MRI Identification of White Matter Reorganization Enhanced by Erythropoietin Treatment in a Rat Model of Focal Ischemia

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Background and Purpose—The objectives of the present study were to: (1) noninvasively identify white matter reorganization and monitor its progress within 6 weeks after the onset of stroke; and (2) quantitatively investigate the effect of recombinant human erythropoietin treatment on this structural change using in vivo measurement of diffusion anisotropy.

Methods—Male Wistar rats were subjected to middle cerebral artery occlusion and treated with recombinant human erythropoietin intraperitoneally at a dose of 5000 U/kg of body weight (n = 11) or the same volume of saline (n = 7) daily for 7 days starting 24 hours after middle cerebral artery occlusion. MRI measurements of T2- and diffusion-weighted images and cerebral blood flow were performed and neurological severity score was assessed at 1 day and weekly for 6 weeks after middle cerebral artery occlusion. Luxol fast blue and Bielschowsky staining were used to demonstrate myelin and axons, respectively.

Results—White matter reorganization occurred along the ischemic lesion boundary after stroke. The region of white matter reorganization seen on the tissue slice coincided with the elevated area on the fractional anisotropy map, which can be accurately identified. The increase in elevated fractional anisotropy pixels corresponded with progress of white matter reorganization and was associated with improvement of neurological function. Treatment with recombinant human erythropoietin after stroke significantly enhanced white matter reorganization, restored local cerebral blood flow, and expedited functional recovery.

Conclusions—White matter reorganization can be detected by fractional anisotropy. Elevated fractional anisotropy pixels may be a good MRI index to stage white matter remodeling and predict functional outcome. (Stroke. 2009;40:936-941.)

Key Words: erythropoietin ■ focal ischemia ■ fractional anisotropy ■ rat ■ white matter reorganization

Diffusion anisotropy, as measured by fractional anisotropy (FA), is sensitive to alteration of white matter fiber integrity and has been successfully used to detect subtle abnormalities in a variety of diseases that involve disruption of white matter fibers, including Wallerian degeneration, multiple sclerosis, traumatic brain injury, and stroke. For both human ischemia and experimental animal models of stroke, the change of diffusion anisotropy within the ischemic area discloses the degree of structural damage in the tissue and indicates functional potential. Loss of structural integrity after stroke and its impact on the outcome of neurological function have been studied in great detail. However, less attention has been paid to structural reorganization in fiber tracts beyond the area of the ischemic lesion, which may contribute to recovery of neurological function. Treatment of stroke with erythropoietin (EPO) promotes brain remodeling and improves neurological function. Reorganization on a structural level is likely enhanced by such treatment and may account for improved functional recovery. However, the progress of structural reorganization in the ischemic brain after EPO intervention, which is an important part of the restorative process, has not been dynamically investigated. The objectives of the present study were to: (1) noninvasively identify white matter reorganization and monitor its progress within 6 weeks after the onset of stroke; and (2) quantitatively investigate the effect of EPO treatment on this structural change after stroke using in vivo measurement of diffusion anisotropy.

Materials and Methods
All experimental procedures were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital.

Animal Model and Treatment Groups
Male Wistar rats (300 to 350 g; The Jackson Laboratory, Bar Harbor, Maine) were used in the present study. Middle cerebral artery occlusion (MCAo) was induced by placement of a single, intact,
fibrin-rich, 24-hour-old, homologous white clot (approximately 1 μL) at the origin of middle cerebral artery. Rats with embolic stroke were treated with recombinant human erythropoietin (rhEPO; epoetin α, AM-GEN) intraperitoneally at a dose of 5000 U/kg of body weight (treated group; n=11) or the same volume of saline (control group; n=7) daily for 7 days starting 24 hours after MCAo. The dosage was chosen based on our previous study, which demonstrated the beneficial effect of rhEPO on functional recovery for this stroke model. All rats were euthanized 6 weeks post-MCAo.

**Tissue Preparation and Histopathology**

Immediately after the final MRI measurement at 6 weeks after stroke, rats were deeply anesthetized and transcardially perfused. The brain was removed shortly after death, immersed and fixed in 4% paraformaldehyde in phosphate-buffered saline for 2 days, and then cut into 7 contiguous coronal blocks 2 mm thick. Coronal sections 6 μm thick were sliced from each block embedded in paraffin and stained for histological evaluation.

To identify structural changes after stroke, double staining for Luxol fast blue and Bielschowsky-stained tissue sections and corresponding fast blue and Bielschowsky-stained tissue sections along the ischemic boundary; G, higher magnification, revealing bundles of myelin and axons aligned along the ischemic boundary; bar=100 μm). This region of structural change appears bright on the FA map (A) and dark on the T2 map (E). A 6-pixel wide ROI adjacent to the edge of the lesion encompasses the region (B) and FA mean ±2 SD, provided by a homologous tissue region on the contralateral side (C), identifies the areas with elevated FA values (D). These elevated FA areas are located in the nonischemic tissue along the lesion boundary (H).

**In Vivo Magnetic Resonance Image Acquisition and Data Processing**

MRI was performed using a 7-T, 20-cm bore superconducting magnet (Magnex Scientific, Abingdon, UK) interfaced to a Bruker console (Bruker, Boston, Mass). Animals were placed on a nonmagnetic holder equipped with a nose cone for administration of anesthetic gases and stereotaxic ear bars to minimize movement of the head. During the imaging procedure, anesthesia was maintained with 1.0% halothane in 69% \( N_2O \) and 30% \( O_2 \) and rectal temperature was kept at 37°C±1.0°C using a feedback-controlled water bath. T2-weighted imaging, diffusion-weighted imaging, and cerebral blood flow (CBF) were measured for all animals in both treated and control groups at 1 day and weekly for 6 weeks after the onset of stroke.

T2-weighted images were acquired using standard 2-dimensional Fourier transform multislice (13 slices, 1 mm thick), multiecho (6 echoes) MRI. Six sets of images (13 slices per set) were obtained using echo times of 15, 30, 45, 60, 75, and 90 ms and a repetition time of 8 s. Images were produced using a 32×32-mm² field of view and a 128×64-image matrix. The total sequence time was approximately 9 minutes.

Diffusion-weighted images were measured using the method described by Le Bihan et al with diffusion gradients in the x, y, and z directions. The spin-echo sequence (13 slices, 1 mm thick, 32×32-mm² field of view, 128×64-image matrix, repetition time =1500 ms, echo time=40 ms) was modified to include 2 10-ms diffusion-weighting gradient pulses, one on either side of the refocusing 180° radiofrequency pulse. The diffusion-weighting gradient was increased in a nonlinear manner from 0 to approximately 83 mT/m to obtain 3 images with gradient b-values of 20, 600, and 1200 s/mm². Each image required a 5-minute scan time, and the entire 3-directional trace map sequence took approximately 15 minutes.

An arterial spin labeling technique was used to quantify CBF in cerebral tissue. Adiabatic inversion of arterial water protons was accomplished through an axial gradient of 0.3 kHz/mm and a 1-second continuous wave radiofrequency power of approximately 0.3 kHz at a frequency offset of 6 kHz. This was followed by a spin echo imaging sequence with repetition time/echo time=1000 ms/20 ms. The labeled slice was 2 cm distal from the imaging slice and 1 mm thick. To eliminate gradient asymmetry in the axial direction, an image average was applied by switching around the gradient polarities. Field of view was 32×32 mm² and the image matrix was 64×64.

Based on the acquired images, we generated 13 equally spaced coronal slices of T2 and FA maps that covered the entire brain in identical slice locations for each animal. On histological evaluation, white matter reorganization after stroke was characterized by oriented bundles of myelin and axons extruding from the corpus callosum into the ipsilateral striatum along the boundary of the ischemic lesion (Figure 1F–G). Careful comparison between Luxol fast blue and Bielschowsky-stained tissue sections and corresponding MRIs demonstrated that the location of this structural reorganization occurred along the lesion boundary (Figure 1F, red arrows) and coincided with the elevated area on the FA map (comparing Figures 1B and F). For the animals studied, a 6-pixel wide ribbon-like region in the nonischemic area immediately adjacent to the edge of the lesion encompassed the area where FA values were elevated (Figure 1B).

**Figure 1.** Identification of tissue areas undergoing white matter reorganization on a slice of FA map measured from a rhEPO-treated animal 6 weeks after MCAo. The upper row (A–D) shows a slice of FA map and the lower row presents the same slice of T2 map (E, H) corresponding Luxol fast blue and Bielschowsky section (F) and a closer view of the section in the boundary region (G). The values of FA are relatively low in the ischemic area (A) determined by the T2 map (E), indicating demyelination induced by ischemia. White matter reorganization, characterized by oriented bundles of myelin and axons extruding from the corpus callosum into the ipsilateral striatum (F, G), takes place along the boundary of the lesion (F, Luxol fast blue and Bielschowsky section, showing a narrow dark area [red arrows] along the ischemic boundary; G, higher magnification, revealing bundles of myelin and axons aligned along the ischemic boundary; bar=100 μm). This region of structural change appears bright on the FA map (A) and dark on the T2 map (E). A 6-pixel wide ROI adjacent to the edge of the lesion encompasses the region (B) and FA mean ±2 SD, provided by a homologous tissue region on the contralateral side (C), identifies the areas with elevated FA values (D). These elevated FA areas are located in the nonischemic tissue along the lesion boundary (H).
To identify the areas of white matter reorganization represented on the FA map, a T2 map was used to detect the ischemic lesion (Figure 1E). The lesion area was specified by those pixels with a T2 value higher than the mean plus twice the SD (mean $+ 2 \text{SD}$) provided by the normal tissue on the contralateral (nonischemic) side.19 The outline of the lesion was copied onto the same slice of the FA map (Figure 1A), and a region of interest (ROI) encompassing the area undergoing structural reorganization was created by expanding the rim of the lesion 6 pixels outward (Figure 1B). The mean value of FA plus 2 SD, measured from homologous tissue area on the contralateral side (Figure 1C), was used as a threshold to identify the elevated FA pixels in the ipsilateral ROI (Figure 1D). For each animal, ROIs were delineated on 13 slices of the FA map based on the corresponding slices of the T2 map. The area of elevated FA in a specific perilesional region on each slice was identified, and the total number of elevated pixels throughout the brain was calculated. The mean value of FA in the identified elevated area for each slice was also measured. To detect changes in CBF in the reorganized area, the identified FA region at the 6-week time point was used as a ROI to track the evolution of CBF within the experimental period. Data were normalized to the contralateral side for each slice to obtain relative FA and CBF and averaged at the same time points for each group.

Behavioral Testing
Neurological severity score (NSS),21 which grades the composite neurological function of an animal on motor, sensory, reflex, and balance tests (normal score, 0; maximum deficit score, 18), was assessed at 1 day and weekly for 6 weeks after MCAo by an examiner blinded to the treatment groups and the corresponding MRI results.

Statistical Analysis
A 2-sample Wilcoxon exact test was used because our data were not normal. Statistical comparisons of MRI measurements between 2 treatment groups, including relative CBF and FA, lesion area, number of slices, number of pixels, and NSS, were performed at each time point. $P < 0.05$ was considered significant. For data illustration, the results are summarized as mean $\pm \text{SE}$ and presented at each time point.

Results
Elevated Area on Fractional Anisotropy Map and White Matter Reorganization
Histological evaluation based on Luxol fast blue and Bielschowsky-stained tissue slices indicated that white matter reorganization occurred within a certain width along the ischemic lesion boundary after stroke (Figure 1F, red arrows). Comparison between the tissue slices and the corresponding FA maps demonstrated that the region of white matter reorganization seen on the tissue slice coincided with the elevated area on the FA map (comparing Figures 1B with F). These elevated areas along the ischemic lesion boundary on the FA map, which can be identified using the methods described in the previous section and illustrated in Figure 1, then represent white matter reorganization.

Figure 2 shows a case comparison between the treated and control animals, showing that white matter reorganization characterized by FA map (red arrows on FA map) occurred earlier in the rhEPO-treated group than in the saline-treated controls. Our data also showed that no observable elevated FA areas were detected in the boundary region at 1 day post-MCAo for both rhEPO-treated and saline-treated ani-
mals. Visually apparent elevated areas on the FA map appeared as early as 1 week after stroke in the treated animals (3 of 11 [27%]), but mostly after 3 weeks in the controls (6 of 7 [86%]). White matter reorganization as indicated by the FA map took place significantly earlier in the rhEPO-treated group than in the nontreated group (P<0.02). These dynamic MRI observations suggest that treatment with rhEPO accelerates white matter reorganization after stroke.

Measurements of Elevated Areas on Fractional Anisotropy Map
FA value of the contralateral hemisphere, which is used to determine the tissue area with structural change (Figure 1), was measured and compared between the treated and control groups. No difference between the 2 groups was detected (P>0.91).

Changes in relative FA, number of slices with elevated FA pixels, and total number of elevated FA pixels throughout the brain are shown, respectively, in Figures 3A–C.

As shown in Figure 3A, relative FA was higher at each time point in the treated group than in the control group, although the difference did not achieve statistical significance. Unlike relative FA, number of slices with elevated FA pixels and total number of elevated FA pixels increased post-MCAo (Figure 3B–C). By 6 weeks, a significantly higher number of slices containing elevated FA pixels were detected in the treated group than in the control group (Figure 3B, 6 weeks, P<0.05). Compared with the MCAo controls, treatment with rhEPO significantly increased the total number of elevated pixels on the FA map from 3 to 6 weeks after stroke (Figure 3C, P<0.05).

Evolution of Cerebral Blood Flow in the Area of Elevated Fractional Anisotropy
As shown in Figure 2, the area of white matter reorganization characterized by the FA map (red arrows on FA map) coincided with the site of CBF restoration in treated animals, whereas no such evidence could be observed in nontreated animals (Figure 2, comparing FA with CBF in elevated FA areas at each time point). More than half of the rhEPO-treated animals (6 of 11 [55%]) and none of the controls exhibited this relationship. Quantitative data measured from 2 groups indicated that relative CBF values in the area of elevated FA were higher in the treated group than in the controls and that a significant difference appeared by 6 weeks after stroke (Figure 3E, P<0.05).

Outcome of Neurological Function
NSSs are given in Figure 3F. Treatment with rhEPO significantly improved NSS during later stages of stroke (5 and 6 weeks) compared with the controls (P<0.05).

Discussion
We investigated the progress of white matter reorganization in the rat brain with and without rhEPO intervention after embolic stroke both noninvasively and dynamically using in vivo MRI measurement of diffusion anisotropy. Our data demonstrate that the area of elevated FA in a specific region along the ischemic boundary after stroke reflects cerebral tissue undergoing white matter reorganization. This structural change can be identified and monitored on an FA map. The increase in elevated FA pixels corresponds with progress of white matter reorganization and is associated with improvement of neurological function and therefore may be a good
MRI index to stage the structural restorative process and predict potential functional outcome.

In the brain, diffusion of water varies significantly with direction, known as anisotropy. This anisotropic property of water diffusion can be quantified by the FA value as proposed by Basser and Pierpaoli.22 FA is a unitless measure, and a higher FA value indicates greater directionality. Anisotropic diffusion is most prominent in the white matter because it is organized into bundles of myelinated axonal fibers running in parallel.2,7 Water diffuses more rapidly along the axon than across it, probably due to the presence of directional subcellular structures, including the axonal membrane and the neurofilamentary cytoskeleton that serve as barriers to diffusion.23 A measure of anisotropy such as FA therefore provides an index of tissue microstructure and could possibly characterize the degree of fiber damage in diseases affecting the white matter.1,2 However, after stroke, structural changes occur not only in the ischemic region, but also in the cerebral tissue area undergoing reorganization.13 Although a growing body of evidence has supported the ability of diffusion anisotropy to detect alteration of fiber tract integrity within the ischemic lesion after stroke,6–10 few data are available regarding structural reorganization at the ischemic boundary as measured by anisotropy.11,12 In addition, the dynamic relationship between structural reorganization and functional outcome is still relatively unknown. In the present study, we demonstrate that FA is a good measure of white matter reorganization and that a larger area of white matter reorganization represented by elevated FA pixels in the perilesional area indicates a better outcome of neurological function after stroke.

Our dynamic measurements of diffusion anisotropy revealed a unilateral asymmetrical increase in FA adjacent to the lesion on the ipsilateral side, and these elevated areas on the FA map identified cerebral tissue undergoing white matter reorganization, which was confirmed histologically (Figure 1). The temporal profile of relative FA in this area for both treated and control groups suggested that white matter reorganization, probably involving restoration and reorganization of intact axonal membranes and reorganized fiber tracts, persisted to 6 weeks after stroke (Figure 3A). This finding is well supported by other studies, which described long-term (3 years) improvement in white matter integrity in the nonlesional brain region after human ischemic stroke.24,25 FA measurements showed not only that structural reorganization occurred earlier in the treated group than in the controls (Figure 2), but also that the area undergoing white matter reorganization was larger in the treated animals than in the control animals (Figure 3C), suggesting that rhEPO promotes structural reorganization after stroke. More importantly, the increase in reorganization area identified by FA map in the rhEPO-treated animals accompanied the improvement of neurological function evaluated by NSS (comparing Figure 3C with Figure 3F). A larger number of elevated FA pixels were associated with a lower NSS, or a better functional outcome. These data seem to imply that the increase in elevated pixels on the FA map characterizes the progress of white matter reorganization and that this structural reorganization contributes to recovery of neurological function. Elevated FA values result from the increase in density and directionality of myelinated fiber tracts consistent with white matter reorganization after stroke. Our data indicated that rhEPO treatment enhanced such structural remodeling (Figure 3C) and, in turn, improved the neurological outcome (Figure 3F).

Previous studies on FA recovery in lesion boundary tissue after transient MCAo were based on the ROIs where T2 recovered.11,12 FA increase resulting from white matter reorganization may have been attenuated, because the ROIs involved white and gray matter. In the present study, we directly identified the elevated FA area that was located in a specific perilesional region and represented white matter reorganization (Figure 1). Discriminating this reorganization area from surrounding tissue allowed us to detect the effect of white matter reorganization on FA (Figure 3A) and, particularly, to monitor the progress of these reorganization areas (Figure 3C) along with time after stroke. The FA data we presented here provide quantitative information about cerebral tissue undergoing structural change after stroke, which cannot be revealed by ROI measurements.

As a member of the Type I superfamily of cytokines, EPO is characterized by pleiotropic functionality.26 The biological activity of EPO extends well beyond erythropoiesis and includes several other important physiological processes, which expands the clinical use of EPO from the treatment of anemia to other pathological conditions such as stroke.26 Administration of EPO after focal ischemia protects cells,17,27 tissues,16,28 and neural vessels29 and promotes angiogenesis,14,17 and neurogenesis,14,16 all of which may expedite structural reorganization after stroke by providing a restorative microenvironment.

The effect of EPO administration on ischemic lesion volume after stroke depends on treatment protocol.14,30,31 Early treatment (eg, ≤6 hours poststroke) with EPO reduces infarct volume,30,31 whereas delayed treatment (eg, 24 hours poststroke) does not.14 Our long-term dynamic measurements confirm previous results that EPO administration (intraperitoneally) initiated at 24 hours poststroke does not significantly alter lesion size (Figure 3D).

Our findings also demonstrate that even without reduction of ischemic lesion size (Figure 3D), rhEPO treatment improves neurological function (Figure 3F) and restores local CBF (Figure 3E), most likely the result of an EPO-induced integrative effect. Animals treated with rhEPO exhibited long-term restoration of CBF in the area of structural reorganization identified by the FA map (Figures 2 and 3E), which may have been due to angiogenesis stimulated and enhanced by rhEPO.14,17 Restoration of local blood supply in an ischemic brain plays a critical role in tissue repair and functional recovery.17 Our observation that CBF restoration and white matter reorganization coexisted strongly in rhEPO-treated animals (Figure 2, comparing FA with CBF at elevated FA areas in the treated animal) suggests that local restoration of CBF may facilitate structural reorganization after stroke. However, such a combined effect was less evident in the controls, probably because angiogenesis was insufficient to restore CBF.

The method we used to identify the area of structural reorganization is an automatic operation without operator bias. However, our data are still subject to errors. The FA
value on the contralateral side provided a baseline in the current study to identify the cerebral tissue undergoing structural reorganization. Although no difference in FA value on the contralateral hemisphere was detected, the threshold measured from a specific narrow tissue region could be affected by the noise of FA map. However, the noise affected all the animals in both groups. Given the restrictions of scan time, 3-direction (x, y, z) instead of 6-direction diffusion-weighted images were chosen to calculate FA. This measurement requires precise and consistent positioning of the animal in the scanner. Although a Tripilot sequence was used to precisely position the animal in the magnet before each scan, errors caused by minor change in position may influence FA. However, our data show that the FA map obtained from the current experimental setting reflects the microstructural changes (Figure 2) as confirmed histologically (Figure 1).

In summary, white matter reorganization in the perilesional area during brain remodeling after stroke can be dynamically detected in vivo by measurement of diffusion anisotropy such as the FA map. Thus, the FA map provides a noninvasive means for real-time visualization of structural reorganization after stroke. The degree of reorganization characterized by the area of elevated FA along the ischemic boundary can be helpful in estimating the potential for functional recovery. Treatment with rhEPO after stroke significantly enhances white matter reorganization, restores local CBF, and expedites recovery of neurological function.

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Disclosures
None.

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