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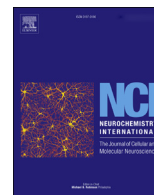

Evidence for the recruitment of autophagic vesicles in human brain after stroke

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ABSTRACT

Autophagy is a homeostatic process for recycling proteins and organelles that is increasingly being proposed as a therapeutic target for acute and chronic neurodegenerative diseases, including stroke. Confirmation that autophagy is present in the human brain after stroke is imperative before prospective therapies can begin the translational process into clinical trials. Our current study using human post-mortem tissue observed an increase in staining in microtubule-associated protein 1 light chain 3 (LC3), sequestosome 1 (SQSTM1; also known as p62) and the increased appearance of autophagic vesicles after stroke. These data confirm that alterations in autophagy take place in the human brain after stroke and suggest that targeting autophagic processes after stroke may have clinical significance.

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1. Introduction

Stroke is the leading cause of long-term disability in adults and a major cause of death worldwide (Lozano et al., 2012). As a disease, stroke has no effective therapy beyond a small percentage of patients who receive thrombolytics. A major reason so few therapies are available is that while the cause of stroke is known, the underlying causes of neuronal death remain poorly understood. Strokes are largely ischemic, resulting from arterial occlusion that prevents perfusion at the core of the infarct and hypoperfusion at the margin of the blood vessels' territory (penumbra). The extent of neurological damage following stroke and the severity of the neurological sequelae depends on the viability of the hypoperfused penumbra and, if the artery occlusion is transient, the reperfusion that follows. Reperfusion triggers molecular pathways leading to programmed cell death (PCD), neuronal loss and consequent disability (Taxin et al., 2014). Additionally, neuronal function is compromised in ischemic injury by energy deficits and consequently damaged proteins, and this debris generation leads to recruitment of cellular protein degradation systems including autophagy (Luo et al., 2013).

Autophagy and the role that it plays in the progression of neuronal injury is an emerging process in the understanding of stroke (Luo et al., 2013; Puyal et al., 2013). Autophagy can be activated in cytodestructive and cytoprotective modes, dependent upon the insult, and autophagic cell death is documented in brain tissue (Higgins et al., 2011; Puyal et al., 2013). The conundrum being that autophagy can be protective in neonatal hypoxic–ischemic injury (Li et al., 2010; Koike et al., 2008) and in models of preconditioning (Sheng et al., 2010), yet cytodestructive in models of middle cerebral artery occlusion (MCAO) (Wen et al., 2008; Shi et al., 2012). Such observations support the concept that the "load" of cellular debris and damaged proteins, as influenced by stroke severity, determine the mode of autophagy and its recruitment, and the pattern of PCD. Despite several studies analysing autophagy in animal models of stroke, there are no published data on human brain tissue. Therefore in the present study we used *post-mortem* brain tissue from patients with a history of stroke to document for the first time the expression and cellular localization

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of three autophagic markers, namely microtubule-associated protein 1 light chain 3 (LC3), sequestosome 1 (SQSTM1; also known as p62) and Beclin 1 (BECN1).

2. Materials and methods

2.1. Human post-mortem brain tissue

All procedures were conducted in accordance with the *National Statement on Ethical Conduct in Human Research* (2007) of the Australian National Health & Medical Research Council, the Victorian Human Tissue Act 1982, the National Code of Ethical Autopsy Practice and the Victorian Government Policies and Practices in Relation to Post-Mortem.

Brain samples from 5 individuals who suffered stroke were obtained from the National Neural Tissue Resource Centre of Australia. Cases were aged between 66 and 90 years (mean 82 years). The *post-mortem* intervals varied between 8.5 and 28.5 h (mean 19.5 h). It is important to emphasise that every patient in this study presented a variety of associated diagnosis (see Table 1 for details). The identification of the infarct regions was performed *post-mortem*, secondary to the main diagnosis, by a neuropathologist (Prof. Catriona McLean), with the age of infarct ranging from 24–48 h to 2–4 weeks prior to death. Serial cryosections were used to directly compare the haematoxylin and eosin staining (serial 1) with the immuno-stained section (serial 2) to clearly identify and cross compare to the area of ischaemic damage. As the immuno-stained sections were counterstained it was also possible to determine the area containing a loss of neurons, thereby supporting the direct cross comparison of ischaemic area as seen on the haematoxylin and eosin sections and confirming the location as being in the correct area. For every patient an area without any sign of infarct was used as internal control.

Since it is challenging to determine areas of infarct in frozen brain tissue the biochemical analysis was performed on brain samples from grey matter cortical tissue in the vicinity of the areas of ischaemic damage identified by haematoxylin and eosin staining (as described above). Four out of the five cases were suitable for this analysis (Table 1 – cases S1–S4).

Control brain samples of 4 individuals, aged between 64 and 90 (mean 80 years), without stroke or other neuropathology were also obtained from the National Neural Tissue Resource Centre of Australia. Clinical information and epidemiological details of all patients are described in Table 1.

2.2. Immunohistochemistry

Human brain paraffin embedded sections (7 μm) were examined to determine the expression and localization of LC3 and SQSTM1 using methods previously described on human postmortem brain tissue (Frugier et al., 2010). Sections were preheated for 30 min at 60 °C and dewaxed by two successive 5-min baths in xylene before microwave treatment for antigen retrieval. Endogenous peroxidase activity was inhibited with a phosphate-buffered saline (PBS) solution containing 3% H_2O_2 for 5 min at room temperature. Then, the sections were blocked for 1 h at room temperature in the following solution: 5% normal serum, 1% bovine serum albumin, 0.1% Tween20 in PBS. Sections were incubated overnight at 4 °C with the primary antibody (1:200) overnight at 4 °C and then incubated with the appropriate secondary biotinylated-antibody (1:500) for 1 h at room temperature. This step was followed by the addition of the ABC kit reagents (Vector Laboratories) for 30 min at room temperature before being visualized using 3,3′-diaminobenzidine (Vector Laboratories).

2.3. mRNA analysis

Total RNA was extracted from fresh frozen brain cortex tissue (100 mg) using a TRIzolPlus RNA purification kit (Life technologies). The concentration and purity of the RNA samples were assessed using a Nanodrop1000 spectrophotometer (Thermo Fisher Scientific) while the RNA integrity was assessed using the Agilent 2100 bioanalyzer (Agilent Technologies). The total RNA fractions of each sample were then converted to cDNA using SuperScript III reverse transcriptase (Life technologies) and oligo $d(T)_{20}$ as primer, and taken as template for real-time quantitative polymerase chain reaction (Q-PCR) to compare gene expression following stroke. Q-PCRs were carried out using TaqMan Universal master mix and the 7900HT Fast Real-Time PCR system (Applied Biosystems). Four control genes were used to calculate the normalization factor needed to achieve relative quantitation by applying the comparative C_T method (ΔC_T): Peptidylpropyl isomerase A (*PPIA*), Hydroxymethylbilane synthase (*HMBS*), Ubiquitin C (*UBC*) and Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The following 9 TaqMan gene expression assays (Applied Biosystems) were used in this study: Hs001777654_m1 (*SQSTM1*), Hs00186838_m1 (*BECN1*), Hs00738808_m1 (*MAP1LC3 alpha*), Hs00917683_m1 (*MAP1LC3 beta*), Hs01374916_m1 (*MAP1LC3 gamma*), Hs99999904_m1 (*PPIA*), Hs00609297_m1 (*HMBS*), Hs00824723_m1 (*UBC*) and Hs99999905_m1 (*GAPDH*).

Table 1
Details of the stroke and control cases. Cases S1–S5: stroke cases. Cases C1–C4: control cases. All brains were obtained at autopsy. PMI, *post mortem* interval (time between death and brain retrieval); M, male; F, female; N/A, not available.

Case	Age (y)	Sex	PMI (h)	Stroke pathology of the brain regions analysed	Cause of death and associated diagnosis
S1	82.5	M	8.5	2–4 week infarct in the frontal cortex	History of multiple cerebrovascular accident (days to years), dementia, severe debility
S2	66.2	M	28.5	2–4 week infarct in the right parietal cortex	Aspiration pneumonia (11 days), multiple sclerosis (28 years), urosepsis (11 days)
S3	85.6	M	19.5	Acute infarct (24–48 h) in the occipital cortex	Spinal cerebellar ataxia (years) and aspiration pneumonia (days)
S4	90.2	F	22	Acute infarct (24–48 h) in the parietal cortex	Right middle cerebral artery infarct (6 days), atrial fibrillation (years), subclinical hyperthyroidism (years)
S5	86.3	F	N/A	2 week infarct in the occipital cortex	Broncho-pneumonia related to neurodegenerative disease for which a ventriculoperitoneal shunt has been performed
C1	64.1	M	24	No	Ischaemic heart disease and coronary artery atherosclerosis
C2	82.7	M	27	No	Unexplained sudden death in a man with metastatic carcinoid tumour of the caecum
C3	82.7	F	28.5	No	Cardiac tamponade – haemopericardium, ruptured acute posterolateral left ventricular myocardial infarction, ischaemic coronary artery disease
C4	90.8	M	32.5	No	Respiratory failure (2 days), pneumonia (7 days), chronic asthma (30 years), chronic renal failure (20 years)

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