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Frequency and function of regulatory T cells after ischaemic stroke in humans

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ABSTRACT

Ischaemic stroke is an important cause of disability and death. Local inflammation in the brain following stroke is thought to enhance damage. Animal studies show that regulatory T cells (Tregs) can downregulate this inflammation and assist recovery, but there are no previous studies of the function of Tregs in human stroke. The current study aimed to quantify Tregs in peripheral blood following stroke and to investigate their function. Treg numbers were significantly increased after stroke, particularly in males. However, the functional capacity of Tregs to inhibit proliferation of autologous cells at low ratios of Treg to responder cells was reduced, particularly in female patients, compared to controls. This study is the first to report gender-specific changes in the numbers and function of Tregs after ischaemic stroke. These changes may affect stroke outcome.

1. Introduction

Ischaemic stroke results from interruption of blood supply to regions of the brain, leading to ischaemia of brain tissue, cell damage and cell death. After stroke, there is microglial activation and inflammation in the brain (McCombe and Read, 2008). T cells also enter the region of acute ischaemic stroke and contribute to tissue injury. This early effect occurs via non-specific mechanisms that do not require antigen recognition (Brait et al., 2010). Suppression of entry of T cells to the brain leads to improved outcome in experimental stroke (Czech et al., 2009; Shichita et al., 2009).

On day 1 after stroke there can be a reduced number of circulating T cells; however, we have recently found that the numbers of both $CD4^+CD25^+$ T cells and $CD4^+CD25^{hi}$ putative T regulatory cells (Tregs) are increased on days 7 and 21 after stroke (Yan et al., 2009). This increased frequency of circulating Tregs could be important in recovery from stroke, since Tregs not only suppress inflammation but have also been reported to be neuroprotective. The mechanisms of neuroprotection by Tregs include modulation of harmful microglial responses (Reynolds et al., 2007) or more direct mechanisms such as production of the growth factors CDNF (ciliary derived neurotrophic factor) and TGF-β (Moalem et al., 2000; Reynolds et al., 2007). In experimental stroke, outcome was improved by transfer of tolerized cells (Becker et al., 2003), by nasal tolerization, where the tolerized cells are thought to be Tregs (Frenkel et al., 2005), and by passive transfer of purified Tregs (Liesz et al., 2009).

Since we had previously seen increases in numbers of $CD4^+CD25^{hi}$ expressing cells following stroke (Yan et al., 2009), the aim of the current investigation was to verify in a larger group of patients that these cells are definitely Tregs, by determining if they also expressed Foxp3 and were $CD127^{dim}$. We also aimed to investigate the functional capacity of these cells to suppress other T cell responses, as this has not previously been measured in human stroke.

2. Material and methods

2.1. Subjects

We studied 77 patients with acute ischaemic stroke and 64 control subjects (Table 1). Acute ischaemic stroke was defined according to the WHO definition (Aho et al., 1980). Patients with subarachnoid haemorrhage, extradural or subdural haemorrhage, transient ischaemic attack (TIA; defined using standard criteria) or neurological deficit due to trauma or neoplasm were excluded. Patients with acute infection after stroke were excluded. We also documented clinical details including the presence of diabetes mellitus, hypertension and the use of statins. This data was obtained by self-report by the patients, or by questioning their relatives.

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Table 1
Clinical details of healthy controls and groups of stroke subjects.

	n	Median age (range)	Hypertension n (%)	Diabetes n (%)	Hyperlipidemia n (%)	Statins n (%)	Median NIHSS (IQR[a])	OSCC[b] n (%)			
								POCI	PACI	LACI	TACI
Controls											
All	64	63 (20–95)	18 (28.1%)	3 (4.7%)	11 (17.2%)	7 (10.9%)	N/A[c]	N/A	N/A	N/A	N/A
Female	31	63 (20–95)	11 (35.5%)	1 (3.2%)	6 (19.4%)	5 (16.1%)	N/A	N/A	N/A	N/A	N/A
Male	33	63 (27–95)	7 (21.2%)	2 (6.1%)	5 (15.2%)	2 (6.1%)	N/A	N/A	N/A	N/A	N/A
Stroke patients											
All	77	66 (27–88)	56 (72.7%)	16 (20.8%)	41 (53.2%)	58 (75.3%)	N/A	15 (20.5%)	27 (37%)	17 (23.3%)	14 (19.2%)
Female	31	66 (28–88)	26 (83.9%)	8 (25.8%)	16 (51.6%)	23(74.2%)	N/A	5 (17.9%)	12 (42.9%)	5 (17.9%)	6 (21.4%)
Male	46	65.5 (27–87)	30 (65.2%)	8 (17.4%)	25 (54.3%)	35(76.1%)	N/A	10 (22.2%)	15 (33.3%)	12 (26.7%)	8 (17.8%)
Day 1											
All	33	66 (32–85)	26 (78.8%)	6 (18.2%)	18 (54.5%)	19 (57.6%)	6 (3–12)	10 (33.3%)	10 (33.3%)	5 (16.7%)	5 (16.7%)
Female	15	66 (40–85)	14 (93.3%)	5 (33.3%)	9 (60.0%)	7 (46.7%)	7 (4.5–14)	4 (33.3%)	4 (33.3%)	2 (16.7%)	2 (16.7%)
Male	18	67.5(32–82)	12 (66.7%)	1 (5.6%)	9 (50.0%)	12 (66.7%)	5 (3–10.5)	6 (33.3%)	6 (33.3%)	3 (16.7%)	3 (16.7%)
Week 1											
All	68	66 (27–88)	48 (70.6%)	15 (22.1%)	36 (52.9%)	50 (73.5%)	3.5 (1.5–7)	14 (21.2%)	25 (37.9%)	14 (21.2%)	12 (18.2%)
Female	26	67.5 (28–88)	20 (76.9%)	7 (26.9%)	12 (46.2%)	18 (69.2%)	3 (0.5–8.5)	4 (16.0%)	11 (44.0%)	4 (16.0%)	5 (20.0%)
Male	42	65.5 (27–87)	28 (66.7%)	8 (19.0%)	24 (57.1%)	32 (76.2%)	4 (2–7)	10 (24.4%)	14 (34.1%)	10 (24.4%)	7 (17.1%)
Week 3											
All	61	66 (27–88)	42 (68.9%)	14 (23.0%)	30 (49.2%)	46 (75.4%)	2 (0–5)	11 (18.6%)	23 (39.0%)	14 (23.7%)	10 (16.9%)
Female	22	67.5 (28–88)	17 (77.3%)	8 (36.4%)	10 (45.5%)	16 (72.3%)	3 (1–5)	4 (19.0%)	9 (42.9%)	4 (19.0%)	3 (14.3%)
Male	39	66 (27–87)	25 (64.1%)	6 (15.4%)	20 (51.3%)	30 (76.9%)	2 (0–4)	7 (18.4%)	14 (36.8%)	10 (26.3%)	7 (18.4%)

[a] IQR = interquartile range.
[b] OSCC not available for 3 females tested on day 1, and 1 female and 1 male tested at week 1 and week 3.
[c] N/A = not applicable.

Blood was intended to be collected from patients with acute ischaemic stroke on day 1, day 7 and day 21 after stroke. In some subjects, there was a delay in collecting samples so that blood was collected at day 1, days 7–10 ("week 1") and between weeks 3 and 6 after stroke (in the figures and legends, "week 3" refers to samples collected between weeks 3 and 6). Blood from the controls was collected once only.

The strokes were classified according to the Oxfordshire stroke classification (Bamford et al., 1991) into total anterior circulation infarcts (TACI), partial anterior circulation infarcts (PACI), posterior circulation infarcts (POCI) and lacunar infarcts (LACI). The NIH stroke scale (NIHSS) (Lyden et al., 1999) was used to measure stroke severity. The full range of the NIHSS is from 0 to 42: the lower the score, the less severe the stroke.

This study was reviewed and approved by Royal Brisbane and Women's Hospital Human Research Ethics Committee and the Medical Research Ethics Committee of the University of Queensland.

2.2. Isolation of PBL, antibody labelling, and flow cytometry

Peripheral blood (50 ml) was collected from participants after informed consent was obtained, and peripheral blood leukocytes (PBL) were purified and labelled as described previously (Yan et al., 2009). Briefly, cells were labelled for 30 min at 4 °C in the dark with fluorescent dye-conjugated monoclonal antibodies (mAb) against the cell surface markers CD3, CD4, CD25, CD69, or CD127 (BD Biosciences, North Ryde, NSW, Australia). Fluorescent-conjugated non-specific isotype-matched mAb were used as negative controls. Cells were then washed with phosphate-buffered saline (PBS) containing 1% serum and 0.1% NaN_3 and analysed on a four colour flow cytometer (FACScalibur, BD Biosciences). For detecting Tregs, cells labelled with anti-CD4, anti-CD25 and anti-CD127 were fixed, permeabilized and stained with fluorochrome-conjugated anti-human Foxp3 mAb (eBioscience, San Diego, CA). For analysis, the cells within the lymphoid cell population were analysed. Percentages of cells stained with a particular antibody are reported after subtraction of the percentage of cells stained with the relevant isotype control antibodies.

2.3. Purification and characterization of Tregs

$CD4^+CD25^+CD127^{dim/-}$ Tregs were isolated using a commercial kit (Miltenyi Biotec, North Ryde, NSW, Australia), as per the manufacturer's instructions. In brief, $CD4^+$ $CD127^{dim/-}$ cells were negatively-selected from PBL, and this population was then positively selected for CD25 expression. Expression of Foxp3 in the purified population was confirmed by flow cytometry.

2.4. Functional assay for Tregs

To evaluate the function of Tregs, autologous PBL (2×10^5/well) were stimulated with bead immobilized anti-CD3/anti-CD28 (Miltenyi Biotec — used according to the manufacturer's instructions) in X-Vivo15 (BioWhittaker, Walkersville, MD) for 5 days in 96-well U-bottom plates (Nunc, Roskilde, Denmark) in the presence or absence of purified irradiated (3000 rad) Tregs at various responder:Treg ratios. One μCi [^{3}H]-thymidine was added during the final 16 h. Cells were then harvested onto glass-fiber mats, and the counts per minute (cpm) determined in a Betaplate counter (Beckman Coulter). The stimulation index (SI) for each sample was calculated using the formula: (mean cpm in presence of stimulus/mean cpm in absence of stimulus)$\times$100. The percentage suppression was calculated using the formula: [1-(SI in the presence of Tregs/SI in the absence of Tregs)]$\times$100.

2.5. Statistical analysis

To determine whether distributions were normal for subsequent statistical analyses, we used Kolmogrov–Smirnov test and D'Agostino and Pearson omnibus normality tests. Depending on whether or not data were normally-distributed, analysis of variance (ANOVA) or the Kruskal–Wallis test were used to compare levels of cell marker expression or functional activity of Tregs among the healthy control and stroke populations at the different time-points. If these gave a $P<0.05$, then post-tests were done to compare individual groups. Power calculations were done using online Java Applets for Power and Sample Size (Lenth, 2006–9).