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Brainstem cytokine changes in healthy ageing and Motor Neurone Disease

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ABSTRACT

Neuroinflammation is linked to healthy ageing, but its role in the development of age-related neurodegenerative diseases is unclear. In this pilot study we used a multiplex assay approach to compare 27 cytokines in 6 young adult and 6 ageing control brainstems with those in 6 MND brainstems. We report that healthy ageing is associated with significantly increased brainstem levels of IL-1β, IP-10 and MIP-1β which co-localise immunocytochemically to astrocytes. MND brainstem is also characterised by a general increase in both pro- and anti-cytokine levels, but fails to show the expected age-related increase in MIP-1β and IP-10. This pilot study is the first to show that MND is associated with a failure of specific features of the normal age-related neuroinflammatory process. We suggest that our pilot data indicates that neuroinflammation during healthy ageing may not always be detrimental to motoneuronal survival and that age-related neurodegenerative diseases, such as MND, may instead result from defective neuroinflammation.

1. Introduction

Motor Neurone Disease (MND) includes several neurodegenerative conditions, namely Amyotrophic Lateral Sclerosis, Progressive Bulbar Palsy, Primary Lateral Sclerosis and Progressive Muscular Atrophy, which are characterised by the loss of upper and lower neurons either singly or in combinations [1]. The single biggest risk factor for approximately 90% of cases (sporadic MND) is advancing age [2]. A characteristic of ageing is an imbalance of inflammatory and anti-inflammatory processes due to dysregulation of initiation and resolution of innate and adaptive immune mechanisms resulting in an overall low-grade pro-inflammatory status in a process that has been termed 'inflammaging' [3–5]. This process also affects the ageing central nervous system (CNS) [6,7], and this has led to the suggestion that aberrant CNS inflammation might underlie the development of age-related neurodegenerative diseases, including MND [8,9] ([10,11], McGeer and [12–15]). Although inflammation involves a complex interplay between multiple chemical mediators [16,17], most studies of inflammation in MND have focussed on increases in single pro-inflammatory cytokines and chemokines, or where multiple cytokines have been studied [18–21], these have focussed on cerebrospinal fluid (CSF) rather than CNS tissue. In MND, elevated levels of IL-1β, IL-2, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17A, G-CSF, FGF, eotaxin, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-α, MIP-1β, RANTES and TNF-α have been reported in cerebrospinal fluid (CSF), serum or spinal cord tissue (Refer Table 1 for references). However, decreased levels of IL-1β IL-2, IL-5, IL-10, FGF, IFN-γ and VEGF have also been found in MND patients (Table 1). Other studies have reported no change in some cytokines, such as IL-4, IL-6, IP-10 and PDGF-BB (Table 1). Thus, a complicated picture emerges of changes in the overall cytokine signature in MND. The extent to which this reflects real differences or differences between different investigators and tissue samples is not clear.

In addition, very few studies include young adults who have died from non-neurological causes to control for the effect of ageing per se on the inflammatory status of the CNS. In this study, we have measured levels of 27 cytokines simultaneously in post-mortem brainstem taken from patients who died from non-neurological causes aged 20–33 years and 72–86 years and compared these with brainstem taken from patients aged 60–78 years who died from sporadic MND. We previously found that facial motoneurons in ageing rats were less likely to die following injury [44] and that this was associated with increased levels of cytokines in the brainstem where the facial nuclei are located [45]. Since, facial motoneurons are affected in MND [46] and we have animal data implicating brainstem cytokines in motoneuronal survival, the present cytokine study has been undertaken on the brainstem in MND and results compared with age-matched and young controls.

2. Methods

Fresh frozen brainstem was obtained from the South Australian Brain Bank from 6 patients (mean age 67 years) who died from sporadic MND, diagnosed clinically and pathologically and from 6 patients (mean age 74 years) of similar age who died from non-neurological causes (old

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Table 1
Cytokine alterations in MND patients.

Cytokine	Classification based on function	Found in	Level of cytokine in MND	Method
IL-1ra	Pro-inflammatory	CSF	↓	MP[1]
IL-1β	Pro-inflammatory	CSF	↓	MP[1]
		Spinal cord tissue	↑	RP[2], MP[3]
IL-2	Pro-inflammatory	CSF	↑	EL[10], MP[5]
		Serum	↓	MP[6]
IL-4	Anti-inflammatory	CSF	–	MP[1]
IL-5	Anti-inflammatory	CSF	↑	MP[12]
		Serum	↓	MP[6]
IL-6	Pro-inflammatory,	Serum, skin	↑	MP[6], EL[7, 8, 10]
	Anti-inflammatory	CSF	↑	EI[4], EL[8], MP[5]
			–	IS[9], MP[1]
IL-7	Pro-inflammatory	CSF	↑	MP[3]
		CSF	–	MP[1]
IL-8	Pro-inflammatory,	CSF	↑	MP[3, 6, 11]
	Chemokine	CSF	–	MP[1]
IL-9	Anti-inflammatory	CSF	↑	MP[3]
IL-10	Anti-inflammatory	CSF	↓	MP[12]
		CSF	–	MP[1]
		Serum	↑	MP[10]
IL-12	Pro-inflammatory	CSF	–	EL[13], MP[3]
		Serum, CSF	↑	EL[14],
IL-13	Anti-inflammatory	Serum	↑	FC[15]
1L-15	Pro-inflammatory	Serum, CSF	↑	EL[14], MP[5, 10]
IL-17A	Pro-inflammatory	Serum, spinal cord tissue, CSF	↑	MP[1, 3, 5, 10], EL[14, 16, 17]
FGF	Growth factor	Spinal cord tissue	↓	RP[18]
		CSF, serum	↑	MP[3, 5, 10]
Eotaxin	Chemokine	CSF, serum	↑	MP[3,11]
G-CSF	Anti-inflammatory, growth factor	CSF, serum	↑	MP[3, 5, 10, 12]
GM-CSF	Pro-inflammatory, growth factor	CSF, serum	↑	MP[5, 10]
IFN-γ	Pro-inflammatory	Blood, CSF, serum	↑	WB[19], EL[21, 24], MP[3,10]
		Plasma	↓	EL[20]
IP-10	Chemokine	CSF	–	MP[11]
		CSF	↑	MP[3]
MCP-1	Chemokine	Spinal cord tissue, CSF, serum	↑	RP[22], EL[10, 23, 25], MP[3, 10, 11]
MIP-1α	Chemokine	CSF, serum	↑	MP[10], EL[26]
MIP-1β	Chemokine	CSF	↑	MP[3, 5, 10]
PDGF-BB	Growth factor	CSF	–	MP[3,10]
RANTES	Chemokine	CSF, serum	↑	EL[27], MP[3]
TNF-α	Pro-inflammatory	Serum plasma, blood	↑	EL[8, 24, 28, 29]
		CSF	–	MP[1]
		CSF	↑	MP[3]
VEGF	Growth factor	CSF, serum	↑	EL[30], RT[31], MP[3, 5, 10]
		Spinal cord tissue	–	EL[30], MP[1]
			↓	EL[32]

(1) [19], (2) [22], (3) [21], (4) [23], (5) [20], (6) [24], (7) [25], (8) [73], (9) [26], (10) [18], (11) [27], (12) [28], (13) [29], (14) [30], (15) [31], (16) [32], (17) [33], (18) [34], (19) [35], (20) [36], (21) [37], (22) [10], (23) [38], (24) [74], (25) [13], (26) [39], (27) [40], (28) [41], (29) [42], (30) [75], (31) [76], (32) [43].
↑ = significantly increased compared to controls, ↓ = significantly decreased compared to controls, – = no change or not detected, MP = multiplex assay, EL = enzyme-linked immunosorbent assay, RP = real time qPCR, EI = electro-immunoassay, WB-western blot, FC = flow cytometry, IS-immunostaining.

control). Fresh frozen brainstem from 6 young adults (mean age 27.2 years) who died from non-neurological causes (young control) was obtained from the Edinburgh Brain Bank, UK (Table 2). The histology of the pons of the MND and age-matched controls were considered normal for the age of the subject. No neuropathological data was available for the young controls.

Table 2
Details of the fresh frozen brain samples used for cytokine assays.

n	Adult control	Ageing control	MND
	6	6	6
Mean age (range) years	27.2 (20 − 33)	74.0 (48–86)	67.0 (62–78)
Male:female	1:1	2:1	1:1
Mean post mortem interval (range) hours	36.7 (20–53)	30.1 (6–48)	24.2 (6–44)
Cause of death [duration of MND (years), disease subtype]	Ischaemic heart disease	Generalised arteriosclerosis	MND (1.1,ALS)
	Suspension by a ligature	MRSA	MND (3, PMA)
	Suspension by a ligature	Pulmonary infarct	MND(14,PMA)
	Cardiomyopathy	Hepatocellular carcinoma	MND(NA,ALS)
	Suspension by a ligature	Metastatic cancer	MND (2,ALS)
	Suspension by a ligature	IHD	MND (2.5,ALS)
Brain bank unique identifiers	SD053/14;BBN24342	SA006	SA0186
	SD042/12;BBN377	SA0112	SA0202
	SD008/12;BBN3771	SA0162	SA0203
	SD006/10;BBN2503	SA0098	SA0212
	SD036/08;BBN2455	SA0214	SA0221
	SD023/08;BBN2442	SA0230	SA0245

PMA-Progressive Muscular Atrophy, ALS-Amyotrophic Lateral Sclerosis, NA-Data not available.

2.1. Cytokine assays

Approximately 2 mm of the mid/lower pons was removed with a sterile scalpel blade, weighed then homogenised at a concentration of 0.1 g/ml in homogenisation buffer made up with PBS, triton-X and protease inhibitors (Roche, complete tablets) in grinding chambers using 10 pestle-strokes for every sample. Homogenised samples were centrifuged (1000*g*) for 15 min at 4 °C, the pellet discarded and the supernatant stored at −80 °C. Bovine serum albumin standards ranging between 0.1 and 10 μg/μl in distilled water were prepared and loaded as triplicates along with blanks and samples into 96-well ELISA plates. The BioRad DC Protein Assay (BioRad, New South Wales), which is a modified Lowry method, was used to quantitate the amount of protein in each sample as per the manufacturer's instructions. Bio-Plex Pro human 27 plex cytokine assay kits (BioRad, New South Wales) were used to measure the concentration of 27 cytokines within each sample. The panel was composed of Interleukin-1β (IL-1β), IL-1 receptor antagonist (IL-1Ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, eotaxin/CCL11, fibroblast growth factor-2 (FGF-2), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), IFN-γ-induced protein 10 (IP-10)/CXCL10, monocyte chemotactic protein-1 (MCP-1)/CCL2, macrophage inflammatory protein-1α (MIP-1α)/CCL3, macrophage inflammation protein-1β (MIP-1β)/CCL4, platelet-derived growth factor BB (PDGF-BB), regulated on activation, normal T cell expressed and secreted (RANTES)/CCL5, tumour necrosis factor-α (TNF-α), and vascular endothelial growth factor (VEGF). Depending on the ability of these cytokines to increase or decrease the inflammatory response they are categorised as being pro-inflammatory or anti-inflammatory respectively [47]. Of the cytokines studied here, IL-1ra, IL-1β, IL-2, IL-7, IL-8, IL-12, IL-15, IL-17A, IFN-ϒ, TNF-α, MIP-1α, MIP-1β, MCP-1, IP-10, RANTES, GM-CSF, VEGF and eotaxin are pro-inflammatory, whereas IL-4, IL-5, IL-9, IL-10, IL-13, FGF, G-CSF and PDGF-BB are anti-inflammatory. IL-6 has shown both pro-inflammatory and anti-inflammatory effects [48]. Samples were loaded onto 96 well plates in duplicates (3 m and 12–18 m rats) and triplicates (24 m rats). Plates were read using a Magpix Luminex multiplexing platform (Abacus-ALS, Queensland, Australia). Experimental data was calibrated against standard samples of all 27 cytokines (BioRad, New South Wales, Australia).