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 (Treg) (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 Treg was lower in patients than in healthy controls. In patients, we found a significant positive relationship between the level of circulating Treg and the FA value in the anterior limb of the internal capsule.

Conclusions: Patients exhibited a decreased frequency of circulating Treg and the degree of reduction correlated with the decrease in the FA value in the internal capsule. Treg 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 Treg in the prevention of post-stroke cerebral damage.

Keywords: Stroke; Magnetic resonance imaging (MRI); Diffusion tensor imaging (DTI); Fractional anisotropy (FA); Regulatory T lymphocyte (Treg)

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. Stroke 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 ≤ 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.

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

| Characteristic | Healthy control subjects (n = 22) | Stroke patients (n = 18) | t or χ² | p |
|----------------|----------------------------------|------------------------|--------|---|
| Age (years)    | 67.2 ± 5.5                       | 70.0 ± 6.7             | 1.46   | 0.15 |
| Female, n (%)  | 4 (18.2)                         | 8 (36.3)               | 0.94   | 0.33 |
| MMSE score     | 29.3 ± 1.0                       | 28.4 ± 1.9             | 1.98   | 0.06 |

Table 2: Fazekas PVH score, n (%).

| Fazekas PVH score, n (%) | 0–2 | 3 | χ²=10.4 | <0.01** |
|--------------------------|-----|---|---------|---------|
| 0–2                      | 13 (72.2) | 22 (100) | - |
| 3                        | 5 (27.8) | 0 (0.0) | 6.98 | <0.01** |

Table 3: Fazekas PVH score, n (%).

| Fazekas PVH score, n (%) | 0–2 | 3 | χ²=10.4 | <0.01** |
|--------------------------|-----|---|---------|---------|
| 0–2                      | 13 (72.2) | 22 (100) | - |
| 3                        | 5 (27.8) | 0 (0.0) | 6.98 | <0.01** |

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; Echo Time (TE)=72 ms; b=0 and
1000 seconds/mm²; 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-correlated. 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 (Wellcome 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/mm². 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).

**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 λ1 - λ3 images, and λ1 - λ3 values were extracted. Axial (λ3) and radial diffusivity ((λ2 + λ3)/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-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 Tregs (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 χ² 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.

| Analysis of covariance † | Stroke patients (n=18) | Healthy controls (n=22) | F (1, 36) | p |
|-------------------------|------------------------|-------------------------|------------|---|
| FA and axial/radial diffusivity | | | | |
| 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     |
| Left anterior limb of internal capsule | | | | |
| FA                      | 0.43 ± 0.08            | 0.50 ± 0.03             | 23.0       | <0.001** |

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).
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 limbs of internal capsule

| Metric                  | Stroke patients (n=18) | Healthy controls (n=22) | F (1, 36) | p     |
|-------------------------|------------------------|-------------------------|-----------|-------|
| 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 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.

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

![Flow cytometry analysis](image-url)

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_reg). The percentage of T_reg 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: Treg 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 (F1,36 = 7.89, p = 0.008). (B) In stroke patients, a significant correlation was observed between the percentage of circulating Tregs 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 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 Tregs 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 Tregs 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. Tregs 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 Tregs [20]. The ability of Tregs to protect the brain and improve stroke outcome was confirmed by Li et al. [21] who studied the effects of post-stroke Treg therapy.

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

Tregs 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 Tregs, the addition of exogenous IL-10 reduced infarct volume [20]. Tregs also limit the immune response by releasing transforming growth factor-β, which may be required for neurogenesis [24]. In addition, Tregs 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 Tregs adoptive treatment inhibited MMP-9 production in the blood and the brain after ischemia [21].

The reason circulating Tregs levels were lower in stroke patients are not clear. People with lower levels of circulating Tregs 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 Tregs after stroke may reflect the migration of Tregs 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 Tregs. Our findings raise the possibility that stroke outcome can be improved by targeting Tregs to protect the brain from tissue damage after stroke.

Whereas Liesz et al. [20] showed a protective effect of Tregs in stroke, Ren et al. [26] found that Tregs had no effect, and Kleinschnitz et al. [27] showed that Tregs 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 Treg/Th17 cells were imbalanced in patients with cerebral infarction: the Th17 frequency increased, and the Treg frequency decreased, relative to the balance in control subjects [28]. Th17 cells orchestrate tissue inflammation, whereas Tregs limit the immune response and prevent tissue damage. An imbalance between Tregs 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 Tregs was reduced in stroke patients. The degree of reduction correlated with the decrease in the FA value in the internal capsule. Tregs might alleviate post-stroke white matter tissue damage by limiting the immune response. Further study of the role of Tregs in preventing post-stroke cerebral damage is needed.

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