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Table 4
Change in psychometric scores and FA values and axial/radial diffusivity over 6 months in patients ($n=18$) and controls ($n=19$).

	10–28 Days after stroke	6 Months after first exam	Paired t-test	p
Patients				
Age (years)	69.2 ± 8.0	–	–	–
Female sex (n, %)	5 (26.3)	–	–	–
mRS score	2.1 ± 0.8	1.6 ± 0.6	$t_{17}=4.12$	< 0.01**
MMSE score	27.6 ± 3.5	29.0 ± 2.1	$t_{17}=2.08$	0.05
SDS score	26.9 ± 5.6	29.2 ± 11.5	$t_{17}=0.93$	0.37
HAM-D score	3.1 ± 2.7	2.8 ± 4.9	$t_{17}=0.21$	0.84
Anterior limb of internal capsule				
FA	0.42 ± 0.06	0.44 ± 0.06	$t_{17}=2.16$	0.05 *
Axial diffusivity (× 10^{-3})	4.13 ± 0.37	4.29 ± 0.45	$t_{17}=1.32$	0.21
Radial diffusivity (× 10^{-3})	3.91 ± 0.34	4.01 ± 0.37	$t_{17}=1.38$	0.37
Controls				
Age (years)	69.1 ± 8.0			
Female sex (n, %)	10 (55.5)			
MMSE score	29.2 ± 1.2	29.7 ± 0.5	$t_{18}=0.95$	0.36
SDS score	22.1 ± 1.8	22.8 ± 2.6	$t_{18}=1.80$	0.10
HAM-D score	0.4 ± 0.6	0.3 ± 0.6	$t_{18}=1.00$	0.33
Anterior limb of internal capsule				
FA	0.49 ± 0.03	0.48 ± 0.03	$t_{18}=0.76$	0.46
Axial diffusivity (× 10^{-3})	4.18 ± 0.66	4.30 ± 0.55	$t_{18}=0.92$	0.37
Radial diffusivity (× 10^{-3})	3.86 ± 0.60	4.00 ± 0.46	$t_{18}=0.75$	0.46

mRS=Modified Rankin Scale. MMSE=Mini-Mental State Examination. SDS=Zung Self-Rating Depression Scale. HAM-D=Hamilton Rating Scale for Depression.
Data are mean ± S.D.
* $p<0.05$.
** $p<0.01$.

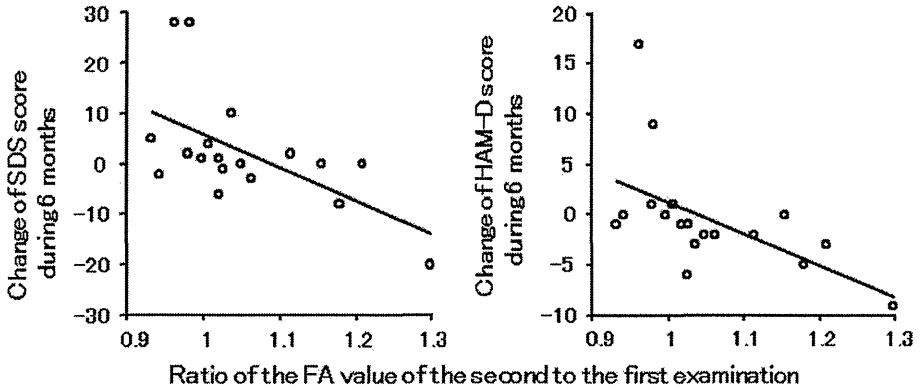

Fig. 2. Scatter plots showing the relationship between the ratio of the FA values of the second to the first examination and the change of depression scale scores among patients ($n=18$) Significant correlations were observed between the ratio of the FA values of the second examination to the first examination, and the changes in depression scale scores ($r=-0.59$, $p=0.01$ for the SDS; $r=-0.88$, $p=0.0001$ for the HAM-D).

the risk of post-stroke depression in conjunction with psychosocial factors following stroke. Further, a significant increase of FA in this region was noted 6 months after the stroke, and an association with a reduction in depression scale scores was revealed. Our findings suggested that the promotion of recovery from the axonal damage of stroke patients might prevent the onset of depressive symptoms. Further investigations are needed to clarify whether and how axonal damage of the internal capsule in stroke patients affects the clinical presentation, treatment response and outcome of depression or other psychiatric conditions in stroke survivors.

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Research Article Open Access

Possible Protective Effect of Regulatory T cells on White Matter Microstructural Abnormalities in Stroke Patients

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Abstract

Background: Despite advances in the understanding of stroke, therapeutic options for stroke are limited. Inflammatory mechanisms activated after brain ischemia are a key target of translational cerebrovascular research. The purpose of the present study was to investigate the existence of microstructure abnormalities in the white matter of stroke patients and their relationship to lymphocyte subsets.

Methods: The study included 18 patients with acute ischemic stroke and 22 healthy subjects. Diffusion tensor scans with magnetic resonance imaging were performed. Whole brain voxel-based analysis was used to compare fractional anisotropy (FA) in the stroke and healthy control groups. Blood samples were obtained from all subjects at the initial examination. The lymphocyte subsets in peripheral blood were evaluated with flow cytometric analysis. Helper T cells ($CD3^+$ and $CD4^+$), cytotoxic T cells ($CD3^+$ and $CD8^+$), B cells ($CD19^+$), natural killer cells ($CD16^+$ or $CD56^+$), and regulatory T cells (T_{regs}) ($CD4^+$, $CD25^+$, and $FOXP3^+$) were identified.

Results: In the voxel-based analysis, FA in the bilateral anterior limbs of the internal capsule was lower in stroke patients than in healthy subjects. These regions exhibited decreased axial diffusivity. The frequency of T_{regs} was lower in patients than in healthy controls. In patients, we found a significant positive relationship between the level of circulating T_{regs} and the FA value in the anterior limb of the internal capsule.

Conclusions: Patients exhibited a decreased frequency of circulating T_{regs} and the degree of reduction correlated with the decrease in the FA value in the internal capsule. T_{regs} might attenuate post-stroke white matter tissue damage by limiting the immune response. Our findings demonstrate the need for further study of the role of T_{regs} in the prevention of post-stroke cerebral damage.

Keywords: Stroke; Magnetic resonance imaging (MRI); Diffusion tensor imaging (DTI); Fractional anisotropy (FA); Regulatory T lymphocyte (T_{reg})

Introduction

Stroke is the third leading cause of death and the most frequent cause of permanent disability in adults worldwide [1]. Despite considerable advances in understanding the pathophysiology of cerebral ischemia, therapeutic options for stroke are limited. Inflammatory mechanisms activated after brain ischemia are a key target of current translational cerebrovascular research. Stoke induces a profound local inflammatory response involving various types of immune cells that transmigrate across the activated blood-brain barrier to invade the brain [2].

In the search for ways to prevent cerebral damage due to stroke, several factors related to inflammation have received considerable attention [3-5]. In particular, T lymphocytes are central to the development of a sustained inflammatory response in brain injury after a stroke. T cells are sources of pro-inflammatory cytokines and cytotoxic substances, such as reactive oxygen species, that likely contribute to neuronal death and poor outcome in stroke. However, recent evidence has indicated a novel role of T cells in promoting brain tissue repair and regeneration in the weeks and months after a stroke [6]. The complex role of T lymphocytes in ischemic stroke remains poorly understood. Further research is needed to understand which T-cell subpopulations produce and prevent damage after a stroke.

The primary aim of the present study was to elucidate the microstructural abnormalities in the white matter circuit in stroke patients and determine their relationship to the levels of circulating T lymphocytes. To identify microstructural abnormalities in stroke

patients, diffusion tensor imaging was performed and whole brain voxel-based analysis was used to compare fractional anisotropy (FA) in acute ischemic stroke patients and healthy control subjects. Furthermore, the circulating T-cell subpopulations in stroke patients and healthy subjects were compared, and the association between T-cell subpopulations and white matter microstructural abnormalities in patients was assessed.

Methods

Subjects

After providing subjects with a complete description of the study, written informed consent was obtained. The study was approved by the medical ethics committee of the National Cerebral and Cardiovascular Center of Japan. The patients were of Japanese ethnicity and were recruited from the neurology unit of the National Cerebral and Cardiovascular Center hospital. The patients had initially been hospitalized for treatment of acute ischemic stroke.

Stroke was diagnosed by neurologists according to the WHO criteria (1989). After the assessment, a group of psychiatrists and neurologists reviewed the data and reached a consensus regarding the presence or absence of psychiatric disease, including dementia, according to DSM-IV criteria. Patients were included if they met the following criteria: 1) a focal lesion of either the right or left hemisphere on magnetic resonance imaging (MRI); 2) absence of other neurologic, neurotoxic, and metabolic conditions; 3) modest ischemic insult (modified Rankin scale $\leq$ 4) with absence of a significant verbal comprehension deficit; and 4) occurrence of stroke 10–28 days before the examinations. The exclusion criteria were: 1) transient ischemic attack, cerebral hemorrhage, subdural hematoma, or subarachnoid hemorrhage; 2) history of a central nervous system disease such as tumor, trauma, hydrocephalus, or Parkinson's disease; and 3) pre-stroke history of depression. Eighteen subjects participated in the MRI study and the analysis of lymphocyte subsets in peripheral blood.

Twenty-two healthy control subjects were recruited for this study from the local area by poster advertisement. Subjects were excluded if they had a history or current diagnosis of any DSM-IV axis I or neurological illness. The major characteristics of this cohort are summarized in Table 1.

Characteristic	Stroke patients (n = 18)	Healthy control subjects (n = 22)	t or χ^2	p
Age (years)	70.0 ± 6.7	67.2 ± 5.5	t=1.46	0.15
Female, n (%)	4 (22.2)	8 (36.3)	χ^2=0.94	0.33
MMSE score	28.4 ± 1.9	29.3 ± 1.0	t=1.98	0.06
History of disease, n (%)				
Diabetes mellitus	5 (27.8)	2 (9.1)	χ^2_1=2.40	0.12
Hyperlipidemia	5 (27.8)	1 (4.5)	χ^2_1=4.19	0.04*
Hypertension	14 (77.8)	5 (22.7)	χ^2_1=12.0	<0.01**
Fazekas DWMH score, n (%)				
0–2	11 (61.1)	22 (100)		
3	7 (38.9)	0 (0.0)	χ^2=10.4	<0.01**
Fazekas PVH score, n (%)				
0–2	13 (72.2)	22 (100)		
3	5 (27.8)	0 (0.0)	χ^2=6.98	<0.01**
mRS score	1.9 ± 0.7	-		
NIHSS score	2.8 ± 0.9	-		
Anti-coagulant/platelet medication, n (%)				
Warfarin	3 (16.7)			
Acetylsalicylic acid	13 (72.2)			
Clopidogrel sulfate	2 (11.1)			
Cilostazol	3 (16.7)			
Number of acute infarcts	1.2 ± 0.5	-		
Volume of acute infarcts (mL)	1.6 ± 0.9	-		
Acute infarct location, n (%)				
Basal ganglia	11 (61.1)	-		
Subcortical white matter	6 (33.3)			
Thalamus	1 (5.6)	-		
Laterality of acute hemisphere infarcts				
Left hemisphere, n (%)	9 (50.0)	-		

Data are the mean ± SD. * p < 0.05, ** p < 0.01; MMSE: Mini-Mental State Examination; DWMH: Deep White Matter Hyperintensity; PVH: Periventricular Hemorrhage; mRS: Modified Rankin Scale; NIHSS: National Institutes of Health Stroke Scale

Table 1: Demographic characteristics of patients and healthy control subjects.

All patients received a neurological examination (modified Rankin Scale (mRS) [7], National Institutes of Health Stroke Scale (NIHSS) [8]) on the day of the MRI scan. Cognitive function was measured with the mini-mental state examination (MMSE) [9] in patients and control subjects. MRIs were conducted for all of the study subjects. The severity of white matter hyper intensity (WMH) was classified using the Fazekas scale, which is a simple visual rating scale used to rate the degree of leukoaraiosis (WMH). It provides an assessment of WMH in the peri-ventricular area (PVH) and in deep white matter (DWMH) on a four-point scale (0–3) [10].

MRI data acquisition

All MRI examinations were performed using a 3-Tesla whole-body scanner (Signa Excite HD V12M4; GE Healthcare, Milwaukee, WI, USA) with an 8-channel phased-array brain coil. Diffusion tensor images were acquired with a locally modified single-shot echo-planar imaging sequence by using parallel acquisition at a reduction (ASSET) factor of 2 in the axial plane. Imaging parameters were as follows: Repetition Time (TR)=17 seconds; Eco Time (TE)=72 ms; b=0 and

1000 seconds/mm^2; acquisition matrix, 128 × 128; field of view (FOV), 256 mm; section thickness, 2.0 mm; no intersection gap; 74 sections. The reconstruction matrix was the same as the acquisition matrix, and 2 mm × 2 mm × 2 mm isotropic voxel data were obtained. A motion probing gradient was applied in 55 directions, and 4144 images were obtained. The acquisition time was 15 min 52 seconds.

To reduce blurring and signal loss arising from field in homogeneity, an automated high-order shimming method based on spiral acquisitions [11] was used before acquiring diffusion tensor imaging scans. FMRIB software (FMRIB Center, Department of Clinical Neurology, University of Oxford, Oxford, England; http://www.fmrib.ox.ac.uk/fsl/) was used to correct for motion and distortion from eddy current and B0 in homogeneity. B0 field mapping data were also acquired with the echo time shift (2.237 msec) method based on two gradient echo sequences.

High-resolution, three-dimensional T1-weighted images were acquired using a spoiled gradient-recalled sequence (TR=12.8 msec; TE=2.6 msec; flip angle=8°; FOV, 256 mm; 188 sections in the sagittal plane; acquisition matrix, 256 × 256; acquired resolution, 1 × 1 × 1 mm). T2-weighted images were obtained using a fast-spin echo (TR=4800 ms; TE=101 ms; echo train length (ETL)=8; FOV=256 mm; 74 slices in the transverse plane; acquisition matrix, 160 × 160; acquired resolution, 1 × 1 × 2 mm).

Image processing

Fractional anisotropy maps, diffusion weighted images, and three eigen values (λ1, λ2, and λ3) were generated for each individual using FMRIB software. First, brain tissue was extracted using the Brain Extraction Tool. Brain maps for each of the 55 directions were eddy-corrected. Subsequently, FA values were calculated at each voxel using the FSL FMRIB Diffusion Toolbox.

Image preprocessing and statistical analysis were carried out using SPM8 software (Welcome Department of Imaging Neuroscience, London, England). Each subject's echo planar image was spatially normalized to the Montreal Neurological Institute echo planar image template using parameters determined from the normalization of the image with a b value of 0 seconds/mm^2. Images were resampled with a final voxel size of 2 × 2 × 2 mm^3. Normalized maps were spatially smoothed using an isotropic Gaussian filter (8-mm full-width at half-maximum).

Voxel-based analysis

Voxel-based analysis was performed using SPM8 software. FA maps of patients and healthy subjects were compared using analysis of covariance (ANCOVA), with age and sex as covariates. Statistical inferences were made with a voxel-level threshold of $p<0.001$, uncorrected, and a minimum cluster size of 100 voxels. The regional FA value was calculated by averaging the FA values for all voxels within the volume of interest (VOI), corresponding to the cluster composed of significant contiguous voxels in the above analysis. The same VOIs were applied to λ_1 - λ_3 images, and λ_1 - λ_3 values were extracted. Axial (λ_1) and radial diffusivity ($[\lambda_2 + \lambda_3]/2$) were compared.

Flow cytometric analysis of lymphocyte subsets in peripheral blood

Blood samples (5 mL) were obtained from all patients and healthy control subjects at the initial examination. The samples were collected into tubes containing sodium heparin. Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll density gradient (Ficoll-Paque PLUS; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's protocol. PBMCs were washed twice with phosphate buffered saline containing 1% fetal calf serum and 2 mM ethylene diamine tetra acetate.

To identify helper T cells ($CD3^+$ and $CD4^+$), cytotoxic T cells ($CD3^+$ and $CD8^+$), B cells ($CD19^+$), and natural killer (NK) cells ($CD16^+$ or $CD56^+$), the PBMCs were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-human CD3 (Beckman Coulter, Orange Country, CA, USA), phycoerythrin-cyanin 5 (PC5)-conjugated anti-human CD4 (Beckman Coulter), phycoerythrin-cyanin 7 (PC7)-conjugated anti-human CD8 (Beckman Coulter), phycoerythrin (PE)-conjugated anti-human CD19 (Beckman Coulter), PC5-conjugated anti-human CD16 (Beckman Coulter), and/or PE-conjugated anti-human CD56 (Beckman Coulter) at 4°C for 20 min. To identify T_{regs} ($CD4^+$, $CD25^+$, and $FOXP3^+$), PBMCs were incubated with FITC-conjugated anti-human CD4 (Beckman Coulter) and PC5-conjugated anti-human CD25 (Beckman Coulter) at 4°C for 20 min. After surface staining, PBMCs were fixed, permeabilized, and stained with PE-conjugated anti-human FOXP3 (Becton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. As negative controls, fluorochrome-conjugated non-specific isotype-matched antibodies (Beckman Coulter) were used. Stained cells were analyzed using a FC500 cytometer and CXP software (Beckman Coulter). The percentage of cells stained with a particular antibody was reported after subtracting the percentage of cells stained with the relevant negative isotype control antibodies.

Statistical analysis

Group differences in the demographic characteristics of patients and healthy controls were examined with an unpaired t-test and Pearson's χ^2 test. To examine group differences in FA values and axial/radial diffusivity in VOIs from the voxel-based analysis, we performed ANCOVA with age and sex as covariates.

We also used ANCOVA with age and sex as covariates to examine differences in the percentage of helper T cells, cytotoxic T cells, regulatory T cells, B cells, and NK cells between patients and healthy controls. For cells that showed significant differences between groups, the correlation between the FA values and the percentage of cells was examined using Spearman's correlation analysis.

All statistical tests were two-tailed and reported at $p<0.05$. The Bonferroni correction was applied to avoid type I errors due to the multiplicity of statistical analyses. Statistical analysis of the data was performed using SPSS for Windows 19.0 (IBM Japan Inc., Tokyo, Japan).

Results

Demographic and clinical data

Table 1 summarizes the demographic and clinical characteristics of the study subjects. Patients did not differ significantly from healthy control subjects in age, sex, or MMSE scores. The occurrence of hyperlipidemia and hypertension was significantly higher in patients than in healthy controls. There were no healthy control subjects with Fazekas scores higher than 3. Table 1 also shows the mRS and NIHSS scores, treatment with anti-coagulant/platelet medication, and the location and volume of the infarct. Patients exhibited some disability

from stroke at the time of the examination. All patients took anti-coagulant and/or anti-platelet medicine. Infarction occurred in the basal ganglia (61.1%), sub-cortical white matter (33.3%), and thalamus (5.6%). There was no significant laterality of hemisphere infarcts. Representative MR images of patients and controls are shown in Figure 1.

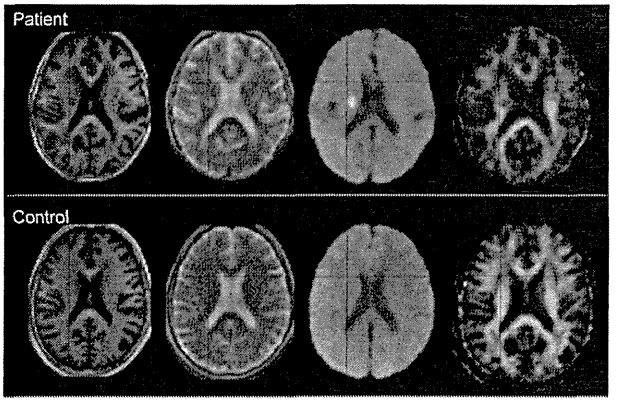

Figure 1: Representative MR images for patients and controls T1-weighted, T2-weighted, diffusion weighted, and fractional anisotropic (FA) images are shown, respectively, from left to right. All figures were spatially normalized to the image template in SPM8, and the axial slices are shown (z=18). The vertical and horizontal lines in the images represent the coordinates of x=-26 and y=12, respectively.

FA values in patient and control groups

In the voxel-based analysis of FA values, the white matter FA values in the left and right anterior limbs of the internal capsule differed in the patient and healthy control groups [left anterior limb of internal capsule: (x, y, z)=(-26,12,18), cluster voxel size=831, T=5.20; right anterior limb of internal capsule: (x,y,z)=(26,16,4), cluster voxel size=487, T=5.24] (Figure 2A). Figure 2B shows scatter plots of the FA values of the anterior limb of the internal capsule. Table 2 shows the FA values and radial/axial diffusivity in the affected regions. Decreased axial diffusivity, but no change in radial diffusivity, was observed in the affected regions.

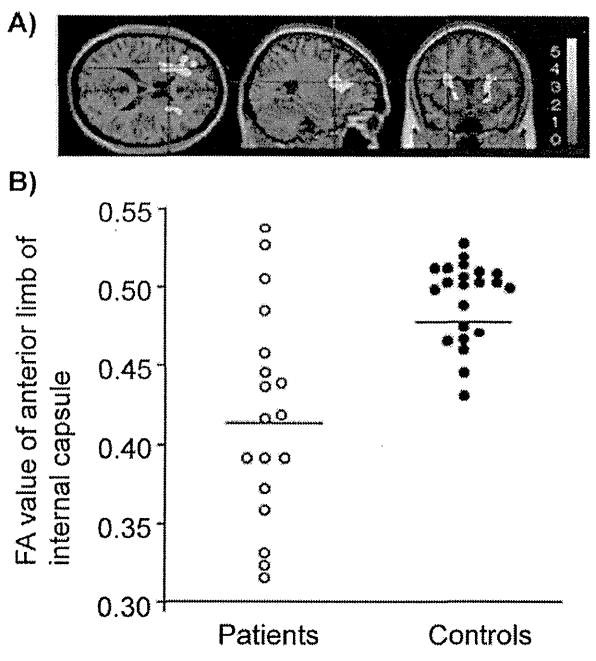

Figure 2: White matter fractional anisotropy (FA) in stroke patients and control subjects. (A) Voxel-based analysis was performed using SPM8 software. FA maps of patients (n=18) and healthy subjects (n=22) were compared using analysis of covariance (ANCOVA), with age and sex as covariates. Statistical inferences were made with a voxel-level threshold of $p<0.001$, uncorrected, and a minimum cluster size of 100 voxels. Statistical parametric mapping projections were superimposed on a representative magnetic resonance image (x=-26, y=12, z=18). FA in the right and left anterior limbs of the internal capsule was reduced in stroke patients. **(B)** Scatter plots of FA values in the regions of FA reduction in stroke patients. The regional FA value was calculated by averaging the FA values for all voxels within the volume of interest corresponding to the cluster composed of significant contiguous voxels in the preceding analysis. In the bilateral anterior limbs of the internal capsule, the FA values of stroke patients were lower than those of healthy subjects ($p<0.01$).

			Analysis of covariance †	
FA and axial/radial diffusivity	**Stroke patients (n=18)**	**Healthy controls (n=22)**	**F (1, 36)**	**p**
Left anterior limb of internal capsule				
FA	0.41 ± 0.08	0.48 ± 0.03	16.4	<0.001**
Axial diffusivity ($\times 10^{-3}$)	4.16 ± 0.32	4.37 ± 0.30	4.24	0.05 *
Radial diffusivity ($\times 10^{-3}$)	3.96 ± 0.30	4.04 ± 0.29	0.48	0.49
Right anterior limb of internal capsule				
FA	0.43 ± 0.06	0.50 ± 0.03	23.0	<0.001**

Axial diffusivity ($\times 10^{-3}$)	4.14 ± 0.33	4.35 ± 0.30	4.03	0.05
Radial diffusivity ($\times 10^{-3}$)	3.93 ± 0.31	4.01 ± 0.30	0.46	0.50
Bilateral anterior limbs of internal capsule				
FA	0.42 ± 0.07	0.49 ± 0.03	20.6	<0.001**
Axial diffusivity ($\times 10^{-3}$)	4.15 ± 0.32	4.36 ± 0.30	4.15	0.05 *
Radial diffusivity ($\times 10^{-3}$)	3.95 ± 0.30	4.02 ± 0.29	0.47	0.50
† Age and sex were entered as covariates; Data are the mean ± SD. * $p < 0.05$, ** $p < 0.01$; FA: Fractional Anisotropy; VOI: Volume of Interest				

Table 2: FA values and axial/radial diffusivity in the VOI in patients and healthy control subjects.

			Analysis of covariance †	
	Stroke patients (n=18)	**Healthy controls (n=22)**	**F (1, 36)**	**p**
Helper T lymphocyte (% $CD3^+$)	67.2 ± 15.1	61.5 ± 12.0	1.00	0.32
Cytotoxic T lymphocyte (% $CD3^+$)	27.9 ± 13.5	33.2 ± 10.6	0.92	0.35
B lymphocyte (% lymphocyte)	17.7 ± 8.3	12.2 ± 7.8	5.42	0.03
NK cell (% lymphocyte)	21.6 ± 11.3	27.5 ± 10.4	1.78	0.19
Regulatory T lymphocyte (% $CD4^+$)	2.1 ± 1.6	3.8 ± 2.3	7.89	0.008 *
† Age and sex were entered as covariates; Data are the mean ± SD. * Significant after correction for multiple statistical tests to avoid type I errors ($p < 0.01$ [0.05/5]); NK: Natural Killer				

Table 3: Percentages of circulating lymphocytes in patients and healthy control subjects.

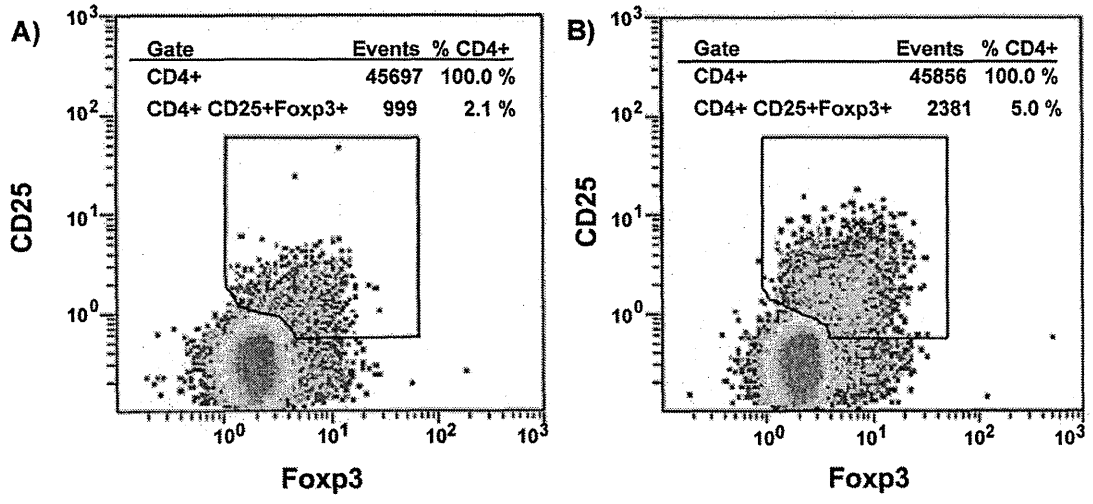

Figure 3: Flow cytometry analysis of the frequency of CD4+CD25+Foxp3+ cells within the CD4+ T-cell population. Representative plots are shown. The framed dots represent $CD4^+CD25^+Foxp3^+$ T cells (T_{regs}). The percentage of T_{regs} in patients **(A)** and controls **(B)** was 2.1% and 5.0%, respectively.

Lymphocyte subsets and their correlation with FA values in patients

The percentage of Tregs was significantly lower in patients than in healthy controls (Table 3). For patients and controls, representative flow cytometry analysis plots of the frequency of Tregs within the CD4+ T cell population are shown in Figure 3. Scatter plots depicting the percentage of Tregs in patients and controls are shown in Figure 4A. In patients, we found a significant positive relationship between the level of circulating Tregs and the FA value in the anterior limb of the internal capsule (r=0.50, p=0.04) (Figure 4B).

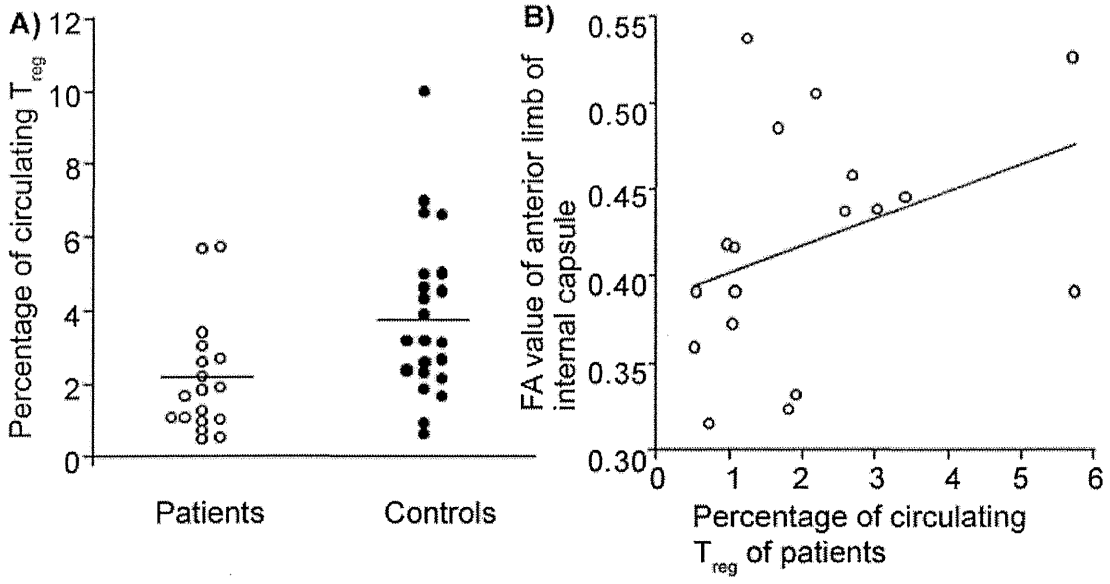

Figure 4: T_{reg} percentages in patients and controls and the relationship between the percentage of circulating Tregs and the FA value of the anterior limb of the internal capsule in patients. (A) The percentage of circulating Tregs differed significantly in the stoke patient and healthy control groups ($F_{1,36} = 7.89$, $p = 0.008$). **(B)** In stroke patients, a significant correlation was observed between the percentage of circulating T_{regs} and the FA value (r=0.50, p=0.04).

Discussion

Our findings showed that stroke patients had lower FA in the bilateral anterior limbs of the internal capsule when compared to healthy control subjects. The reduced FA in stroke patients was associated with decreased axial diffusivity. Axonal damage leads to a marked decrease in axial diffusivity, and demyelination leads to an increase in radial diffusivity [12]. Therefore, our finding of the reduced FA was a result not of demyelination but of a gross reduction in axonal number and/or size, possibly reflecting Wallerian degeneration secondary to neuronal loss due to stroke [13]. From an anatomical perspective, the anterior limb of the internal capsule represents the intercept point in the course of the frontal-subcortical circuits [14]; it has extensive connectivity with the cortical and subcortical areas. The reduced FA in the anterior limb of the internal capsule may reflect the conjunctive focus of degeneration due to stroke in spatially different sites of the cortical and subcortical areas [15].

Our results demonstrated that the percentage of circulating T_{regs} was lower in stroke patients than in healthy controls. The degree of reduction correlated with the decrease in the FA value in the internal capsule. This may indicate that a decrease in T_{regs} is associated with axonal damage in the internal capsule in stroke patients. After an ischemic stroke, activated T lymphocytes infiltrate the brain and function as a source of pro-inflammatory cytokines and cytotoxic substances [16-18]. However, not all T-cell subtypes are detrimental to acute stroke outcome, and there is evidence that T cells promote brain tissue repair and regeneration. T_{regs} are an important T-cell subtype; they support brain tissue repair and regeneration [19]. Infarct volume and neuronal dysfunction increased when mice were treated with an anti-CD25 monoclonal antibody to neutralize T_{regs} [20]. The ability of T_{regs} to protect the brain and improve stroke outcome was confirmed by Li et al. [21] who studied the effects of post-stroke T_{reg} therapy.

Although the interaction between the brain and the immune system subsequent to ischemic stroke has only recently been documented, the functional role of T_{regs} in other pathological conditions has been studied extensively. Most studies have ascribed the protective effect of T_{regs} to the alleviation of an excessive inflammatory response. It is reasonable to attribute the neuroprotective effect of T_{regs} in stroke to a similar mechanism [22].

T_{regs} limit the immune response by releasing IL-10, an anti-inflammatory cytokine [23]. In experimental brain ischemia with anti-CD25 monoclonal antibody-mediated depletion of T_{regs}, the addition of exogenous IL-10 reduced infarct volume [20]. T_{regs} also limit the immune response by releasing transforming growth factor-β, which may be required for neurogenesis [24]. In addition, T_{regs} conferred protection against stroke by blunting the increase in metalloproteinase-9 (MMP-9) [21]. Stroke-induced MMP-9 production resulting from neutrophil infiltration contributed to the breakdown of the blood-brain barrier and promoted leukocyte

infiltration and brain damage [25], whereas T_{reg} adoptive treatment inhibited MMP-9 production in the blood and the brain after ischemia [21].

The reason circulating T_{reg} levels were lower in stroke patients are not clear. People with lower levels of circulating T_{regs} may be more likely to develop stroke and experience severe axonal damage because the activity of their inflammatory system is excessive. Alternatively, the reduction in circulating T_{regs} after stroke may reflect the migration of T_{regs} to brain tissue for the repair of cerebral neuronal injuries. Our findings are based on cross-sectional data without immunohistochemical analysis, which limits our ability to determine which explanation is correct. However, our results are consistent with previous reports describing the brain-protective and outcome-improving effects of T_{regs}. Our findings raise the possibility that stroke outcome can be improved by targeting T_{regs} to protect the brain from tissue damage after stroke.

Whereas Liesz et al. [20] showed a protective effect of T_{regs} in stroke, Ren et al. [26] found that T_{regs} had no effect, and Kleinschnitz et al. [27] showed that T_{regs} exacerbated brain injury early after transient ischemia. However, the animal models used in the studies differed in several aspects, including the duration of ischemia and the methods for Treg depletion. In our study, the stroke patients predominantly suffered a modest ischemic insult, and their circulating lymphocytes were studied after 10–28 days. Differences in the severity and stage of ischemic insult may account for the conflicting results.

Our study has a few limitations. First, patients with significant comprehension deficits were excluded because clinical verbal interviews could not be conducted. Second, because our study was an *in vivo* human brain study, immunohistochemical analysis of brain tissue was not possible. Third, all of the patients took anti-coagulant/platelet medicine. Of note, 13 patients took acetylsalicylic acid, which has an anti-inflammatory effect and may have affected our results. However, the extent to which the results were affected by medication remains uncertain. Finally, we did not investigate the other functionally unique $CD4^+$ T-cell subsets (Th1, Th2, and Th17) in this study. A previous study reported that T_{regs}/Th17 cells were imbalanced in patients with cerebral infarction: the Th17 frequency increased, and the T_{reg} frequency decreased, relative to the balance in control subjects [28]. Th17 cells orchestrate tissue inflammation, whereas T_{regs} limit the immune response and prevent tissue damage. An imbalance between T_{regs} and Th17 cells in stroke patients may contribute to tissue damage [28]. Further analysis, taking these points into consideration, is needed to confirm our present findings.

In conclusion, the present study suggests that FA is reduced in the bilateral anterior limbs of the internal capsule in stroke patients. In addition, the percentage of circulating T_{regs} was reduced in stroke patients. The degree of reduction correlated with the decrease in the FA value in the internal capsule. T_{regs} might alleviate post-stroke white matter tissue damage by limiting the immune response. Further study of the role of T_{regs} in preventing post-stroke cerebral damage is needed.

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ORIGINAL ARTICLE

Microstructural abnormality in white matter, regulatory T lymphocytes, and depressive symptoms after stroke

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Key words: *diffusion tensor imaging, fractional anisotropy (FA), magnetic resonance imaging (MRI), post-stroke depression, regulatory T lymphocytes (T_{reg}), stroke.*

Abstract

Background: The purpose of the present study was to investigate the existence of microstructure abnormalities in the white matter circuit in stroke patients and its relationship to depressive episodes. To target the prevention of depression, we also investigated the relationship between lymphocyte subsets and cerebral abnormalities in patients.

Methods: Participants included 18 patients with acute ischemic stroke and 22 healthy control subjects. Diffusion tensor imaging was performed. Whole-brain voxel-based analysis was used to compare fractional anisotropy (FA) between groups. Blood samples were obtained, and the lymphocyte subsets were evaluated using flow cytometry. Follow-up examinations were conducted on 12 patients at 6 months.

Results: FA was decreased in the bilateral anterior limb of the internal capsule in stroke patients. At the 6-month follow-up examination, there was a significant increase in FA, which was associated with a lower depression scale score. Patients showed a decreased percentage of circulated regulatory T lymphocytes, and the degree of reduction was related to the decrease in the FA value in the internal capsule.

Conclusions: FA reductions in the anterior limb of the internal capsule cause abnormality in the frontal-subcortical circuits and confer a biological vulnerability, which in combination with environmental stressors results in the onset of depression. Our findings also demonstrated the possibility of preventing post-stroke depression by targeting the role of regulatory T lymphocytes in brain tissue repair and regeneration after stroke.

INTRODUCTION

Stroke is the third leading cause of death and the most frequent cause of permanent disability in adults worldwide.[1] Depression is common and serious emotional symptom following stroke and is associated with excess disability, cognitive impairment, and mortality.[2] Despite considerable advances in understanding the pathophysiology of cerebral ischemia, therapeutic options for stroke and its related emotional symptoms are still limited. Inflammatory mechanisms activated after brain ischemia represent a key target of current translational cerebrovascular research. Stroke induces profound local inflammatory response involving various types of immune cells that transmigrate across the activated blood–brain barrier to invade the brain.[3]

In attempts to target the prevention of cerebral damage due to stroke, several factors related to inflammation have received considerable attention.[4–6] T lymphocytes are especially central to the development of a sustained inflammatory response in brain injury after a stroke. T cells are sources of pro-inflammatory cytokines and cytotoxic substances, such as reactive oxygen species, in the brain after a stroke; these likely contribute to neuronal death and poor outcomes. However, recent evidence has

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indicated a novel role of T cells in promoting brain tissue repair and regeneration in the weeks and months after a stroke.[7] The role of T lymphocytes in ischemic stroke is complex and remains poorly understood. More research is needed to gain a greater understanding of which T-cell subpopulations produce and prevent damage after a stroke.

The primary aim of the present study was to elucidate the microstructural abnormalities in the white matter circuit in stroke patients, as well as their relationship with depressive symptoms after a stroke. When abnormalities were found and a statistical association with post-stroke depressive symptoms was demonstrated, we investigated their relationship with circulating T lymphocytes. For identification of the microstructural abnormalities in stroke patients, diffusion tensor imaging was performed, and whole-brain voxel-based analysis was used to compare fractional anisotropy (FA) between acute ischemic stroke patients and healthy control subjects. Furthermore, we examined which circulating T-cell subpopulations showed differences in stroke patients when compared to healthy subjects and how such T-cell subpopulations are associated with microstructural abnormalities in the white matter of patients.

METHODS

Subjects

After the study was described to subjects, written informed consent was obtained. The study was approved by the medical ethics committee of the National Cerebral and Cardiovascular Center of Japan. The patients, all of whom were of Japanese ethnicity, were recruited from the neurology unit of the National Cerebral and Cardiovascular Center Hospital. These patients had initially been hospitalized for treatment of acute ischemic stroke.

Stroke was diagnosed by neurologists according to the World Health Organization criteria (1989). After the assessment, a group of psychiatrists and neurologists reviewed the data and reached a consensus regarding the presence or absence of psychiatric disease, including dementia according to the Diagnostic and Statistical Manual, 4th edition criteria. Patients were included if they met the following criteria: (i) a focal lesion of either the right or left hemisphere on magnetic resonance imaging (MRI); (ii) absence of other neurologic, neurotoxic, or metabolic conditions; (iii) modest ischemic insult (modified Rankin scale ≤4) with absence of a significant verbal comprehension deficit; and (iv) occurrence of stroke 10–28 days before the examinations. Exclusion criteria included the following: (i) transient ischemic attack, cerebral haemorrhage, subdural haematoma, or subarachnoid haemorrhage; (ii) history of a central nervous system disease such as tumour, trauma, hydrocephalus or Parkinson's disease; and (iii) pre-stroke history of depression. Eighteen subjects met the criteria and participated in this study.

Twenty-two healthy control subjects were recruited locally for this study based on their response to a poster seeking subjects. Exclusion criteria for healthy subjects were a history or present diagnosis of any Diagnostic and Statistical Manual, 4th edition axis I or neurological illness. Major characteristics of this cohort are summarized in Table 1.

All patients were subjected to a neurological examination using the modified Rankin scale (mRS) and the National Institutes of Health Stroke Scale (NIHSS) on the day of the MRI scan.[8,9] A quantitative measurement of cognitive function, the Mini-Mental State Examination (MMSE), and of depressive symptoms, the Hamilton Rating Scale for Depression (HAM-D), was carried out in patients and control subjects. MRI were conducted for all study subjects.[10,11]

At 6 months, follow-up MRI were conducted for 12 of the 18 patients. There were no changes in medication use between baseline and follow-up, and no patients were on antidepressant treatment during the examinations. All patients were subjected to a series of quantitative measurements of depressive symptoms (HAM-D), cognitive function (MMSE), and neurological examination (mRS, NIHSS) on the day of the follow-up MRI scan.[8–11]

Data acquisition of MRI

All MRI examinations were performed with a 3-Tesla whole-body scanner (Signa Excite HD V12M4; GE Healthcare, Milwaukee, WI, USA) with an eight-channel phased-array brain coil. Diffusion tensor images were acquired with a locally modified single-shot echo-planar imaging sequence that used parallel acquisition at a reduction factor of 2 in the axial plane. Imaging parameters were as follows: repetition time = 17 s; echo time = 72 ms; b value = 0, 1000 mm^2/s, acquisition matrix, 128 × 128; field of view, 256 mm; section thickness, 2.0 mm; no intersection gap; 74 sections. The reconstruction matrix was the same as

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Table 1 Demographic characteristics of patients and healthy control subjects

Characteristic	Stroke patients (n = 18)	Healthy control subjects (n = 22)	t or χ^2	P-value
Age (years)	70.0 ± 6.7	67.2 ± 5.5	t = 1.46	0.15
Female sex (n)	4 (22.2%)	8 (36.3%)	χ^2 = 0.94	0.33
MMSE score	28.4 ± 1.9	29.3 ± 1.0	t = 1.98	0.06
HAM-D score	2.4 ± 2.4	1.0 ± 1.5	t = 2.20	0.03*
History of disease (n)				
Diabetes mellitus	5 (27.8%)	2 (9.1%)	χ^2_1 = 2.40	0.12
Hyperlipidaemia	5 (27.8%)	1 (4.5%)	χ^2_1 = 4.19	0.04*
Hypertension	14 (77.8%)	5 (22.7%)	χ^2_1 = 12.0	<0.01**
mRS score	1.9 ± 0.7	—		
NIHSS score	2.8 ± 0.9	—		
Anticoagulant or anti-platelet medication (n)				
Warfarin	3 (16.7%)			
Acetylsalicylic acid	13 (72.2%)			
Clopidogrel sulfate	2 (11.1%)			
Cilostazol	3 (16.7%)			
Acute infarcts	1.2 ± 0.5	—		
Volume of acute infarcts (mL)	1.6 ± 0.9	—		
Location of acute infarcts (n)				
Basal ganglia	11 (61.1%)	—	0.611	
Subcortical white matter	6 (33.3%)			
Thalamus	1 (5.6%)	—	0.056	
Laterality of hemisphere infarcts				
Left hemisphere (n)	9 (50.0%)	—		

Data are mean ± SD. *P < 0.05, **P < 0.01.
HAM-D, Hamilton Rating Scale for Depression; MMSE, Mini-Mental State Examination; mRS, modified Rankin scale; NIHSS, National Institutes of Health Stroke Scale.

the acquisition matrix, and 2 × 2 × 2 mm isotropic voxel data were obtained. Motion-probing gradient was applied in 55 directions, the number of images was 4144, and the acquisition time was 15 min 52 s.

To reduce blurring and signal loss arising from field inhomogeneity, we used an automated high-order shimming method based on spiral acquisitions before acquiring diffusion tensor imaging scans.[12] To correct for motion and distortion from eddy current and B_0 inhomogeneity, FMRIB software (FMRIB Center, Department of Clinical Neurology, University of Oxford, Oxford, UK; http://www.fmrib.ox.ac.uk/fsl/fslwiki/) was used. B_0 field mapping data were also acquired with the echo time shift (2.237 ms) method based on two gradient echo sequences.

High-resolution 3-D, T_1-weighted images were acquired with a spoiled gradient-recalled sequence (repetition time = 12.8 ms, echo time = 2.6 ms, flip angle = 8, field of view, 256 mm; 188 sections in the sagittal plane; acquisition matrix, 256 × 256; acquired resolution, 1 × 1 × 1 mm). T_2-weighted images were obtained with a fast-spin echo (repetition time = 4800 ms; echo time = 101 ms; echo train length = 8; field of view = 256 mm; 74 slices in the transverse plane; acquisition matrix, 160 × 160, acquired resolution, 1 × 1 × 2 mm).

Image processing

Fractional anisotropy (FA) maps and three eigenvalues (λ1, λ2, and λ3) were generated from each individual with FMRIB software. First, brain tissue was extracted using the Brain Extraction Tool. Brain maps for each of the 55 directions were eddy corrected, subsequent to which FA values were calculated at each voxel with the FSL FMRIB Diffusion Toolbox.

Image pre-processing and statistical analysis were carried out using SPM8 software (Wellcome Department of Imaging Neuroscience, London, UK). Each subject's echo planar image was spatially normalized to the Montreal Neurological Institute echo planar image template using parameters determined from the normalization of the image with a b value of 0 mm²/s and the echo planar image template in SPM8. Images were resampled with a final voxel size of 2 × 2 × 2 mm³. Normalized maps were spatially smoothed using an isotropic Gaussian filter (8-mm full-width at half-maximum).

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Voxel-based analysis

Voxel-based analysis was performed using SPM8 software. FA maps were compared between patients and healthy subjects with ANCOVA, with age and sex as covariates of no interest. Statistical inferences were made with a voxel-level threshold of $P < 0.001$, uncorrected, with a minimum cluster size of 100 voxels. The regional FA value was calculated by averaging the FA values for all voxels within the volume of interest corresponding to the cluster composed of significant contiguous voxels. The same volumes of interest were applied to λ_1–λ_3 images, and λ_1–λ_3 values were extracted. Axial (λ_1) and radial diffusivity ($(\lambda_2 + \lambda_3)/2$) were compared.

Flow cytometric analysis of lymphocyte subsets in peripheral blood

Blood samples (5 mL) were obtained from all of the patients and healthy control subjects at the initial examination. The samples were collected into tubes containing sodium heparin. Peripheral blood mononuclear cells (PBMC) were isolated using a Ficoll density gradient (Ficoll-Paque PLUS; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's protocol. PBMC were washed twice with phosphate-buffered saline containing 1% foetal calf serum and 2-mM EDTA.

To identify helper T cells (CD3+ & CD4+), cytotoxic T cells (CD3+ & CD8+), B cells (CD19+) and natural killer cells (CD16+ or CD56+), we incubated the PBMC with fluorescein isothiocyanate-conjugated anti-human CD3 (Beckman Coulter, Orange Country, CA, USA), phycoerythrin-cyanin (PC)5-conjugated anti-human CD4 (Beckman Coulter), PC7-conjugated anti-human CD8 (Beckman Coulter), phycoerythrin-conjugated anti-human CD19 (Beckman Coulter), PC5-conjugated anti-human CD16 (Beckman Coulter), and/or phycoerythrin-conjugated anti-human CD56 (Beckman Coulter) at 4°C for 20 min. To identify regulatory T lymphocytes (T_{reg}) (CD4+, CD25+ & FOXP3+), we incubated the PBMC with fluorescein isothiocyanate-conjugated anti-human CD4 (Beckman Coulter) and PC5-conjugated anti-human CD25 (Beckman Coulter) at 4°C for 20 min. After surface staining, PBMC were fixed, followed by permeabilization and staining with phycoerythrin-conjugated anti-human FOXP3 (Becton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. As negative controls, fluorochrome-conjugated non-specific isotype-matched antibodies (Beckman Coulter) were used. Stained cells were analyzed using a FC500 cytometer and CXP software (Beckman Coulter). Percentages of cells stained with a particular antibody are reported after subtraction of the percentage of cells stained with the relevant negative isotype control antibodies.

Statistical analysis

Group differences in demographic characteristics between patients and healthy controls were examined by unpaired *t*-test and Pearson's χ^2 test. To examine the group differences in FA values and axial/radial diffusivity in volume of interest shown in the voxel-based analysis, we performed ANCOVA with age and sex as covariates.

Paired *t*-tests were performed 6 months after the initial examinations to determine changes in patients' mRS, NIHSS, MMSE, and HAM-D scores and FA values. We performed Pearson's correlation analysis to examine the relationship between FA values and depressive symptoms at the first assessment and at the assessment performed 6 months later. To examine the relationship between the change in depression scale scores and the ratio of the FA values (FA values at second vs initial examination) in patients, we performed Pearson's correlation analysis.

To examine whether the ratio of the FA values was related to the change in depression scale scores (HAM-D scores at second examination minus initial examination), we performed multiple regression analysis after adjustment for age and gender. The change in depression scale scores was the dependent variable, and the ratio of the FA values was the independent variable.

Additionally, we performed ANCOVA with age and sex as covariates to examine the differences in the numbers of helper T cells, cytotoxic T cells, regulatory T cells, B cells, and natural killer cells between patients and healthy control subjects. For the cells showing significant differences between groups, we examined the correlation between FA values and cell number by Spearman's correlation analysis. To determine whether FA values were related to cell number, we performed multiple regression analysis with the FA values as dependent variable and cell number as independent variables, after adjustment for age and gender.

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All statistical tests were two-tailed and reported at $P < 0.05$. Bonferroni correction was applied to avoid type I errors due to the multiplicity of statistical analyses. Statistical analysis of the data was performed using SPSS for Windows 19.0 (IBM Japan Inc., Tokyo, Japan).

RESULTS

Demographic and clinical data

Table 1 summarizes the demographic and clinical characteristics of the study subjects. Patients did not differ significantly from healthy control subjects with regard to age, sex, and MMSE scores. As to the history of disease, the ratio of the history of hyperlipidaemia and hypertension was significantly higher in the patients than in the healthy controls. mRS and NIHSS score, anticoagulant or anti-platelet medication, and the location and volume of the infarction among the patients are also shown in Table 1. Patients showed some disability from the stroke at the time of the examination. All of the patients took anticoagulant and/or anti-platelet medicine. Infarctions were located in the basal ganglia (61.1%), subcortical white matter (33.3%), and thalamus (5.6%). There was no significant laterality of hemisphere infarcts.

Comparisons of FA values between groups

In the voxel-based analysis of FA values, the patient and healthy control groups differed in white matter FA values in the left and right anterior limbs of the internal capsule (left anterior limb of internal capsule: $(x, y, z) = (-26, 12, 18)$, cluster voxel size = 831, T value = 5.20; right anterior limb of internal capsule: $(x, y, z) = (26, 16, 4)$, cluster voxel size = 487, T value = 5.24) (Fig. 1a). Figure 1b shows the scatter diagrams of the FA values of the anterior limb of the internal capsule. Table 2 shows the quantification of the differences in FA values and radial/axial diffusivity in these affected regions. These regions revealed decreased axial diffusivity but no change in radial diffusivity.

No patients had lesions in the location of the anterior limbs of the internal capsule. Using Pearson's correlation analysis, we found no significant relationship ($P > 0.05$) between the FA value in the anterior limb of the internal capsule and the volume of infarcts or the severity of stroke shown with mRS and NIHSS scores.

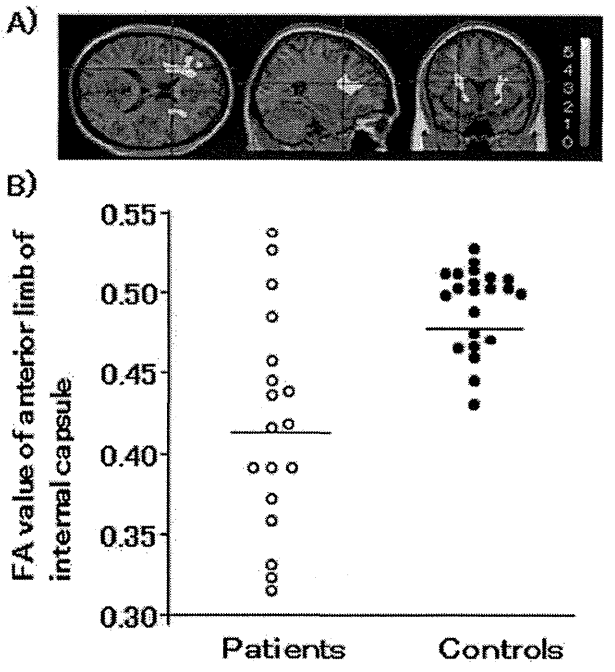

Figure 1 (a) White matter fractional anisotropy (FA) differences in voxel-based analysis comparisons between stroke patients ($n = 18$) and control subjects ($n = 22$). Images are presented in radiological orientation. Statistical parametric mapping projections were superimposed on a representative magnetic resonance image ($x = -26$, $y = 12$, $z = 18$). Patients showed reduced FA in the right and left anterior limbs of the internal capsule. Statistical inferences were made with a voxel-level statistical threshold ($P < 0.001$), uncorrected, with a minimum cluster size of 100 voxels. (b) Scatter plots of FA values in the region of FA reduction of patients and control subjects. Patients' FA values were lower than those of healthy subjects in the bilateral anterior limb of the internal capsule ($P < 0.01$).

Change in FA values of patients after 6 months

Patients showed significantly increased FA values in the anterior limb of the internal capsule 6 months after the infarction (Table 3, Fig. 2a).

There were no significant changes in MMSE and HAM-D scores for either group 6 months after the initial examination (Table 3). There was no significant relationship between FA values and depressive symptoms at the first assessment and at the assessment performed 6 months later. However, we found a significant negative correlation between the increased ratio of the FA values and the change in the scores of depression scales of HAM-D at follow-up 6 months later ($r = -0.67$, $P = 0.02$) (Fig. 2b).

When multiple regression analysis was used to evaluate whether the increased ratio of FA values was

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Table 2 Differences in values of FA and axial/radial diffusivity in VOI between patients and healthy control subjects

FA and axial/radial diffusivity	Stroke patients (n = 18)	Healthy control subjects (n = 22)	ANCOVA†	
			$F_{1,36}$	P-value
Left anterior limb of internal capsule				
FA	0.41 ± 0.08	0.48 ± 0.03	16.4	<0.001**
Axial diffusivity (×10^{-3})	4.16 ± 0.32	4.37 ± 0.30	4.24	0.05*
Radial diffusivity (×10^{-3})	3.96 ± 0.30	4.04 ± 0.29	0.48	0.49
Right anterior limb of internal capsule				
FA	0.43 ± 0.06	0.50 ± 0.03	23	<0.001**
Axial diffusivity (×10^{-3})	4.14 ± 0.33	4.35 ± 0.30	4.03	0.05
Radial diffusivity (×10^{-3})	3.93 ± 0.31	4.01 ± 0.30	0.46	0.50
Bilateral anterior limb of internal capsule				
FA	0.42 ± 0.07	0.49 ± 0.03	20.6	<0.001**
Axial diffusivity (×10^{-3})	4.15 ± 0.32	4.36 ± 0.30	4.15	0.05*
Radial diffusivity (×10^{-3})	3.95 ± 0.30	4.02 ± 0.29	0.47	0.50

†Age and gender are entered as covariates. Data are mean ± SD. *P < 0.05, **P < 0.01.
FA, fractional anisotropy; VOI, volume of interest.

Table 3 Change in psychometry scores, FA values, and axial/radial diffusivity over 6 months in patients (n = 12)

	10–28 days after stroke	6 months after first exam	Paired t-test	P-value
Patients				
mRS score	1.9 ± 0.5	1.6 ± 0.5	t_{11} = 2.35	0.04*
NIHSS score	2.8 ± 1.0	1.8 ± 0.7	t_{11} = 4.00	0.002**
MMSE score	29.0 ± 1.5	29.7 ± 0.5	t_{11} = 1.54	0.15
HAM-D score	3.7 ± 2.9	2.3 ± 3.0	t_{11} = 1.13	0.28
Anterior limb of internal capsule				
FA	0.40 ± 0.06	0.43 ± 0.06	t_{11} = 2.26	0.04*
Axial diffusivity (×10^{-3})	4.22 ± 0.30	4.13 ± 0.2	t_{11} = 0.74	0.48
Radial diffusivity (×10^{-3})	4.01 ± 0.26	3.87 ± 0.25	t_{11} = 1.50	0.16

Data are mean ± sd. * P < 0.05, ** P < 0.01.
FA, fractional anisotropy; HAM-D, Hamilton Rating Scale for Depression; MMSE, Mini-Mental State Examination; mRS, modified Rankin scale; NIHSS, National Institutes of Health Stroke Scale.

related to the change in depression scale scores (HAM-D) after 6 months, the ratio of FA values was found to be negatively related to the change in the HAM-D scores (β = –0.46, P = 0.04).

Lymphocyte subsets and their relation to FA values in patients

Patients showed significantly decreased numbers of T_{reg} compared with healthy controls (Table 4, Fig. 3a). We also found a significant positive relationship between the level of circulating T_{reg} and the FA value in the anterior limb of the internal capsule in the patients (r = 0.50, P = 0.04) (Fig. 3b). There was no significant relationship between the level of circulating T_{reg} and the HAM-D scores.

When multiple regression analysis was used to evaluate whether the level of circulating T_{reg} was related to the FA value in the anterior limb of the

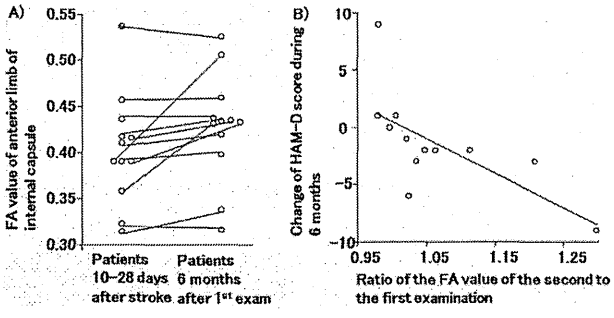

Figure 2 (a) Scatter plots of fractional anisotropy (FA) values in the region of FA reduction among stroke patients (n = 12) at 10–28 days after the stroke and at the 6-month follow-up. A significant FA increase was observed in the patients at the 6-month follow-up (P < 0.05). (b) Scatter plots showing the relationship between the ratio of the FA values of the second to the first examination and the change in depression scale scores among patients (n = 12). Significant correlations were observed between the ratio of the FA values of the second to the first examination and the changes in depression scale scores (r = –0.67, P = 0.02). HAM-D, Hamilton Rating Scale for Depression.

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Table 4 Differences in percentage of lymphocytes in the circulation between patients and healthy control subjects

	Stroke patients (n = 18)	Healthy control subjects (n = 22)	ANCOVA†	
			$F_{1,36}$	P-value
Helper T lymphocyte	67.2 ± 15.1	61.5 ± 12.0	1.00	0.32
Cytotoxic T lymphocyte	27.9 ± 13.5	33.2 ± 10.6	0.92	0.35
B lymphocyte	17.7 ± 8.3	12.2 ± 7.8	5.42	0.03
NK cell	21.6 ± 11.3	27.5 ± 10.4	1.78	0.19
Regulatory T lymphocyte	2.1 ± 1.6	3.8 ± 2.3	7.89	0.008*

†Age and sex are entered as covariates. Data are mean ± SD. *Significant after correction for multiple statistical tests to avoid type I errors (P < 0.01 (0.05/5)). NK, natural killer.

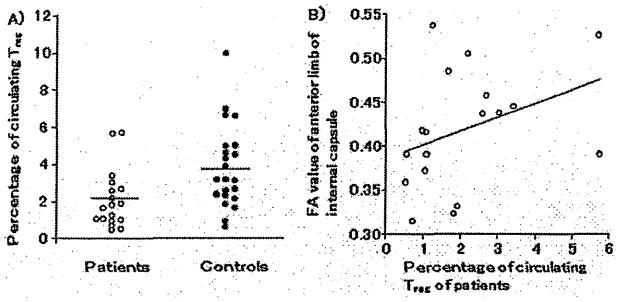

Figure 3 (a) Scatter diagrams showing the differences in circulating T_{reg} between patients and controls. A significant difference in the percentage of T_{reg} was observed between groups ($F_{1,36}$ = 7.89, P = 0.008). (b) The relationship between the percentage of circulating T_{reg} and FA values of the anterior limb of the internal capsule in patients. A significant correlation was observed between the percentage of T_{reg} and FA values (r = 0.50, P = 0.04). FA, fractional anisotropy; T_{reg}, regulatory T lymphocytes.

internal capsule, the T_{reg} level was found to be positively related to the FA values (β = 0.59, P = 0.02).

DISCUSSION

Our findings showed that stroke patients had lower FA in the bilateral anterior limb of the internal capsule relative to healthy control subjects. Six months after initial assessment, a significant increase in FA was noted, and it revealed an association with a reduction in depression scale scores. Our findings are not the result of direct neuronal damage caused by the infarction located on the internal capsule, as no patients had a lesion in this location. Also, there was no direct relationship between the FA value in this region and the volume of infarcts or the severity of stroke.

Reduced FA level was associated with decreased axial diffusivity. Axonal damage leads to a marked decrease in axial diffusivity, while demyelination leads to an increase in radial diffusivity.[13] Therefore, our finding was not a result of demyelination but of gross reduction in axonal number and/or size, possibly reflecting Wallerian degeneration secondary to neuronal loss due to stroke.[14] From an anatomical perspective, the anterior limb of the internal capsule represents the intercept point in the course of the frontal-subcortical circuits,[15] and it has extensive connectivity with the cortical and subcortical areas. Its reduced FA may reflect the conjunctive focus of degeneration due to stroke in the spatially different sites of cortical and subcortical areas.

The frontal-striatal-thalamic-cortical circuits, connected by the anterior limb of the internal capsule, play an important role in behavioural regulation,[16] and based on MRI, microstructural change of the anterior limb of the internal capsule is related to the severity of depressive symptoms in adults with major depressive disorder.[17] Degeneration in this region may relate to a loss of white matter integrity of these neural circuits,[18] and this abnormality might trigger the onset of negative mood change. Our findings on the association between the change in FA values of the internal capsule and depression scale scores might reflect an association between axonal damage of the internal capsule and depressive mood in stroke patients.

Our findings demonstrate that patients had reduced amounts of circulating T_{reg}, with the degree of reduction being related to the decrease in FA value in the internal capsule. This may indicate that a decrease in T_{reg} is related to the axonal damage of the internal capsule in stroke patients. Our findings showed no direct relationship between T_{reg} level and depression scale scores, but T_{reg} may indirectly affect post-stroke depressive symptoms via its effect on the cerebral damage.

An ischemic stroke caused T lymphocytes to become activated, infiltrate the brain, and then function as sources of pro-inflammatory cytokines and cytotoxic substances.[19–21] However, not all T-cell

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subtypes are detrimental to acute stroke outcome, and recent evidence indicates a novel role of T cells in promoting brain tissue repair and regeneration. T_{reg} cell is an important T-cell subtype, and it supports brain tissue repair and regeneration.[22] T_{reg} cells act to limit the immune response by releasing transforming growth factor-β and interleukin-10,[23] and they have also been reported to be required for neurogenesis.[24] Infarct volume and neuronal dysfunction were significantly increased in mice treated with an anti-CD25 monoclonal antibody to neutralize T_{reg} compared with controls.[23] Furthermore, this protection was observed only 7 days after a modest ischemic insult.[23] These findings of the brain-protecting and outcome-improving effects of T_{reg} were also confirmed by Li *et al.* using post-stroke T_{reg} cell therapy.[25]

One possible explanation for our results of T_{reg} is that people with lower circulated T_{reg} are more likely to develop stroke and tend to have severe axonal damage after stroke. Another possibility is that the reduction in circulated T_{reg} after stroke might be induced by the consumption of T_{reg} to repair cerebral neuronal injuries, including axonal damage. Our findings of lower circulated T_{reg} are based on cross-sectional data, which provide limited ability to infer which explanation is right. In any case, our results are consistent with previous reports of the brain-protecting and outcome-improving effects of T_{reg}. In principle, our findings showed the possibility of improving stroke outcome by targeting the role of T_{reg} in protecting brain tissue damage after a stroke.

Ren *et al.* and Kleinschnitz *et al.* respectively demonstrated no role or an opposite role for T_{reg} in exacerbating brain injury early after transient ischemia.[26,27] The animal model they used was different from that of the Liesz study in several aspects, including the duration of ischemia and methods for T_{reg} depletion. Furthermore, the late stage effect of T_{reg} depletion was not addressed in their studies. In our study, stroke patients were predominantly of modest ischemic insult, and their circulating lymphocytes were studied after 10–28 days. There is a possibility that the differences in the severity and stage of the ischemic insult caused different results regarding the role of T_{reg} in their studies and ours.

Our study has some limitations. First, patients with significant comprehension deficits were excluded because clinical verbal interviews could not be conducted. Second, all of the patients took anticoagulant or anti-platelet medicine. Specifically, 13 patients took acetylsalicylic acid, which has an anti-inflammatory effect, and this may have affected our results. However, the extent to which our findings relate to medication remain uncertain. Further analysis, inclusive of considerations of these points, is needed to confirm our present findings.

In conclusion, the present study suggests that FA reduction in the bilateral anterior limb of the internal capsule is evident in stroke patients. This regional damage relates to abnormality of neuroanatomical pathways in frontal-subcortical circuits and renders a biological vulnerability, which then gives rise to the onset of depressive symptoms. Our findings also demonstrate that patients have reduced amounts of circulating T_{reg}, with the degree of reduction being related to the decrease in FA value in the internal capsule. T_{reg} cells might have a role in improving post-stroke white matter tissue damage by limiting the immune response and promoting neurogenesis.

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EFFECTS OF INTRAVENOUS ADMINISTRATION OF UMBILICAL CORD BLOOD CD34$^+$ CELLS IN A MOUSE MODEL OF NEONATAL STROKE

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Abstract—Neonatal stroke occurs in approximately 1/4000 live births and results in life-long neurological impairments: e.g., cerebral palsy. Currently, there is no evidence-based specific treatment for neonates with stroke. Several studies have reported the benefits of umbilical cord blood (UCB) cell treatment in rodent models of neonatal brain injury. However, all of the studies examined the effects of administering either the UCB mononuclear cell fraction or UCB-derived mesenchymal stem cells in neonatal rat models. The objective of this study was to examine the effects of human UCB CD34$^+$ cells (hematopoietic stem cell/endothelial progenitor cells) in a mouse model of neonatal stroke, which we recently developed. On postnatal day 12, immunocompromized (SCID) mice underwent permanent occlusion of the left middle cerebral artery (MCAO). Forty-eight hours after MCAO, human UCB CD34$^+$ cells (1 × 10^5 cells) were injected intravenously into the mice. The area in which cerebral blood flow (CBF) was maintained was temporarily larger in the cell-treated group than in the phosphate-buffered saline (PBS)-treated group at 24 h after treatment. With cell treatment, the percent loss of ipsilateral hemispheric volume was significantly ameliorated (21.5 ± 1.9%) compared with the PBS group (25.6 ± 5.1%) when assessed at 7 weeks after MCAO. The cell-treated group did not exhibit significant differences from the PBS group in either rotarod (238 ± 46 s in the sham-surgery group, 175 ± 49 s in the PBS group, 203 ± 54 s in the cell-treated group) or open-field tests. The intravenous administration of human UCB CD34$^+$ cells modestly reduced histological ischemic brain damage after neonatal stroke in mice, with a transient augmentation of CBF in the peri-infarct area. **© 2014 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: neonatal stroke, neonatal encephalopathy, brain injury, umbilical cord blood, CD34$^+$ cell, cell-based therapy.

INTRODUCTION

Perinatal/neonatal stroke occurs in 1/2800 to 1/5000 live births and results in life-long neurological impairments: e.g., cerebral palsy, mental retardation, and epilepsy (Nelson and Lynch, 2004; Chabrier et al., 2011). The current treatment for infants with stroke is predominantly supportive, as there is no evidence-based specific treatment available (Roach et al., 2008; Chabrier et al., 2011). The onset of neonatal stroke is antenatal in some cases and is unknown in others. Hence, treatments that have a narrow therapeutic window, such as tissue plasminogen activator, are not feasible for perinatal/neonatal stroke. Cell-based therapy has attracted much attention as a novel treatment for a number of neurological diseases, including neonatal encephalopathy (NE) (Bennet et al., 2012), which encompasses stroke and hypoxic–ischemic encephalopathy (HIE) (Dammann et al., 2011). Apart from its possible regenerative properties, its wide therapeutic time window, up to days after the insult (Yasuhara et al., 2010; Donega et al., 2013), is one of the most attractive features of this therapy. This is astonishing considering the fact that almost all candidate drugs examined in animal models exhibit neuroprotection only when administered before or within a few hours after the insult.

Human umbilical cord blood (hUCB) contains many stem cell types, i.e., hematopoietic stem cells, endothelial progenitor cells, and mesenchymal stem cells (MSCs) (Ingram et al., 2004; Lee et al., 2004). CD34 is widely used as a marker of hematopoietic stem

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Abbreviations: ANOVA, analysis of variance; CBF, cerebral blood flow; GDNF, glial cell line-derived neurotrophic factor; HIE, hypoxic–ischemic encephalopathy; hUCB, human umbilical cord blood; HuNu, anti-human nuclei antibody; MCAO, middle cerebral artery occlusion; MNC, mononuclear cell; MSC, mesenchymal stem cell; NE, neonatal encephalopathy; PBS, phosphate-buffered saline; ROI, region of interest; SCID, severe combined immunodeficiency; UCBC, umbilical cord blood cell; VEGF, vascular endothelial growth factor.

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