Apparent Diffusion Coefficient Images in Subacute Stroke Detects DNA Fragmentation

T. A. Kent, Z. GU, J. Wei, M.J. Quast, J.R. Perez-Polo
Departments of Neurology, Human Biological Chemistry & Genetics, The Marine Biomedical Institute
The University of Texas Medical Branch, Galveston, Texas 77555

INTRODUCTION

The evolution of ischemic stroke is readily visualized by detection of alterations in the apparent diffusion coefficient (ADC) of water using diffusion-weighted imaging (DWI). The sensitivity of DWI is related to the detection of alterations in water compartmentalization or motion, and becomes less diffusible after various pathological conditions. Histological analyses of DWI lesions after stroke indicate cellular swelling and cytotoxic lesions. However, the process of cell death in stroke may also involve apoptotic cell death. The precise nature of apoptosis in the nervous system remains controversial, and may differ from that in other cell types such as immune cells, and may be less clearly distinct from necrotic counterparts. The hippocampus seems particularly sensitive to ischemic stress and DNA fragmentation and some form of apoptosis can be detected there after modest ischemic insults. It is not known whether DWI also reflects CNS apoptosis, although, given the "spectrum" of apoptotic and necrotic features found in CNS apoptosis, we hypothesized that apoptotic neurons undergo alterations in diffusion characteristics that are detectable by DWI.

We set out to develop a level of ischemia/reperfusion that is not severe enough to cause necrosis and hinder interpretation but will produce oxidative stress. The luminal suture model used here has sufficient variation and produces varying degrees of perfusion restriction. We varied the duration of ischemia in 18 rats in order to obtain a broad spectrum of outcomes to ischemia. We also set out to reliably and specifically measure apoptotic cell death with high spatial resolution so as to correlate with topographic MR images. We chose to detect apoptotic cell death by *in situ* nick translation using the KLENOW-FragEL™ DNA Fragmentation Detection Kit. This method detects 3' free DNA strand breaks generated in response to apoptotic signals ("sticky-ends") and catalyzes the template-dependent addition of deoxynucleotides using exogenous Klenow DNA polymerase. Here, we used rats at 3 days post-ischemia. This time point insured that the apoptotic process had started and that the ischemic injury was detectable by histological analysis.

METHODS

Animal model

Fifteen male Sprague-Dawley rats weighing 300 to 350 g were anesthetized with 3% halothane, and a 14-0 catheter was used for endotracheal intubation. Rats were artificially ventilated with 0.5 to 1.0% halothane in 30% 02 and 70% N_2 during surgery and MRI procedures. The rats were respirated via a Brain circuit coupled to a modified, compressed air-power clinical pressure ventilator to minimize dead space. The femoral artery and vein were cannulated with PE-5O polyethylene catheters for monitoring MABP, blood pH, $PaCO_2$, PaO_2. Rectal temperature was maintained at 37 to 37.8 C with a heating pad and during MRI the rat core temperature was maintained by circulating warm air through the magnet bore.

Focal cerebral ischemia was induced by occluding the origin of the middle cerebral artery using the intraluminal suture insertion method with some modifications (1). The insertion length of the suture was 15 mm from the base of the pterygopalatine artery. The right middle cerebral artery was occluded transiently for 1.0 to *2.5* hours, at which time the suture was removed and the internal carotid artery was tied off to allow recirculation via the circle of Willis. A sham-operated group was prepared by ligation of the internal carotid artery only.

MRI Measurements

Proton MRI was performed with a 4.7 T horizontal bore imaging system (SISCO/Varian, Palo Alto). At 3 days after occlusion rats were imaged with a 5-cm surface coil tuned to 200 MHz. The MRI protocol consisted of spin echo imaging with 3 different diffusion weightings. Multislice images with a 60 mm field of view (FOV), 128 or 256 phase encode steps and a *256* x *256* digital matrix, resulting in 12 contiguous transverse slices covering a 20 mm length from the cerebellum to the olfactory lobe with a slice thickness of 1.6 mm. Echo time (TE) was 65 ms, repetition time (TR) was 3.0 s. In DWI, the b factor ranged between 10 in T2-weighted images (T2WI) and 1500 s/mm^2 in diffusion weighted images (DWI). The complete MRI protocol took 1 h.

Apparent diffusion coefficient (ADC) images were calculated from the slopes of the MRI signal intensity versus b value. Regional analysis of MRI images was performed by determining the presence of hyperintensity in T2WI or areas of restricted diffusion in ADC images. The regions analyzed consisted of the hippocampus, the striatum, the parietal cortex, the piriform/insular cortex and the basal forebrain.

DNA Fragmentation Detection

Right after MRI, rats were deeply anesthetized and perfusion-fixed. Histochemical staining for DNA fragments of apoptotic cells was performed by ***in situ*** nick translation using KLENOW-FragEl™ DNA fragmentation detection kit (Oncogene, Cambridge, MA) with some modifications described here. In brief, Klenow enzyme *(E. coil* DNA polymerase I) within the Klenow labeling reaction mixture binds to exposed nick ends of DNA fragments generated in response to apoptotic signals and catalyzes the template-dependent addition of biotin-labeled and unlabeled deoxynucleotides. The reaction was stopped with the Stop Buffer, followed by incubation with Blocking Buffer for 10 mm and then conjugated with streptavidin-horseradish peroxidase for 30 mm. The staining was visualized with DAB solution. The sections were counterstained with methyl green. For generation of a positive control, cerebellum sections were incubated with 1 mg/ml DNase I (Amersham). The negative brain section controls were using the procedure except for the omission of the Klenow enzyme.

RESULTS

Several rats appeared to have the desired degree of perfusion restriction, resulting in no obvious T2-weighted hyperintensity lesion, but regions of restricted diffusion on the ADC image. There was a remarkably distinct laminar band of diffusion restriction in sub-regions of the hippocampus, corresponding to CA1 and CA3, as well as in some cortical regions. Histological sections revealed prominent dark-brown irregularly shaped labeling on the ischemia/reperfusion hemisphere, but not on the control side, where non-specific light brown staining was evident. This specific staining was visible in CA1, CA3, and regions of the cortex, all spatially corresponding to the restricted regions on the ADC image.

Overall, in serial sections, restricted diffusion was seen in the hippocampus in 12 sections, all of which also showed positive FragEL staining, while there was no restricted diffusion seen in 11, none of which had positive FragEL staining (p=.0001; chi-square).

CONCLUSION

High resolution ADC images revealed heterogeneity of the hippocampus and cortical areas demonstrating regions of restricted diffusion. These regions co-localized spatially with cells in which histological sections were positive for the presence of "sticky ends", a feature of DNA fragmentation in cells undergoing apoptosis. The specificity of any single measure of apoptosis requires confirmation by independent techniques, and that is under way. Nevertheless, intracellular events associated with apoptosis that could potentially restrict water diffusion include: the uncoiling of DNA that restricts water due to entropic effects; the formation of apoptotic blebs and other inclusion bodies; and the dissolution of nuclear membranes altering hydrophilic interactions.

REFERENCE:

1. Quast MJ, Huang, N, Hillman GR, Kent TA, Magnetic Resonance Imaging 11:46-471 (1993).