had died from a non-neurological disease served as controls. All patients and controls were autopsied with 8 hr of death. Cortical tissue from area 4 (leg area) [5, 4] was used for this study. From each patient, two blocks of brain tissue of area 4 (leg area) approximately 1 cm thick were snap frozen in liquid nitrogen and stored at -76°C until use. The adjacent part of area 4 was fixed in 10% formalin and embedded in paraffin.

Histology and histochemistry

Paraffin embedded $8\text{ }\mu\text{m}$ sections were stained with haematoxylin and eosin (H & E), Klüver-Barrera, Nissl, Bodian and Holzer techniques. Cryostat sections with a thickness of $5\text{ }\mu\text{m}$ were stained with oil-red-O, sudan black-B, non-specific esterase (NE) and acid phosphatase.

Immunocytochemistry

The monoclonal antibodies (MoAbs) and polyclonal antibodies (PoAbs) used in this study are listed in Table 2 and 3. The term perivascular cells was used for all cells

located around small or medium-sized blood-vessels. The immunohistochemical demonstration of the cell types was carried out according to a three step indirect immunoperoxidase procedure on serial $5\text{ }\mu\text{m}$ cryostat sections [26]. Enzyme activity was developed with the use of 3-amino-9-ethyl carbazole and H_2O_2 . Sections were lightly counterstained with haematoxylin. Control experiments consisted of substituting the primary antibody with an irrelevant antibody of similar isotype; these controls were invariably negative.

For the immunohistochemical demonstration of glial fibrillary acid protein (GFAP), the peroxidase anti-peroxidase (PAP) technique was carried out on $5\text{ }\mu\text{m}$ cryostat sections fixed in absolute ether. An immunoenzyme double staining method was used to visualize pairs of cellular epitopes in one tissue section simultaneously according to the procedures principally described in the literature [20, 30, 36]. Alkaline phosphatase activity was visualized in blue [7]; peroxidase activity was visualized in red [14]. The presence of two epitopes on one and the same cell was seen as a purple mixed-colour. The following pairs of antibodies were used in this study: GFAP/HLA-DR, GFAP/KP1, GFAP/ $\alpha_1\text{ACT}$, KP1/HLA-DR, KP1/ $\alpha_1\text{ACT}$, HLA/ $\alpha_1\text{ACT}$, NSE/Tal-1B5, NSE/HLA-DR (all rabbit/mouse combinations), and HLA-DR/KP1 (IgG2a/IgG1 combination).

Results

Histological and cytochemical findings

The best way to detect neuronophagia in the cerebral cortex with conventional methods appeared to be a simple Nissl stain on paraffin sections. The Nissl stain combined good morphology with an adequate detection of the foamy brownish pigment within macrophages (Figure 1). The process of neuronophagia appeared not to be restricted to severely damaged or dead neurons. In cases 1 and 2, many well preserved neurons with clear nuclei and a normal cytoplasm were found surrounded and partly covered by rounded phagocytes containing brownish pigment. Elongated microglial nuclei with coarse chromatin were sometimes observed close to the neurons affected by phagocytosis. With the Nissl stain, scattered rounded phagocytes, either single or in clusters were found throughout the parenchyma of the grey matter. Similar small clusters of phagocytes were also found without any trace of a neuron. Small numbers of oil-red-O and sudan

MoAbs	CD	Specificity	Working dilution	Source
LCA	45	Leucocyte common antigen on haematopoietic cells [40]	1:100	Dako
HLA ABC	—	Common framework of MHC [8]	1:200	Dako
HLA DR	—	MHC-class II antigen [23]	1:100	B&D
Tal 1B5	—	Common framework of MHC	1:20	Bodmer
VIC-Y1	—	Invariant gamma chain of HLA DR	1:20	Quaranta
OKM 1	11b	Monocytes/macrophages [33]	1:20	Ortho
OKM 5	36	Monocytes/macrophages [33]	1:50	Ortho
Leu M1	15	Granulocytes/monocytes/activated T cells	1:20	B&D
Leu M3	14	Monocytes/macrophages	1:20	B&D
MAC 387	—	Granulocytes/monocytes/tissue histiocytes	1:400	DAKO
Kp1	68	Macrophages/monocytes	1:50	DAKO
P150/95	11c	Macrophages	1:50	DAKO
HAM-56	—	Monocytes/macrophages [13]	1:1000	ENZO

Table 2. Monoclonal antibodies (MoAbs) used in the study

Dako, Dakopatts. B&D, Becton and Dickinson. Ortho, Ortho-diagnostics Systems. SBA, Southern Biotechnology Associates.

Table 3. Polyclonal antibodies (PoAbs) used in the study

<i>PoAbs</i>			
GFAP—glial fibrillary acidic protein			DAKO
NSE—neuron specific enolase			DAKO
α_1 ACT— α_1 antichymotrypsin			DAKO
<i>Antibody/enzyme conjugate</i>			
Goat anti-mouse Ig, alkaline phosphatase	1:10	TAGO	
Goat anti-rabbit Ig, peroxidase	1:50	DAKO	
Goat anti-mouse IgG1, alkaline phosphatase	1:20	SBA	
Goat anti-mouse Ig2, peroxidase	1:200	SBA	
Swine anti-rabbit Ig, biotin	1:400	DAKO	

Dako, Dakopatts. B&D, Becton and Dickinson. Ortho, Ortho-diagnostics Systems. SBA, Southern Biotechnology Associates.

black B-positive rounded macrophages were found in the white matter and in the perivascular spaces. The grey matter occasionally contained a single oil-red-O or sudan black B-positive rounded macrophage. Several clusters of macrophages were found in the cortex, but precise identification was not possible due to the non-specific esterase and acid phosphatase activity in neurons; these cytochemical stains were, therefore, not very useful.

Immunohistochemistry

Macrophage markers

All ALS cases had very similar numbers of parenchymal macrophages, perivascular cells, and microglial cells in

the cortex and white matter (the results are summarized in Table 4).

Perivascular cells in white and grey matter

In general, the number of perivascular cells was higher in the white than in the grey matter. However, the phenotype, as determined by immunocytochemistry, was the same.

Most perivascular cells were reactive with antibodies directed against HLA-DR, α_1 ACT, leucocyte common antigen (LC), and showed oil-red-O and acid phosphatase activity. Some cells were reactive with OKM 5 and occasionally also with OKM 1. All perivascular cells were reactive with KP 1 (Figure 2). With the KP1/HLA-DR, KP1/ α_1 ACT and HLA/DR/ α_1 ACT double staining techniques, a number of perivascular cells were labelled. The vessel walls were mildly reactive with HAM 56 and strongly with HLA-ABC. Control brains contained a few perivascular cells which showed the same staining pattern as those in ALS but consistently lacked immunoreactivity for HLA-DR.

Parenchyma: white matter

Many cells with ramifying processes and closely resembling microglia were dispersed diffusely in the white matter, and were stained intensely with HLA-DR and KP1. These cells were stained only lightly by LC, Leu M1 and CD 11c. It was not possible to detect whether or not rounded macrophages positive for HLA-DR or KP1 were present

Figure 1. Normal Betz cell and small group of phagocytes (arrow) in the motor cortex of patient 4. Nissl. Bar represents 30 μ m.

Figure 2. Macrophages around vessel wall and in the parenchyma (arrows), in the deeper layers of the motor cortex of patient 2. Immunocytochemistry: KP 1. Bar represents 100 μ m.

Figure 3. Microglial cells in the white matter of patient 2. Immunocytochemistry: HLA-DR. Bar represents 70 μ m.

Figure 4. α_1 ACT-positive cells surround and partly cover a large pyramidal cell in the motor cortex of patient 2. Immunocytochemistry. Bar represents 50 μ m.

	PVC	Neuronophages	Microglia	Neurons	Astrocytes
α_1 ACT	+	+	—	—	—
LC	+	±	±	—	—
HLA ABC	—	—	—	—	±
HLA DR	+	+	+	±	±
Tal 1B5	+	+	+	±	—
VIC-Y1	+	+	+	±	—
OKM 1	±	—	—	—	—
OKM 5	±	±	—	—	—
Leu M1	—	—	±	—	—
Leu M3	±	±	—	—	—
MAC 387	—	—	—	—	—
Kp1	+	+	+	—	+
P150/95	±	±	±	—	—
HAM-56	—	—	—	—	—

Table 4. Cell staining properties with macrophage antibodies

PVC, Perivascular cells. +, Many positive cells. ±, Occasional weakly stained cell. —, No staining.

amongst the many microglial cells. The HLA-DR positive microglial cells were stained in a fine granular pattern (Figure 3). KP1, in contrast, showed a coarse granular cytoplasmic staining. A faint immunoreactivity of microglia for LC and incidentally for HLA-DR was found in the white matter of the control tissue. The KP1 showed a wide-spread reactivity on microglia in controls but not to the same extent as in ALS. With double labelling techniques, many KP1/HLA-DR positive dendritic microglial cells and a single GFAP/HLA-DR astrocyte were found. The other double-labelling experiments did not reveal any double labelled cells.

Parenchyma: grey matter

HLA-DR and KP1 stained many dendritic cells in the cortex. These cells were not restricted to layer 5 (Betz cell layer) but were dispersed without a sharp boundary line over the third and fifth cortical layer. Around neurons, there were clusters of intensely KP1, α_1 ACT and HLA-DR positive rounded macrophages. KP1 seemed to stain all microglial cells and all rounded macrophages (Figure 4). HLA-DR stained microglia and the majority of the rounded macrophages. α_1 ACT selectively stained the rounded macrophages both in perivascular regions and in areas of neuronophagia (Figures 4 and 5). It was clear that neuronophagic clusters contained many more α_1 ACT positive macrophages than dendritic microglial cells. Patchy HLA-ABC expression was found in all cortical layers in the parenchyma of three patients. Some GFAP/HLA-DR positive astrocytes were found. However, the majority of the GFAP-stained astrocytes were not stained with HLA-DR. GFAP/KP1 and GFAP/ α_1 ACT were negative. Microglial

cells were stained with KP1/HLA-DR. Several rounded macrophages were double stained with α_1 ACT/HLA-DR and with KP1/HLA-DR.

Neurons

Staining with antibodies directed against MHC Class II antigens, i.e. HLA-DR, Vic and Tal 1 B5 showed that some cortical neurons expressed HLA-DR (Figure 6). However, the number of these neurons was low. With NSE/HLA-DR and Tal 1 B5/NSE sporadic double labelled triangular cells were found. HLA-DR positivity was seen on the cell membrane, whereas NSE stained the cytoplasm.

Discussion

In neuronophagia the neurons were found to be virtually covered by KP1 positive cells. The macrophages involved in neuronophagia probably originate from perivascular cells. No distinction could be made between microglia and other macrophages with KP1. On the other hand, perivascular macrophages but not microglia were stained with α_1 ACT. Since the majority of macrophages around neurons in the cortex expressed α_1 ACT, the majority of neuronophages are phenotypically identical to perivascular macrophages and not to microglial cells. Considering the number of perivascular macrophages in neuronophagia, phagocytosis and the removal of cell debris may be a major task of the perivascular macrophages. However, resident microglial cells could also change both in morphology and the expression of antigens, which could make the resident microglia the source of these macrophages. The possibility of change in morphology and antigen

Figure 5. Several clusters of phagocytes in the cerebral motor cortex of patient 2. Immunocytochemistry: α , ACT. Bar represents 100 μ m.

Figure 6. Class II antigen positive neurons (arrows) in the cerebral cortex near a vessel wall (asterisk), patient 2 immunocytochemistry: HLA-DR. Bar represents 100 μ m.

expression has been demonstrated before in microglia, migrating during development into the CNS [32]. Leu M3 failed to stain macrophages in the parenchyma and also failed to stain the majority of the perivascular macrophages. This contradicts the findings of Franklin [10] who stated that 100% of the perivascular macrophages in brain tissue are Leu M3 positive.

The exact trigger for phagocytosis is not known. Both perivascular macrophages and microglia in neuronophagia were HLA-DR positive, but double labelling also showed several rounded macrophages which were not stained with HLA-DR. In view of the low numbers of T cells in ALS cortex [39], presentation of exogenous proteins by antigen-presenting cells (APC) to CD4⁺ T lymphocytes does not seem to be a major factor in neuronophagia in ALS. This would require the association of the lymphocytes with class II major histocompatibility complex (MHC) molecules, e.g. HLA-DR [21], as a possible pathway of destruction. Furthermore, the majority of the lymphocytes found in the cerebral cortex in ALS belong to the CD8⁺ subset, and are located perivascularly; they are not present in the parenchyma [39]. The capacity of

infiltrating T cells to release interferon gamma (IFN gamma) within the brain tissue and the amount of HLA-DR antigens on antigen presenting cells (APCs) may not be critical for the process of cell destruction. It has been shown that viruses can convert HLA-DR negative APCs to HLA-DR positive cells by IFN gamma-unrelated mechanisms [28]. Therefore it is possible that the HLA-DR upregulation on microglia in ALS is unrelated to the production of lymphokines by lymphocytes.

The functional properties of microglial cells may also contribute to the mechanism of neuronophagia. Two important macrophage functions of microglia are known: antigen presentation and tumour cytotoxicity [12, 19]. In addition, the production of a neural cytotoxic factor by microglia is a possibility. It has been shown that unstimulated microglial cells show no tumour cytotoxicity, whereas upon stimulation with IFN gamma and lipopolysaccharide (LPS) [10] microglial cells do show cell cytotoxicity.

In ALS, microglia are strongly activated [17, 39] and neurons with a morphologically normal appearance are phagocytosed by perivascular macrophages and microglia.

It might therefore be hypothesized that the activated microglia in ALS produce a cytotoxic product by which the neuron is lysed. The subsequent phagocytosis and removal of cell debris is performed by the PVM which has the amoeboid properties necessary for the removal of the cellular debris from specific sites.

The role of microglia in neuronophagia is obscure. The clearcut upregulation of HLA-DR expression by microglia both in white and grey matter in ALS is also seen in other neurodegenerative diseases like Alzheimer's disease [1, 31]. The precise guidance from perivascular macrophages and microglia to the neurons which are to be digested could be integrin-dependent. Integrins are transmembrane glycoproteins with an intracellular domain linked to the cytoskeleton. The extracellular domain attaches to receptors on membranes or matrix proteins [16]. Integrins play a key role in immune function by providing guidance of cells to their correct location and promoting their adherence to target structures [16]; they may play a role in the guidance of the perivascular macrophages to the target neurons. The presence of β_2 -integrins on microglia has been demonstrated by Akayima [1] in normal brain tissue and in Alzheimer's disease, but their distribution has not yet been investigated in ALS.

The primary goal of the present study was to identify the phagocytes in ALS neuronophagia. It was found that neurons in ALS incidentally express class II MHC antigens. Since an unequivocal identification of cells is difficult in conventional cryostat sections due to their limited morphological resolution, we took advantage of double labelling techniques. With this method and the use of different antibodies directed against class II antigens we here demonstrated that MHC class II antigens are expressed by a small number of neurons. This demonstration is reinforced by the observations that: (i) with different antibodies against class II antigens the same results were observed; (ii) double labelling with NSE/HLA-DR showed double labelled cells in the cortex.

The observation of HLA-DR expression on neurons is not in accordance with earlier findings in the CNS where HLA-DR expression has been exclusively found on microglia, macrophages, astrocytes and vessel walls [24, 35]. However, in experimental animal models it has been shown that interferon-gamma can induce MHC molecule expression on neurons [41]. Possible explanations for this discrepancy are: (i) HLA-DR levels on neurons in other studies have been below the level of detection by immunocytochemical methods in previous studies; (ii) the

influence of autopsy delay is more important than previously thought [35], and (iii) HLA-DR expression could be a transient event occurring only for a very short period in the course of the disease. When it is not a simple cross or bystander reaction, the *de novo* expression of MHC class II antigens indicates that, in certain neurological conditions, neurons may be antigen presentors. If that is the case, autoimmune or infectious hypothesis for ALS would be strongly supported by this observation.

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