Imaging the Ischemic Penumbra with $^{18}$F-Fluoromisonidazole in a Rat Model of Ischemic Stroke

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**Background and Purpose**—The ischemic penumbra is a major focus of stroke research. $^{18}$F-fluoromisonidazole ($^{18}$F-FMISO), a positron emission tomography (PET) marker of hypoxic cells, has shown promise as a technique to image the penumbra in humans. Our aim was to delineate the pattern of $^{18}$F-FMISO binding in a rat middle cerebral artery transient thread-occlusion model, and correlate this with tissue outcome at 24 hours. We hypothesized that the pattern of $^{18}$F-FMISO binding would mimic that seen in humans.

**Methods**—Thirty-eight rats underwent 2 hours transient middle cerebral artery (MCA) occlusion, and then received $^{18}$F-FMISO at time points from 0.5 to 22 hours post-MCA occlusion and were killed 2 hours later. Autoradiographic assessment of $^{18}$F-FMISO binding and assessment (triphenyltetrazolium chloride) of the area of infarction were performed on tissue slices.

**Results**—Until 1 hour after MCA occlusion, $^{18}$F-FMISO binding was increased in the entire MCA territory, with little or no infarction visible. Over the next 5 hours, the pattern of binding evolved to a small rim of intensely binding tissue surrounding the infarct core, which itself showed reduced binding compared with the contralateral hemisphere. By 24 hours, there was minimal accumulation of $^{18}$F-FMISO binding and a large area of infarction.

**Conclusions**—The pattern of $^{18}$F-FMISO binding rats reproduced the pattern seen in humans, consistent with this tracer being a marker of the ischemic penumbra in both species. This technique may have application in studying the ischemic penumbra in animal models, and correlating this with similar studies in humans. (Stroke. 2004;35:975-980.)

Key Words: stroke ☐ imaging ☐ animal models of human disease ☐ nitroimidazoles ☐ autoradiography

The concept of the ischemic penumbra is critical to current thinking about ischemic stroke. The penumbra is the severely hypoperfused, but potentially salvageable region surrounding the infarct core. It is structurally intact, but has lost electrical and protein synthetic function, the latter 2 features being potentially reversible with reperfusion and/or neuroprotection. This potential for salvage is both time- and cerebral-blood-flow–(CBF) dependent; the penumbra is, therefore, an evolving entity.

Despite its importance, reliably identifying the ischemic penumbra remains problematic. In humans, multitracer positron emission tomography (PET) measuring blood flow and metabolism is the current gold-standard imaging technique for penumbral identification, but is technically demanding and requires invasive arterial sampling. Diffusion/perfusion-weighted magnetic resonance imaging (MRI) and perfusion computerized tomography (CT) are now becoming more commonly used because of their simplicity and suitability for repeat studies; however, they may not differentiate infarct, penumbra, and oligemia reliably early after stroke. Cerebral blood flow autoradiography has also been used to predict penumbra in experimental animals; however, while prediction of tissue destined to infarction was quite accurate, the eventual fate of the ‘penumbral’ tissue was heterogeneous. More precise, yet applicable, ways of identifying infarction and penumbra are needed, both in humans and experimental models.

$^{18}$F-FMISO PET was developed to overcome some of the shortcomings of existing penumbral imaging approaches. Nitroimidazole compounds such as fluoromisonidazole (FMISO) diffuse freely across cell membranes, and are reduced by intracellular reductases in living cells. In normoxic conditions these compounds are rapidly reoxidised and diffuse back out of cells. Under hypoxic conditions, further reduction occurs, and the compounds become irreversibly bound to intracellular molecules. Thus, nitroimidazoles are

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trapped within hypoxic cells, but not in necrotic tissues. Tracer binding appears to be independent of blood flow. FMISO has already been used extensively as a marker of hypoxic tissue within tumors and myocardium. In studies of human ischemic stroke there was binding surrounding the infarct core in the acute phase (<48 hours); however, binding was not seen in the late phase (>48 hours), and some of the hypoxic tissue progressed to infarction, while some did not. There was also a correlation between neurological deterioration in the week following stroke and the proportion of the initially hypoxic region, which progressed to infarction. The advantages of this technique are that it provides a simple, direct image of the penumbra, is less demanding than the current gold-standard multitracer technique, and is therefore more feasible in acute stroke patients. Further, it specifically targets cells under hypoxic stress rather than relying on extrapolation from regional perfusion and metabolic rates.

Our aim was to study the pattern of FMISO binding during stroke evolution using a rat transient middle cerebral artery occlusion (MCAO) model to test the hypothesis that the pattern and evolution of FMISO binding in the rat would mimic that seen in humans. Specifically, we hypothesized that binding of FMISO would occur within the territory of the occluded middle cerebral artery (MCA), surround the infarct core in the acute phase, and not be seen in the subacute phase. Further, the area of hypoxic tissue in early phase cohorts would be larger than final infarct size in the late phase cohort, implying that some of the initially bound tissue is destined to infarction, while some is not.

Materials and Methods

Animals

All animal experimentation was performed with the approval of the animal ethics committee of our institution, and followed institutional guidelines. Thirty-eight male Sprague-Dawley rats weighing 270 to 320 g were used. Anesthesia was induced using 2 mL enflurane in an ether jar, and maintained with 2% isoflurane administered in a 50/50 air/oxygen mix. Atropine 120 μg was administered intraperitoneally immediately following induction. Temperature was maintained at 37°C throughout the procedure using a rectal temperature regulated heating pad.

Focal Ischemia

MCA thread occlusion was performed using the method of Belai et al (after Zea Longa et al). The tip of a preprepared poly-L-lysine-coated 40 nylon monofilament was blunted by heat to a diameter of 0.42 to 0.48 μm and a mark made at 18 mm from the tip. After ligation of branch arteries including the pterygopalatine artery and creation of an external carotid artery stump, the thread was passed via the right external carotid, and up the internal carotid artery 17 to 18 mm depending on animal size, until resistance was felt. Animals were subjected to 2 hours MCAO, and then the MCA-occluding monofilament was retracted into the external carotid stump under anesthesia to reopen the MCA. Each animal received 3.7MBq FMISO (330 μCi/kg in 250 μL) via a tail vein injection, and was killed 2 hours later. Six cohorts of rats received FMISO at 0.5, 1.0, 2.0, 3.0, 6.0, or 22.0 hours after MCAO. Animals with no evidence of infarction on triphenyltetrazolium hydrochloride (TTC) sections were excluded from further analysis. In the 0.5 hours cohort, only animals without both TTC evidence of stroke and focal FMISO uptake were excluded.

FMISO was synthesized at the PET center of Austin Health by using the method of Cherif et al.

Tissue Processing

Animals were killed by anesthetic overdose, followed by decapitation. The brains were removed and placed in ice-cold normal saline for 5 minutes. Coronal sections 8 x 2 mm thick were prepared using a rat brain template. These sections were immediately immersion-stained in a freshly prepared 3% solution of the neuronal integrity marker 2,3,5 TTC in normal saline brought to 37°C for 5 minutes before use. TTC was kept in the dark at all times. Staining was stopped by immersion in phosphate buffered saline containing 0.16% paraformaldehyde and 0.5% glutaraldehyde, and digital photographs were taken. Autoradiography was performed by 40 minutes exposure of the TTC stained sections to phosphor imaging plates that were read using a Fuji BAS 3000 (Fuji Photo Film Ltd). The areas of FMISO uptake and TTC pallor were outlined manually using the MCID software package (Imaging Research). The sum of areas of FMISO binding or TTC staining from all tissue slices from each animal was calculated, and means and standard errors calculated. At later time points, areas of reduced uptake at the infarct core were noted visually, and this was confirmed by demonstrating a relative optical density <0.8 times that in the corresponding homotopic region of the contralateral hemisphere using the 1 mm diameter circular selection tool of the MCID program.

Areas of infarction and FMISO binding were expressed as sum totals of areas from all 2 mm tissue slices (7 or 8, depending on animal size). We chose not to present calculated volumes, because of the errors inherent in measures made from tissues compressed in autoradiography cassettes and compounded by the high tissue penetration of FMISO.

Statistics

In order to determine whether the profiles of TTC and FMISO were parallel over time, a mixed-model analysis of variance (ANOVA) was performed. First, the main effects of TTC and FMISO were extracted. Then the TTC x FMISO interaction was used to test the null-hypothesis of parallelism of profiles over time. The data are presented as means and standard errors. The level of statistical significance was set at P<0.05. The statistical analyses were performed using SYSTAT v. 10 (SPSS Inc).

One-way ANOVA with Tukey’s test was applied to evaluate the differences between individual time points for FMISO binding and TTC pallor. A paired Students’ T-test was used to evaluate the statistical significance of areas of reduced FMISO binding at the infarct core and of islands of increased binding at later time points. In each case, these areas were compared with paired homotopic regions in the contralateral hemisphere.

Results

Eight animals were excluded because of no evidence of infarction (including one in the 0.5 hour group with neither evidence of infarction nor increased FMISO uptake). The pattern of FMISO uptake progressed as predicted in terms of distribution and area (Figure 1). At early times there was a large area of FMISO uptake in the ipsilateral MCA territory (195.9±16.9 mm² at 0.5 hour, 201.5±23.7 mm² at 1.0 hour). With time, the area of FMISO uptake became smaller (117.7±33.3 mm² at 2.0 hours, 50.1±29.8 mm² at 3.0 hours), with more intense binding at the edges of the infarct territory. In the 3.0 hour cohort, there was still a thin rim of increased uptake around the infarct in most animals, and an area of reduced uptake at the lesion core was also seen in all animals (Figure 2). By later points in time, there was minimal or no residual FMISO uptake (35.6±19.1 mm² at 6.0 hours, 7.8±2.9 mm² at 22.0 hours). In those animals with small areas of uptake, FMISO uptake was confined to the edge of the infarct (Figure 3 and 4). All animals in the 6 and 22 hour cohorts showed reduced FMISO uptake at the core of the infarct.
(P≤0.005), and all also had at least one area at the edge of the infarct with increased uptake >1.25 times the homotypic region of the contralateral hemisphere (P≤0.005 and 0.05 respectively).

The area of infarction demarcated by TTC staining increased with time, and progressed outwards from striatum to involve the cortex with increasing time intervals. By six hours, striatal infarction was complete, while there was still a trend for continued infarct growth in the cortex (Figure 5).

**Discussion**

The pattern of 18F-FMISO binding in this rat model of stroke largely replicated that previously seen in humans. In the hyperacute (0.5 to 1.0 hour) phase there was increased 18F-FMISO binding in the entire ipsilateral MCA territory. Increased binding surrounded the infarct core in the acute phase (2 to 3 hours), and was negligible in the subacute phase (6 to 22 hours). Although the area of hypoxic tissue in hyperacute cohorts was larger than final infarct size in the subacute cohort, this difference did not achieve statistical significance. This might be explained by interanimal variability in infarct size that limited the statistical power of the study, or because the 2 hour MCAO produced a near-complete MCA territory infarct in this species. So, in the absence of neuroprotection, spontaneously recovering areas were necessarily small.

Interestingly, the area of 18F-FMISO binding seen at 1.0 hour was the same as that seen at 0.5 hour. Because 18F-FMISO relies on active cellular metabolism for the reduction reaction that results in trapping, this implies that up to this point in time, there is still active cellular metabolism (and potential for salvage) within virtually the entire ischemic territory. Other investigators have shown that 0.5 or 1 hour transient MCAO produces a pattern of selective neuronal necrosis involving isolated cortical neurons or small focal areas of pannecrosis, but this does not produce the widespread pannecrosis seen after permanent MCAO. Our results are consistent with these data, suggesting a potential for salvaging much of the ischemic territory after short duration ischemia in the rat, which is consistent with it being penumbral.

Evolution of stroke is believed to occur more rapidly in rats than in humans, yet there were still small areas of increased 18F-FMISO binding at 6 and 22 hours. There are strong parallels with primate and human PET studies using CBF/CMRO2 and 18F-FMISO, showing small regions of penumbra persisting for up to 16 to 48 hours post-stroke in some subjects. Hence, there may be small areas of potentially salvageable tissue later in the evolution of stroke than has generally been thought. It is not clear what importance the salvage of small areas of penumbra may have, but...
it may enhance the plasticity and subsequent recovery of function. A direct penumbral marker, such as $^{18}$F-FMISO, is well suited to the study of small areas of penumbra, and may be a useful tool to test this hypothesis. Persistence of increased $^{18}$F-FMISO binding after the removal of the MCA-occluding suture at 2 hours may, at first, appear to be biologically implausible since reperfusion should have returned normoxic conditions. However peri-infarct edema, small vessel occlusion, or the "no-reflow" phenomenon are possible explanations for persistent local hypoxia after large vessel reperfusion.

There appeared to be a trend for later progression to infarction in the cortex than in the striatum (Figure 5). This is consistent with previous rat MCAO data suggesting that ischemic changes evolve more rapidly in the caudate-putamen than in the cortex. The different rate of progression in cortex versus striatum has been hypothesized as due to a greater reduction in CBF to the striatum. Interestingly, CT and PET studies in humans and primates also show striato-capsular damage hours before the appearance of cortical damage, and the penumbra has been qualitatively shown to evolve more rapidly in central than peripheral regions.

There are some limitations to this study, particularly due to the radiochemical properties of $^{18}$F-FMISO. Fluorine-18 has a short half-life (1.83 hours), and a delay of at least 2 hours is necessary between injection and animal sacrifice to allow adequate washout of $^{18}$F-FMISO from normal tissues. Thus, autoradiography could not be delayed for the time necessary for tissue fixation and was performed on fresh tissues. This resulted in some tissue distortion due to compression in the autoradiography cassettes (see Figures 2 and 4); thus, while TTC staining allowed assessment of infarction in the same slices used for autoradiography, it was not practical to overlay both sets of images without computer transformation of the images. Further, direct comparison by superimposing the two sets of images would not be meaningful because the autoradiographic image resulted from the trapping of $^{18}$F-FMISO 2 hours prior to TTC staining. It is presumed that the process of infarction evolved further over these 2 hours. TTC staining is not reliable earlier than 24 hour after occlusion in permanent MCAO models; however, TTC has been reported to be more effective at the early detection of necrosis in transient
rather than permanent MCAO models, with detection of infarcted tissue as early as 2.5 hours after artery occlusion. Nevertheless, all methods for pathological identification of “irreversibly damaged tissue” early after stroke have limitations, demonstrating a functional correlation with salvage of the ischemic penumbra that is considered an important criterion for the presence of penumbra. We have not presented the results of behavioral tests, as this study was not designed to assess penumbral salvage with the same MCAO time in each cohort. Moreover, results of behavioral testing for the early cohorts were likely to be confounded by residual anesthetic effects.

One previous study has investigated a related hypoxic marker, 3H-misonidazole, in a gerbil model of stroke. Increased uptake was found in the stroke hemisphere, compared with the contralateral hemisphere in a widespread but patchy distribution. Another hypoxic marker, 123I-iodozaymcin arabinoside (IAZA), has been investigated in a rat MCA thread-occlusion model in combination with a measure of blood flow and MRI scanning. Increased uptake occurred in regions with moderately reduced blood flow, but not those with either severe or mild flow reductions. Within the area of reduced diffusion on MRI, there were some areas of increased IAZA uptake, and some with low IAZA uptake. These early results suggest that hypoxic probes may be effective in differentiating penumbra from both necrosis and oligemia.

We have demonstrated that FMISO is suitable for autoradiographic studies of hypoxic tissues in the temporary rat MCAO model. Taken concurrently with previous human PET studies using the same marker, we conclude that FMISO shows great promise as a research tool for the study of penumbra in ischemic stroke. As one of the few penumbral markers that may be imaged easily in both animals and humans, important parallels in the characteristics of the ischemic penumbra may be drawn between the two species and, thus, enhance our understanding of its genesis.

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**References**

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