Positron Emission Tomography Imaging of Poststroke Angiogenesis

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Background and Purpose—Vascular endothelial growth factor (VEGF) and VEGF receptors (VEGFRs) play important roles during neurovascular repair after stroke. In this study, we imaged VEGFR expression with positron emission tomography (PET) to noninvasively analyze poststroke angiogenesis.

Methods—Female Sprague-Dawley rats after distal middle cerebral artery occlusion surgery were subjected to weekly MRI, 18F-FDG PET, and 64Cu-DOTA-VEGF121 PET scans. Several control experiments were performed to confirm the VEGFR specificity of 64Cu-DOTA-VEGF121 uptake in the stroke border zone. VEGFR, BrdU, lectin staining, and 125I-VEGF165 autoradiography on stroke brain tissue slices were performed to validate the in vivo findings.

Results—T2-weighted MRI correlated with the “cold spot” on 18F-FDG PET for rats undergoing distal middle cerebral artery occlusion surgery. The 64Cu-DOTA-VEGF121 uptake in the stroke border zone peaked at ∼10 days after surgery, indicating neovascularization as confirmed by histology (VEGFR-2, BrdU, and lectin staining). VEGFR specificity of 64Cu-DOTA-VEGF121 uptake was confirmed by significantly lower uptake of 64Cu-DOTA-VEGFmutated in vivo and intense 125I-VEGF165 uptake ex vivo in the stroke border zone. No appreciable uptake of 64Cu-DOTA-VEGF121 was observed in the brain of sham-operated rats.

Conclusions—For the first time to our knowledge, we successfully evaluated the VEGFR expression kinetics noninvasively in a rat stroke model. In vivo imaging of VEGFR expression could become a significant clinical tool to plan and monitor therapies aimed at improving poststroke angiogenesis. (Stroke. 2009;40:270-277.)

Key Words: positron emission tomography ■ stroke ■ vascular endothelial growth factor receptor

Stroke remains the number one cause of disability among Americans each year. Besides thrombolytic treatments, which have a very narrow time window and limited availability,1 there are currently no other therapeutic approaches. Promising novel therapeutic strategies, such as cell transplantation,2 are moving toward clinical trials, but there is a significant lack of clinically applicable methods to guide and monitor such therapies. Molecular imaging, a recently emerged field that seeks to quantitatively measure biological processes at the cellular and molecular level in vivo,3 can be a valuable tool for these applications. Major areas of molecular imaging in stroke research include metabolic imaging without contrast agents,4 labeling methods for tracking stem cells and macrophages migrating into ischemic brain regions,5 probes that can reflect the metabolic status,6,7 and probes that binding specifically to stroke markers.8

One potentially important repair mechanism after stroke is angiogenesis,9,10 the formation of new blood vessels. Increased expression of vascular endothelial growth factor (VEGF) and VEGF receptors (VEGFRs) plays important roles during neurovascular repair after stroke.11 Human neural stem cells overexpressing VEGF have been shown to provide neuroprotection and enhance angiogenesis.12 Because ischemia and cell transplantation can enhance VEGFR expression,13,14 in vivo imaging of VEGFR could be a valuable indicator for the repair mechanisms occurring after stroke.

Positron emission tomography (PET) has been widely used in the clinic for diagnosis and treatment monitoring.15 We recently developed a tracer, 64Cu-DOTA-VEGF121 (DOTA denotes 1,4,7,10-Tetraazacyclododecane-N,N′,N″,N‴-tetraacetic acid), which can enable PET imaging of VEGFR expression in various disease models.16,17 Herein, we demonstrate for the first time to our knowledge the in vivo imaging of VEGFR expression after acute experimental stroke.
Materials and Methods

Distal Middle Cerebral Artery Occlusion Surgery
All experimental protocols performed on animals were approved by the Stanford University Administrative Panel on Laboratory Animal Care. Focal cerebral ischemia (distal middle cerebral artery occlusion [dMCAo] model) was generated as described previously.18 Sham-operated animals (controls) underwent the same surgery without occlusion of the blood vessels. Daily intraperitoneal injections with 5-bromo-2'-deoxyuridine (BrdU) were administered in 3 mice for 7 days after dMCAo.

Receptor Binding Assay
The detailed procedure of a competitive cell binding assay has been reported previously.16,19 VEGF121, VEGFmutant (the amino acid residues 63, 64, 67, 82, 84, and 86 in the VEGFR binding sites were mutated to alanine), and their DOTA conjugates were prepared as previously reported.16,20 VEGFR-1 and VEGFR-2 binding affinities of VEGF121, VEGFmutant, and the DOTA conjugates were evaluated by porcine aorta endothelial cells transfected with human VEGFR-1 and porcine aorta endothelial cells transfected with human VEGFR-2 cell-binding assays using [125I]-VEGF165 (GE Healthcare) as the radio-ligand. The best-fit IC50 values were calculated by fitting the data by nonlinear regression using GraphPad Prism Software. Experiments were performed with triplicate samples.

MRI Protocol
The MR frame consisted of a nonmagnetic stereotactic wrist coil with a cylindrical surface coil (5-cm internal diameter) positioned directly over the rat head. MR imaging was performed in a 3-T whole-body MR scanner (GE Healthcare). Rats were anesthetized during imaging using 1% to 2% inhaled isoflurane. The brain of each rat was imaged weekly with T2-weighted fast-spin echo sequence (receiver bandwidth=16 kHz; repetition time=5000 ms; echo time=86 ms; echo train length=8; field of view=4×4 cm; matrix=256×256; 16 slices; slice thickness=1 mm; number of signals averaged=3; total imaging time=8 min). The MR images were analyzed using Image J software (http://rsb.info.nih.gov/ij/). The stroke area in each MR image was traced and measured to calculate the stroke volume.

MicroPET Studies
Weekly PET scans of the rats were performed on a microPET R4 rodent model scanner (Siemens Medical Solutions).16,21 64CuCl2 (10 mCi) was diluted in 300 μL of 0.1 mol/L sodium acetate buffer (pH=6.5) and added to 100 μg DOTA-VEGF121 or DOTA-VEGFmutant.16,22 The reaction mixture was incubated for 1 hour at 40°C with constant shaking.64Cu-DOTA-VEGF121 and 64Cu-DOTA-VEGFmutant were then purified by size exclusion chromatography using phosphate-buffered saline as the mobile phase. The radiochemical purity was >98%.

Each rat was intravenously injected with ~1 mCi tracer (18F-FDG, 64Cu-DOTA-VEGF121, or 64Cu-DOTA-VEGFmutant). For 18F-FDG, the rats were scanned at 1 hour after injection. For 64Cu-DOTA-VEGF121 or 64Cu-DOTA-VEGFmutant, the rats were scanned at multiple time points in pilot studies (1, 2, and 4 hours after injection); 2 hours after injection was chosen because of the best contrast observed. For each PET scan, 3-dimensional regions of interest were drawn over the stroke area on decay-corrected images. The average radioactivity concentration was obtained from the mean pixel values within the region of interest volume, which were converted to counts per milliliter per minute by use of a predetermined conversion factor. Given a tissue density of 1 g/mL, the counts per milliliter per minute were converted to counts per gram per minute, and the values were divided by the injected dose to obtain the imaging region of interest derived percentage injected dose per gram (%ID/g).

Autoradiography
Detailed procedure of autoradiography was reported earlier.23 The perfused and acetone fixed rat brain slices (with or without the stroke area) were sectioned at 50 μm and mounted onto glass slides. Autoradiography was performed as previously described.23

Figure 1. Characterization of the stroke model. A, Axial and coronal T2-weighted MR images of the rat brain at various days after surgery. B, The stroke size based on MRI. C, Hematoxylin and eosin–stained rat brain tissue after dMCAo surgery. D, Weekly 18F-FDG PET scans of the dMCAo rats. E, 18F-FDG uptake in the stroke area and the contralateral brain. Arrowheads indicate the stroke area in all cases. n=4; *P<0.05; **P<0.01; ***P<0.001.
area; 20-μm thick) were incubated with 125I-VEGF165 (0.1 μCi per slice) for 1 hour at 20°C. After washing with phosphate-buffered saline 3 times, the slices were placed onto a chilled autoradiography cassette containing a Super Resolution screen (Packard) and stored for 3 days at −20°C. Screens were then laser-scanned with the Packard Cyclone system and displayed at pseudocolor scale.

**Histology**

Stroke rats at different days after surgery were transcardially perfused with saline, followed by 3% paraformaldehyde. The brains were removed and postfixed for 24 hours in 3% paraformaldehyde, followed by cryo-preservation in 3% paraformaldehyde/20% sucrose for 48 hours. Subsequently, the brains were embedded in optimal cutting temperature compound (OCT) and cut into 20-μm sections.

All primary antibodies were diluted in phosphate-buffered saline/0.3% Triton containing 1% bovine serum albumin (BSA). The following antibodies were used: rabbit anti-VEGFR-1 (Laboratory Vision Corp; 1:50), rabbit anti-VEGFR-2 (Laboratory Vision Corp; 1:50), mouse anti-lectin (Vector Labs; 1:250), mouse anti-BrdU (Vector Labs; 1:250), mouse anti-GFAP (Advanced ImmunoChemicals; 1:800), mouse anti-NeuN (R&D Systems; 1:500), FITC donkey anti-rabbit (Jackson ImmunoResearch; 1:500), and Cy3 goat antimouse (Jackson ImmunoResearch; 1:500). For double staining of
lectin and BrdU, the lectin stain was performed and the tissue postfixed in 3% paraformaldehyde for 10 minutes at 4°C. After thorough washes, the sections were treated with 3 mol/L HCl for 30 minutes at 37°C. After blocking and washing, the sections were incubated with mouse anti-BrdU, followed by a secondary antibody. 4',6-Diamidino-2-phenylindole was used to label the nuclei (Sigma-Aldrich; 1:1000).

Sections were analyzed on a confocal laser scanning microscope (Zeiss) with split panel and z-axis analysis to confirm marker colocalization. VEGFR-2 expression was quantified in 3 high-power fields in the stroke border zone at 2, 10, and 17 days after stroke. The percent area in each high power field covered by VEGFR-2 immunoreactivity was measured using Image J.

Statistical Analysis
Quantitative data were expressed as mean±SD. Means were compared using 1-way analysis of variance and Student t test. P<0.05 was considered statistically significant.

Results

Stroke Morphology and Size
A group of 4 dMCAo rats underwent weekly T2-weighted MRI, 18F-FDG, and 64Cu-DOTA-VEGF121 scans. A successful stroke surgery and focal cortical stroke in the left hemisphere was confirmed for all animals on T2-weighted MRI (Figure 1A). The stroke volume based on MRI was 244.7±33.5, 153.6±41.9, and 152.6±42.1 mm³ at days 1, 8, and 15, respectively (Figure 1B; n=4). Typically, the stroke size is overestimated on T2-weighted MRI early after surgery because of edema, which appears hyperintense in the images. Hence, the lesion size significantly decreased from day 1 to day 8 after surgery but remained stable thereafter, with the true stroke size being ~150 mm³ (Figure 1B). Hematoxylin and eosin staining confirmed the stroke presence (Figure 1C). Sham-operated rats were also prepared and no stroke was observed in their brains based on MRI.

18F-FDG PET
Normal brain tissue has prominent uptake of 18F-FDG because of the constant need for glucose in the brain. A “cold spot” was seen in the 18F-FDG PET of dMCAo rat brain, which corresponds to the stroke area delineated by MRI (Figure 1D). The contralateral brain uptake of 18F-FDG

Figure 3. Control experiments with 64Cu-DOTA-VEGFmutant. A, Cell-binding assay reveals that VEGF121 has high affinity binding to VEGFR-2 (IC50: 2.8 nmol/L), whereas VEGFmutant does not. B, Both VEGF121 and VEGFmutant bind to VEGFR-1, with the IC50 values being 5.4 and 11.3 nM, respectively. C, T2-weighted MRI and 18F-FDG PET confirmed stroke presence. D, Axial and coronal PET images of the dMCAo rat brain at 2 hours postinjection of ~1 mCi of 64Cu-DOTA-VEGFmutant. Note that there is virtually no uptake in the stroke area. n=3.
remained constant at \( \approx 1.0 \) to 1.1% ID/g, whereas that of the stroke area was significantly lower at all time points examined (Figure 1E; \( P<0.05; n=4 \)). The uptake in the stroke area was \( \approx 0.5 \) to 0.6% ID/g at days 1, 15, and 22. At day 8, the stroke area appeared to be smaller and the uptake was higher (0.76±0.11% ID/g). Such phenomenon is likely attributable to inflammation in the stroke area because \(^{18}\text{F-FDG}\) has been well-established to be taken up by inflamed tissue (which does not significantly affect the MR images).25,26

**\( ^{64}\text{Cu-DOTA-VEGF}_{121} \) PET**

The day after the MRI and \(^{18}\text{F-FDG}\) scans, the rats were subjected to \( ^{64}\text{Cu-DOTA-VEGF}_{121} \) PET. Uptake in the stroke area could be clearly seen as early as day 2 (0.24±0.02% ID/g; n=4; Figure 2A,B). The uptake in the stroke area peaked at day 9 (0.46±0.11% ID/g) and remained elevated at day 16 (0.29±0.10% ID/g). The uptake in the contralateral brain was low at all time points (<0.10% ID/g). At day 23, the uptake in the stroke area was no longer statistically significantly different from the background (0.06±0.04% ID/g vs 0.03±0.02% ID/g, respectively; n=4). The stroke-to-normal tissue uptake ratio was 12.5±1.6, 9.0±1.8, 3.2±1.6, and 2.1±1.9 at days 2, 9, 16, and 23, respectively (n=4; Figure 2C). The uptake of \(^{64}\text{Cu-DOTA-VEGF}_{121} \) in the surgical wound was prominent in most of the images because angiogenesis is also a key process during wound healing.

To validate VEGFR expression in the stroke border zone, \(^{125}\text{I-VEGF}_{165}\) autoradiography was performed on brain slices at day 2 and day 9 after surgery because good stroke-to-normal tissue ratios were observed at these time points. Clearly, VEGFR expression was seen at the proximity of the stroke area but not in the contralateral brain or the normal brain tissue (Figure 2D). The site of VEGFR overexpression correlates to the area with \(^{64}\text{Cu-DOTA-VEGF}_{121} \) uptake in the PET scans.

**\( ^{64}\text{Cu-DOTA-VEGF}_{\text{mutant}} \) PET and Sham-Operated Rats**

Several control experiments were performed to confirm VEGFR specificity of \(^{64}\text{Cu-DOTA-VEGF}_{121} \) uptake. The VEGF\text{mutant} does not bind to VEGFR-2 with an IC\text{50} value of >10 μmol/L (2.8 nmol/L for VEGF\text{121}; Figure 3A). For VEGFR-1, the IC\text{50} values of VEGF\text{121} and VEGF\text{mutant} were 5.4 nmol/L and 11.3 nmol/L, respectively (Figure 3B). A group of 3 dMCAo rats (Figure 3C) were prepared and subjected to \(^{64}\text{Cu-DOTA-VEGF}_{\text{mutant}} \) scans at day 10 after surgery (when the uptake of \(^{64}\text{Cu-DOTA-VEGF}_{121} \) was the highest in the stroke border zone). The uptake of \(^{64}\text{Cu-DOTA-VEGF}_{\text{mutant}} \) in the stroke border zone was significantly lower than that of \(^{64}\text{Cu-DOTA-VEGF}_{121} \) (0.20±0.08% ID/g vs 0.46±0.11% ID/g; \( P<0.05 \); Figure 3D).

Three sham-operated rats were prepared and both MRI and \(^{18}\text{F-FDG} \) PET confirmed the lack of stroke in the rat brain (Figure 4A). Subsequent \(^{64}\text{Cu-DOTA-VEGF}_{121} \) scans revealed that the tracer uptake was in the wound, but not in the brain (<0.10% ID/g at days 9 and 16; n=3; Figure 4B). The wound uptake was the highest at day 2, and was significantly lower at day 9 and day 16. Taken together, these 2 sets of control experiments confirmed that the \(^{64}\text{Cu-DOTA-VEGF}_{121} \) uptake in the stroke border zone was VEGFR-specific (Figure 4C).

**Histology**

Stroke morphology as seen on hematoxylin and eosin–stained sections correlated well with that observed in MRI and PET (Figure 1C). VEGFR-2 was expressed in the stroke border zone as early as 2 days after surgery with predominantly cellular expression (Figure 5A). At 10 days after surgery, a mixed cellular and vascular expression pattern was noted (Figure 5B,D), which decreased significantly at day 17 (Figure 5C). Phenotypic characterization showed colocalization of VEGFR-2 with astrocytes (GFAP; Figure 5E), neu-
rons (NeuN; Figure 5F), and blood vessels (Lectin; Figure 5G) at day 10 after stroke. Quantification of VEGFR-2 expression in the stroke border zone confirmed the PET findings with a peak expression at day 10 (Figure 5H). The overall expression between day 2 and day 10 was not significantly different, but there was markedly less vascular expression at day 2 after stroke. VEGFR-2 expression in the contralateral brain and normal brain tissue was minimal and VEGFR-1 staining of the dMCAo rat brain slices was also weak at all time points (data not shown). BrdU staining at day 10 demonstrated numerous BrdU-positive cells in the stroke border zone (Figure 6A,B). Colocalization of lectin and BrdU further substantiated the presence of poststroke angiogenesis (Figure 6B,C).

**Discussion**

No treatment currently exists to restore the lost neurological function after stroke. Increased vascularization in the stroke border zone within a few days after stroke is associated with neurological recovery. Stem cells, erythropoietin, chemokines, and gene therapy, among others, have been shown to induce angiogenesis after stroke. Noninvasive molecular imaging of poststroke angiogenesis, such as the one demonstrated here, could be extremely valuable not only
as a prognostic factor but also as a measurement of success to help guide proangiogenic therapies. Different scenarios can be envisioned. To date we do not know the right timing or the ideal place for cell or drug delivery in future clinical trials with stroke patients. It is conceivable that molecular imaging techniques will not only help us monitor the therapy effects but also help us defining patient specific treatment strategies. Moreover, it is also possible that early molecular and biological events (assessable by molecular imaging) might be predictors of clinical outcome.

Upregulation of VEGFR was already found at 2 days poststroke, indicating that signals to induce angiogenesis appear very rapidly after stroke. The uptake of $^{64}$Cu-DOTA-VEGF$_{121}$ in the stroke border zone peaked at $\sim$10 days after surgery (significantly higher than the baseline uptake at days 2, 9 and 16, but not at day 23), confirmed by both histology and autoradiography, which showed that VEGFR-2 expression peaked at $\sim$10 days after stroke (Figures 2 and 5). Previous studies have demonstrated peak VEGF expression between 12 and 24 hours, preceding VEGFR-2 expression.$^{32}$ Whereas VEGFR-1 mRNA level returned to baseline after 7 days, the VEGF-2 mRNA level persisted for 7 days.$^{32}$ $^{64}$Cu-DOTA-VEGF$_{121}$ binds to both receptors; therefore, it cannot differentiate between VEGFR-1 and VEGFR-2. However, the significantly lower uptake of $^{64}$Cu-DOTA-VEGF$_{mutant}$ which does not bind to VEGF-2 but binds to VEGF-1 with lower affinity than $^{64}$Cu-DOTA-VEGF$_{121}$ (Figure 3A,B), at day 10 in the stroke border zone suggests that VEGFR-2 may play a more dominant role over VEGFR-1 in poststroke angiogenesis. In agreement with previous studies, VEGF$^{2}$ expression was concentrated around the stroke border zone$^{32}$ and was found to colocalize with the blood vessels, astrocytes, and neurons (Figure 5).$^{33}$$^{34}$ BrdU staining further confirmed the active formation of new blood vessels (Figure 6).

Because there is a blood–brain barrier instability after stroke, partially influenced by VEGF, it is possible that our imaging not only represents endoluminal but also represents some cellular expression of VEGFR. Recently, VEGFR upregulation in the ventral premotor hand representation and the M1 hind limb representation in squirrel monkeys 3 days after stroke were reported.$^{35}$ This underscores the potential importance of VEGF/VEGFR pathway not only in acute stroke but also in poststroke plasticity and perhaps long-term rehabilitation.

Imaging VEGF and VEGFR expression are both important for diagnosis and monitoring of angiogenesis-related treatment efficacy. Although increased expression of growth factors and cytokines occurs in many stroke models, many of the signaling pathways that are responsible for revascularization have only been subjectively proposed based on ex vivo/in vitro studies. Much research will be needed to develop blood–brain barrier-penetrating tracers for imaging VEGF expression in the (stroke) brain. We envision that examining the stroke brain in the same animals or patients with both VEGF- and VEGFR-targeted tracers may give important insights about the expression kinetics of VEGF and VEGFR and may shed new light on the disease/recovery mechanisms based on VEGF/VEGFR signaling. Generation of VEGFR-1–specific or VEGFR-2–specific VEGF$_{mutant}$ with-out any affinity to the other receptor may also help us better understand poststroke angiogenesis in future studies. Besides VEGF/VEGFR imaging, other angiogenesis-related targets such as integrin $\alpha_\beta$ should also be studied in the future.$^{36}$

In this study, the wound in the rat skin is close to the stroke area. In pilot studies, we also created a group of stroke rats using a suture model in which the wound is not directly adjacent to the stroke area. However, we found that the stroke size varies to a great extent between animals, which led to less reproducible results in MRI, $^{18}$F-FDG, and $^{64}$Cu-DOTA-VEGF$_{121}$ PET scans. Therefore, we chose the dMCAo model in which the stroke size in each rat is quite consistent. Recent development of hybrid PET/MR scanners may facilitate the coregistration of the MR and PET images in future studies for differentiation between stroke and normal brain tissue. Nonetheless, control experiments in sham-operated rats, the use of a VEGF$_{mutant}$, autoradiography, and histology all clearly demonstrated that the uptake in the stroke border zone is attributable to VEGFR overexpression in poststroke angiogenesis.

**Summary**

Multimodality imaging was used to assess the temporal profile of the lesion size, metabolic activity, and angiogenesis in the same animals after stroke. For the first time to our knowledge, we successfully evaluated the time-course of VEGF expression noninvasively by PET in a rat stroke model. The ability to visualize VEGFR expression level in vivo could provide new opportunities to document angiogenesis after stroke, which may have significant clinical implications in guiding future therapies aimed at improving angiogenesis. Future studies of imaging VEGFR expression before, during, and after new stroke therapies are warranted.

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None.

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