Changes in hyaluronan production and metabolism following ischaemic stroke in man

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The extent of recovery from stroke is dependent on the survival of neurons, particularly in peri-infarcted regions. Angiogenesis is critical for the development of new microvessels and leads to re-formation of collateral circulation, reperfusion and better recovery. Hyaluronan (HA) is an important component of the brain extracellular matrix and a regulator of cellular differentiation, migration, proliferation and angiogenesis. We have found that the production of total HA and low molecular mass 3–10 disaccharides of HA (o-HA) was increased in post-mortem tissue and in the serum of patients 1, 3, 7 and 14 days (peaking at 7 days) after ischaemic stroke. Hyaluronidase activity was also increased in serum samples (peaking after 3 days), which might explain the subsequent increase in o-HA. Affinity-histochemical staining was performed using a HA-specific biotinylated binding protein, and it showed enhanced deposition of HA in blood vessels and intracellularly as well as in the nuclei of peri-infarcted neurons. Western blotting and immunohistochemistry demonstrated upregulation of HA synthases (HAS1 and 2) and hyaluronidases (HYAL1 and 2) in inflammatory cells from both stroke and peri-infarcted regions of the brain. HYAL1 was upregulated in microvessels and intracellularly in neurons, whilst HAS2 became translocated into the nuclei of neurons in peri-infarcted areas. Receptor for HA-mediated motility was observed intracellularly and in the nuclei of neurons, in the tunica media of larger blood vessels and in the endothelial cells of microvessels in stroke-affected tissue, whilst expression of other receptors for HA, CD44 and tumour necrosis factor-stimulated gene 6 (TSG-6) were mainly increased in infiltrating mononuclear cells from inflammatory regions. The data presented here demonstrate that HA breakdown is a feature of the acute stage of stroke injury. Increased o-HA production soon after stroke may be detrimental through enhancement of the inflammatory response, whilst activation of HA and/or o-HA-induced cellular signalling pathways in neurons and microvessels may impact on the remodelling process by stimulating angiogenesis and revascularization, as well as the survival of susceptible neurons.

Keywords: RHAMM; hyaluronan; hyaluronidase; hyaluronan synthase; ischaemic stroke

Abbreviations: BSA = bovine serum albumin; EC = endothelial cells; ECM = extracellular matrix; HA = hyaluronan; o-HA = oligosaccharides of hyaluronan; HABP = hyaluronan binding protein; HAS = hyaluronan synthase; HYAL = hyaluronidase; IS = ischaemic stroke; PBS = phosphate-buffered saline; RHAMM = receptor for hyaluronan-mediated motility; RT–PCR = reverse transcription–polymerase chain reaction; SDS–PAGE = sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SSS = Scandinavian Stroke Scale; TSG-6 = tumour necrosis factor-stimulated gene 6

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Introduction

Stroke is a leading cause of death and disability in the Western world and usually occurs as a result of progressing atherothrombosis, cardiac or artery–artery embolism (Rubio et al., 2005) resulting in loss of membrane integrity, proteolysis, the ability to synthesize proteins and altered signal transduction (Lipton, 1999). The survival of neurons,
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Material and methods

Patient details

This study was approved by the local Ethical Committee, and consent was obtained according to the Declaration of Helsinki. Serial blood samples were collected from indwelling intravenous catheters on admission (Day 1) and on Days 3, 7 and 14, from patients aged between 50 and 93 years, admitted with IS ($n = 54$; 27 male and 27 female). A full clinical examination was carried out on admission, and the patients were scored according to the 58-point Scandinavian Stroke Scale (SSS) (Slevin et al., 2000). Lesions were evaluated by CT or MRI and classified as being large infarct (LI; infarct > 4 cm; SSS < 30, $n = 14$), moderate infarct (MI; >1.5 cm and <4 cm; SSS > 30, $n = 8$) or small infarct (SI; <1.5 cm; SSS > 40, $n = 32$). Routine blood parameters including peripheral leucocytosis, cholesterol, fibrinogen, urea and glucose levels were also determined on admission. Excluded from the study were patients with previous transient ischaemic attack or stroke; recent history of head trauma; major cardiac, renal, hepatic, or cancerous disease; and obvious signs of infection after admission.

A set of healthy, age-matched control sera ($n = 24$) from patients who had no recent infections or history of serious illness or recent head trauma, and who were subsequently shown to be disease-free, were obtained from the National Blood Service, Manchester, UK.

Tissue samples were obtained from nine patients who died 3–37 days after stroke following middle cerebral artery occlusion (Table 1). Immediately after death the bodies were routinely refrigerated at 4°C and tissue was collected within 8 h. Tissue samples were taken from grey and white matter and from infarcted and peri-infarcted areas. The peri-infarcted region was defined as the area of tissue adjacent to the ischaemic core, which has previously been found to exhibit stroke-related changes including neuronal apoptosis and angiogenesis and was characterized by tissue oedema and discolouration, morphology of the neurons and maintenance of structural integrity (Krupinski et al., 2000). Sections were stained with 2,3,5-triphenyltetrazolium chloride, which stains active neurons and oligodendrocytes, and has a role in astrocyte motility, neurite migration and axonal growth (Lynn et al., 2001). Tumour necrosis factor-stimulated gene 6 (TSG-6) is upregulated in many cell types in response to proinflammatory stimulation and growth factors (Milner and Day, 2003). It is activated during blood vessel injury and can modulate cell proliferation and, interestingly, CD44 expression, where it increases the binding affinity of HA (Lesley et al., 2002). We hypothesize that HA turnover increases in stroke tissue, and our aim in the present study was to investigate the serum and tissue levels of HA and associated enzymes together with the tissue expression of HA receptors CD44, RHAMM and TSG-6 in human subjects after ischaemic stroke (IS).

<table>
<thead>
<tr>
<th>Table 1 Primer sequences used in PCR</th>
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<tr>
<td>HAS1-F</td>
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<td>RHAMM-F</td>
</tr>
<tr>
<td>RHAMM-R</td>
</tr>
<tr>
<td>TSG-6-F</td>
</tr>
<tr>
<td>TSG-6-R</td>
</tr>
</tbody>
</table>
at −70°C. A sample of each tissue was processed for histology and stained with haematoxylin and eosin to determine tissue morphology. The value of post-mortem samples in studies involving measurement of RNA and protein expression has been previously identified (Castennson et al., 2000; Love et al., 2003; Mitsios et al., 2006).

**Measurement of HA in serum and tissue**

The method was based on that of West et al. (1995) with minor modifications. Serum and tissue samples were digested with pronase E (0.1 cm-3; 1 mg/cm3 in phosphate-buffered saline (PBS)), boiled for 30 min and centrifuged (3000 g, 20 min). The supernatant was divided into two, and one sample was used to determine total HA concentration. The second sample was centrifuged through a Sephacryl S100 Filtron column (6000 g, 30 min) with a 50 kDa cut-off and oligosaccharides of hyaluronan (o-HA) (<50 kDa) were collected.

HA concentration was measured by enzyme-linked immunosorbent assay (ELISA) (West et al., 1997). A 96-well ELISA plate was coated overnight at room temperature (RT) with 200 μl of HA solution (100 μg/cm²), and after washing in PBS the wells were blocked with bovine serum albumin (BSA) (2% in PBS containing 0.05% Tween-20; PBST) for 2 h, and washed again with PBS. Duplicate 100 μl aliquots of samples and standards were applied and 100 μl of biotinylated-HA binding protein (B-HABP; 1 μg/cm²) in PBST, prepared and characterized as described elsewhere; West et al., 1997) was added to all wells except the blank. Plates were sealed and incubated at RT, washed in PBS (×4) and incubated with alkaline phosphatase–streptavidin (diluted 1:3000 in PBST containing 1% w/v BSA). Plates were washed (×4) with PBST and developed with p-nitrophenylphosphate (1 mg/cm²) in 100 mM diethanolamine, pH 9.8, containing 2 mM CaCl₂. Absorbance was read at 405 nm using an ELISA plate reader with Titresoft software.

**Measurement of serum HYAL**

The method of Natowicz et al. (1996) was used. Briefly, serum (10 μl) was incubated with 250 μl buffered substrate solution (0.1 M sodium formate, 0.1 M NaCl, 250 mg/l HA and 1.5 mM saccharic acid 1,4-lactone; 4 h at 37°C). The reaction was terminated by the addition of 50 μl of borate buffer, the samples were heated for 20 min at 37°C and the absorbance was read at 585 nm. A standard curve was prepared with concentrations of N-acetylgalactosamine from 0 to 1 mM. One unit of hyaluronidase activity was defined as the amount of enzyme producing 1 μmol of N-acetylgalactosamine per minute at 37°C.

**RNA extraction**

RNA extraction was performed using standard Clontech procedures (BD Biosciences, Hertfordshire, UK). RNA quality was measured using a spectrophotometer.

**Reverse transcription–polymerase chain reaction (RT–PCR)**

Gene expression was examined by RT–PCR, in samples of contralateral, peri-infarcted and infarcted tissue, using primers designed against the gene of interest. PCR conditions were as follows: an initial denaturation step of 10 min at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C, and a final extension step of 10 min at 72°C. The products were visualized following agarose gel electrophoresis (1.5%) and DNA stained with ethidium bromide (10 μg/ml). Samples without cDNA were used as negative controls.

All experiments were carried out twice and at 20, 30 and 40 cycles to ensure equal loading of cDNA and proportional cycling. Results for 30 cycles are shown. S16 housekeeping gene was used to normalize the results (forward: 5′-GGC AGA CGG AGA TGA ATC CTC A-3′ and reverse: 5′-CAG GTC CAG GGG TCT TGG TCG TCC-3′). Sense and antisense oligonucleotide primers containing 27 nt based on previously reported mRNA sequences in the GenBank depository were designed with the aid of the Primer3 Output Program (Version 0.2). Invitrogen plc. (Paisley, UK) synthesized the primer sets.

**Western blotting**

Proteins were extracted from tissue samples (~50 mg) and 20 μg from each sample (determined by Biorad protein estimation) was separated by SDS–PAGE gel electrophoresis as described elsewhere (Slevin et al., 1998). Proteins were electroblotted onto nitrocellulose filters (Hoefler, San Francisco, CA, USA), blocked overnight with 1% BSA in Tris-buffered saline-Tween (TBS-Tween) and incubated for 4 h at RT with fully characterized antibodies to the following proteins: rabbit polyclonal HAS1 and HAS2 (gifts of Dr P. Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden; Usui et al., 2000), mouse monoclonal HYAL1 (a gift from Dr B. Triggs-Raine, Department of Biochemistry and Medical Genetics, University of Manitoba, Canada; Shuttleworth et al., 2002), rabbit polyclonal HYAL2 (a gift from Professor R. Stern, Department of Pathology, University of California, San Francisco, USA; Bourguignon et al., 2004), rabbit polyclonal RHAMM (a gift from Dr E. Turley, Department of Physiology, University of Manitoba, Winnipeg, Canada; Lynn et al., 2001), rat monoclonal CD44 (which binds the membrane N-terminal portion of the receptor; Calbiochem, CA, USA) and mouse monoclonal TSG-6 (a gift from Dr Katalin Mikecz, Department of Orthopedic Surgery, Rush University Medical Centre, Chicago, USA; Lesley et al., 2002). All antibodies were used at a dilution of 1:1000 except anti-c-CD44 (1:500).

Filters were washed in TBS-Tween before staining with antibodies to peroxidase-conjugated secondary antibody. Proteins were detected using the ECL western blotting detection system (Amersham, UK). The intensity of staining relative to α-actin was measured with an LKB densitometer. Results are semi-quantitative and presented as a fold change compared with the contralateral tissue, which was given an arbitrary value of 1.0. All experiments were performed at least twice and a representative example is shown. If a protein was not detected in the control, the weakest band on the gel, visible by eye, was assigned a value of 1.0. Specificity of the antibodies was confirmed by measuring expression in human tissue cell lysates (HeLa, T98G and Jurkat cells) and bands of the expected mass (HYAL1, 57 kDa; HYAL2, 60 kDa; HAS1, 75 kDa; HAS2, 65 kDa; RHAMM, 55 kDa; CD44, 80 kDa; and TSG-6, 36 kDa) were observed.

**Affinity-histochemical staining of HA using biotinylated HABP**

Sections were deparaffinized in xylene, rehydrated, washed in PBS, pH 7.4, and blocked in 1% H₂O₂ for 5 min. Sections were incubated
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with 1% BSA for 30 min and then overnight with biotinylated HABP (3 μg/cm² in 1% BSA in PBS; a gift from the Seikagaku Corporation, Tokyo, Japan). Sections were washed in PBS, treated with avidin–biotin peroxidase complex (ABC, CA, USA; 1:200 dilution, 1 h at RT) and then incubated with 3,3-diaminobenzidine (DAB, Sigma, Poole, UK; 0.5% w/v) containing 0.03% H₂O₂ for 5 min. Slides were counterstained with haematoxylin and then dehydrated and mounted in DPX. Positive staining was indicated by the presence of a characteristic brown end-product at the site of the target antigen. Controls pre-treated with Streptomyces hyaluronidase (100 turbidity reducing units/cm³ in 50 mM sodium acetate, pH 5.0, for 3 h) were prepared in parallel.

### Immunohistochemistry

Sections were deparaffinized and rehydrated as above. Sections were boiled for 1 min in concentrated citric acid, pH 6.0, to unmask the antigens. Slides were then incubated in 0.5% H₂O₂ in methanol for 10 min followed by addition of the primary antibodies HAS1/2, HYAL1/2 and RHAMM (1:50) or CD44 (1:25) in Tris–HCl, pH 7.2, for 4 h. Sections were then incubated with streptavidin-peroxidase-labelled polymer for 30 min and the colour developed by exposure to Vector DAB solution for 3–20 min. For double labelling, sections were subsequently stained with rabbit polyclonal antibodies to β-tubulin (1:50; Autogen Bioclear, Wiltshire, UK) or glial fibrillary acidic protein (GFAP; 1:50; Sigma Chemicals, Dorset, UK), recognizing neurons and glia, respectively, and developed with Vector blue. Tissue was counterstained with haematoxylin (except in double-labelled sections). Slides were dehydrated and mounted in DPX. Positive staining was indicated by the presence of a characteristic brown end-product at the site of the target antigen. Control slides in which the primary antibody was replaced with PBS were run in parallel (data not included).

### Statistical analysis of serum HA/HYAL

Statistical analysis was based on the assumption that the data were not normally distributed (Slevin et al., 2000) and was performed using non-parametric tests for paired groups (Wilcoxon) and unpaired groups (Mann–Whitney U-test). The analysis was two-tailed unless otherwise specified. Data are expressed as means ± standard deviation. Values were considered statistically different when P < 0.05. The relationships between o-HA, HA, other proteins and all measured blood parameters were determined by linear regression analysis (Spearman’s ρ). The SPSS statistical package was used for all analyses.

### Results

#### HA and o-HA concentrations are increased in the serum after stroke

The concentration of HA was significantly increased in serum at all time points after IS (P < 0.01 in all cases; Fig. 1A, i). The highest measured levels occurred 7 days after stroke (145.6 ± 97 ng/cm³). Mean HA level in the age-matched controls (n = 24) was 21.6 ± 19.1 ng/cm³.

The concentration of o-HA was significantly increased in serum at all time points after IS (P < 0.01 in all cases; Fig. 1B, i). Highest levels were 7 days after stroke (54.0 ± 47.6 ng/cm³). The mean o-HA concentration in the control group was 7.4 ± 4.6 ng/cm³.

#### HYAL activity is increased in the serum after stroke

Total serum HYAL activity was significantly increased in IS patients at all time points (P < 0.01 in all cases), reaching a maximum of 4.3 ± 2.5 μM/cm³/min 3 days after stroke (P < 0.05 in all cases; Fig. 1C, i). The mean activity in the control group was 1.8 ± 1.0 μM/cm³/min. HYAL activity was still elevated compared with controls, after 14 days.

#### Correlation studies

There was no relationship between patient HA or o-HA serum levels and blood parameters (markers of inflammation), including peripheral leucocyte number (Fig. 1; A–C, ii) and fibrinogen concentration (Fig. 1A–C, iii). HYAL activity correlated with leucocyte number after 3 days (Fig. 1C, iii; P = 0.011). The expression of HA also correlated with FGF-2 expression in these patients (Fig. 1D; P = 0.014). There was no significant difference in hyaluronidase activity between patients with large or small vessel disease or in association with clinical recovery based on SSS measurement.

#### HA and o-HA are increased in stroke-affected tissue

The production of HA and o-HA was investigated in stroke-affected tissue samples in three groups containing three samples each: (i) Serum levels of HA in patients 1–14 days after IS. Data are expressed as mean ± standard deviation. *P < 0.01 compared with the control. (ii) Serum levels of o-HA in patients 1–14 days after IS. Data are expressed as mean ± standard deviation. +P < 0.01 compared with the control. (iii) Circulating fibrinogen (data show analysis 3 days after stroke; Spearman’s P > 0.05). (B) Serum levels of o-HA in patients 1–14 days after IS. Data are expressed as mean ± standard deviation. *P < 0.01 compared with the control. (ii) Positive correlation with peripheral leucocyte concentration and (iii) circulating fibrinogen (data show analysis 3 days after stroke; Spearman’s P > 0.05). (C) Serum levels of HYAL in patients 1–14 days after IS. Data are expressed as mean ± standard deviation. +P < 0.01 compared with the control. (ii) Positive correlation with peripheral leucocyte concentration and (iii) circulating fibrinogen (data show analysis 3 days after stroke; Spearman’s P > 0.05). (D) Positive correlation of FGF-2 expression with o-HA in the serum of patients 3 days after IS (P = 0.014; Spearman’s ρ). (E) Tissue levels of HA in patients at different times after IS. Data are expressed as mean ± standard deviation. *P < 0.01 compared with the control. (F) Tissue levels of o-HA in patients at different times after IS. Data are expressed as mean ± standard deviation. +P < 0.01 compared with the control.
which approximately correspond to the periods of inflammation, angiogenesis and revascularization and tissue remodelling after stroke. Two to six days after stroke, the mean HA concentration was 17.6 ± 2.0 μg/g in the contralateral tissue, 50.0 ± 3.8 μg/g in the infarcted core and 45.6 ± 1.7 μg/g in the peri-infarcted area. HA levels remained increased in the 9–16 days survival group and reached a maximum of 65.3 ± 1.8 μg/g in stroke tissue and 55.1 ± 4 μg/g in peri-infarcted tissue, compared with 16.0 ± 1.4 in the control, in the longest surviving group (Fig. 1D). At all time points there was a significant increase in HA deposition in stroke and peri-infarcted tissue (P < 0.05 in all cases).

There was a significant elevation of α-HA (P < 0.05 in all cases) in tissue from the same time points, with the maximum values occurring after 26–37 days. The values were 52.6 ± 2 μg/g in stroke tissue, 40.6 ± 2.5 μg/g in peri-infarcted tissue and 8.1 ± 1.4 μg/g in the contralateral tissue (Fig. 1E).

**HA is increased in peri-infarcted neurons and blood vessels after ischaemic stroke**

The localization of HA in affected brain tissue from selected patients listed in Table 2 was investigated. Patchy staining for HA was evident in grey matter from the contralateral tissue and was more pronounced around blood vessels (Fig. 2A, Patient 20). Increased staining was seen in infarcted and peri-infarcted grey matter 3 days after stroke, and was particularly noticeable in the microvessels in this region (Fig. 2B, Patient 20). Strong staining of microvessels together with nuclear staining of neurons near to the infarcted core was also observed in peri-infarcted regions, in a patient who survived for 5 days after stroke (Fig. 2C and D; Patients 13 and 20, respectively). Increased staining persisted in the grey matter up to 37 days after stroke. Staining was abolished following pre-treatment of a control section by hyaluronidase (Fig. 2E, Patient 13).

**HYAL expression is increased in the inflammatory phase of stroke**

RT–PCR and western blotting showed upregulation of HYAL1 gene and protein expression, respectively, in the majority of patients in both peri-infarcted and infarcted regions (Fig. 3A and B). Western blotting demonstrated increased expression of HYAL1 protein in infarcted grey matter in all nine patients examined compared with the contralateral control tissue (range: 2.4- to 10.0-fold). The increase was more modest in peri-infarcted tissue (4 out of 9 patients; range: 2.0- to 7.5-fold). In white matter, there was an increase in 6 out of 9 samples in peri-infarcted tissue (range: 1.8- to 10.2-fold) and in all samples from the stroke tissue (range: 1.5- to 4.2-fold). All the changes are reported in Table 3. In HYAL1-stained western blots, a weak band was sometimes observed at around 50 kDa. This may represent a proteolytic cleavage fragment, which could help release it from its GPI anchor (Stern, 2003), or most likely, could be the result of a slight amount of tissue degeneration in our post-mortem tissue samples, since it was expressed in equal proportions in each sample, and our unpublished data demonstrated staining of only one band in fresh rat MCAO tissue (data submitted elsewhere). Immunohistochemistry showed that HYAL1 was expressed intracellularly, although only weakly, in normal-looking neurons in all layers in the contralateral tissue samples. There was weak but consistent staining in non-neuronal cells, possibly perineuronal oligodendrocytes (Fig. 3C, i; Patient 20). Astrocytes showed an absence of detectable labelling (Fig. 3C, ii). There was no observable staining in the white matter. In peri-infarcted grey matter HYAL1 staining was increased both intracellularly and in the axons of abnormal-looking neurons 3–6 days after stroke (Fig. 3D and E) as well as in microglia and mononuclear inflammatory cells (Fig. 3F, Patient 20). At later time points (10–37 days after stroke) HYAL1 expression was still observed in inflammatory peri-infarcted and infarcted regions.

### Table 2 Clinical details of patients with IS

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<thead>
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<th>Patient*</th>
<th>Age</th>
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<th>Survival time (days)</th>
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<th>Hypercholesterolaemia†</th>
<th>Stroke type (Oxfordshire classification)</th>
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<td>37</td>
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<td>PACI</td>
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</table>

*Prior to first stroke, patients were not on anti-platelet or statin treatment; †serum cholesterol concentration > 5.5 mmm/l; PACI, partial anterior circulation infarct; TACI, total anterior circulation infarct; LACI, lacunar infarct; §according to the hospital death certificate and clinical history, the main cause of death was extensive stroke. The intermediate causes of death are listed in the Table; ¶Patient 8 was used only in the HA tissue analysis study.

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**HYAL expression is increased in the inflammatory phase of stroke**

RT–PCR and western blotting showed upregulation of HYAL1 gene and protein expression, respectively, in the majority of patients in both peri-infarcted and infarcted regions (Fig. 3A and B). Western blotting demonstrated increased expression of HYAL1 protein in infarcted grey matter in all nine patients examined compared with the contralateral control tissue (range: 2.4- to 10.0-fold). The increase was more modest in peri-infarcted tissue (4 out of 9 patients; range: 2.0- to 7.5-fold). In white matter, there was an increase in 6 out of 9 samples in peri-infarcted tissue (range: 1.8- to 10.2-fold) and in all samples from the stroke tissue (range: 1.5- to 4.2-fold). All the changes are reported in Table 3. In HYAL1-stained western blots, a weak band was sometimes observed at around 50 kDa. This may represent a proteolytic cleavage fragment, which could help release it from its GPI anchor (Stern, 2003), or most likely, could be the result of a slight amount of tissue degeneration in our post-mortem tissue samples, since it was expressed in equal proportions in each sample, and our unpublished data demonstrated staining of only one band in fresh rat MCAO tissue (data submitted elsewhere). Immunohistochemistry showed that HYAL1 was expressed intracellularly, although only weakly, in normal-looking neurons in all layers in the contralateral tissue samples. There was weak but consistent staining in non-neuronal cells, possibly perineuronal oligodendrocytes (Fig. 3C, i; Patient 20). Astrocytes showed an absence of detectable labelling (Fig. 3C, ii). There was no observable staining in the white matter. In peri-infarcted grey matter HYAL1 staining was increased both intracellularly and in the axons of abnormal-looking neurons 3–6 days after stroke (Fig. 3D and E) as well as in microglia and mononuclear inflammatory cells (Fig. 3F, Patient 20). At later time points (10–37 days after stroke) HYAL1 expression was still observed in inflammatory peri-infarcted and infarcted regions.
Fig. 2 Distribution of HA in the stroked brain. (A) Normal grey matter (Patient 20) showing weak staining for HA around blood vessels (×100; insert: ×200, arrows; (i) blood vessel; (ii) neurons). (B) The boundary zone between infarcted and peri-infarcted grey matter (Patient 20) showing more intense staining in the peri-infarcted region and in infarcted blood vessels (×40; insert: ×200, arrows). (C) Infarcted grey matter showing strong staining around blood vessels in Patient 13 (×100; insert: ×200, arrows). (D) Infarcted grey matter from Patient 20 showing increased nuclear and intracellular staining of neurons (×100; insert: ×200, arrows). (E) Staining was abolished following pre-treatment with hyaluronidase (Patient 13). Staining was carried out with biotinylated-HA and sections were developed using avidin–biotin peroxidase.
HYAL1 expression after IS. (A) RT–PCR showing increased gene expression of HYAL1 in peri-infarcted and infarcted samples. Example shows the grey matter from Patient 20 who survived for 3 days after stroke. Lane 1, contralateral hemisphere; Lane 2, peri-infarcted region; Lane 3, infarcted region; Lane 4, positive control; Lane 5, external control; and Lane 6, negative control (−cDNA). (B) Western blots of HYAL1 expression in Patients 20 and 23. (i) Antibody specificity was demonstrated using HeLa cell lysate. (ii) Increased expression was seen in peri-infarcted and infarcted regions. Lanes 1–3 are grey matter and Lanes 4–6 are white matter; C, control; PI, peri-infarcted; and I, infarcted region. α-Actin loading gels are also shown. Each experiment was carried out at least twice and a representative example is shown. (iii) Bar charts show relative protein expression compared with the control, given an arbitrary value of 1.0. (C–F) Immunohistochemical analysis of HYAL1 distribution in stroke tissue. (C) Normal-looking grey matter showing weak intracellular staining in neurons (i, arrow) and possibly oligodendrocytes (i, broken arrow), but an absence of detectable labelling of astrocytes in double-labelled sections (ii, arrow; DAB for GFAP and Vector blue for HYAL1; broken arrow shows a weakly labelled neuron (×100; inserts: ×200). (D–E) Strong intracellular and axonal staining of abnormal-looking neurons from the peri-infarcted region of Patient 20 (×100; insert: ×200). (F) General positive staining of infiltrating inflammatory cells and microglia in peri-infarcted grey matter tissue from Patient 24 (×100; insert: ×200).
HYAL2 was increased in the majority of patients mainly in grey matter infarcted regions (2 out of 9 in peri-infarcted tissue, 2.5- to 8.5-fold; 5 out of 9 in stroke tissue, 1.6- to 9.4-fold). Representative RT–PCR results and western blots are shown (Fig. 4A and B). Weak intracellular HYAL2 staining was observed mainly in neurons in normal-looking grey matter (Fig. 4C), but extensive intracellular and nuclear neuronal staining as well as an increase in positively stained mononuclear inflammatory cells was seen in both peri-infarcted and infarcted tissue in patients 3–10 days after stroke (Fig. 4D and E). In infarcted grey matter, staining for HYAL2 was also associated with microvessels after 3 days, and this pattern of staining persisted in patients surviving for 25 days (Fig. 4E).

HAS expression is increased in neurons and inflammatory cells after stroke

HAS1 was upregulated in 3 out of 9 samples (range: 1.5- to 10.8-fold) in grey matter peri-infarcted tissue and 3 out of 9 samples in grey matter stroke tissue (range: 1.5- to 9.4-fold). HAS1 staining was observed mainly in neurons in normal-looking grey matter (Fig. 4C), but extensive intracellular and nuclear neuronal staining as well as an increase in positively stained mononuclear inflammatory cells was seen in both peri-infarcted and infarcted tissue in patients 3–10 days after stroke (Fig. 4D and E). In infarcted grey matter, staining for HAS1 was also associated with microvessels after 3 days, and this pattern of staining persisted in patients surviving for 25 days (Fig. 4E).

HAS2 was upregulated to a greater extent in grey matter (5 out of 9 in infarcted tissue, range: 1.6- to 9.5-fold; 6 out of 9 in peri-infarcted tissue, range: 1.8- to 7.1-fold) than white matter (3 out of 9 in stroke tissue, range: 2.0- to 5.0-fold, and 3 out of 9 in peri-infarcted tissue, range: 1.7- to 6.0-fold). In infarcted grey matter, staining for HAS2 was increased in neurons, where it was often translocated to the nucleus (Fig. 4D and E), and in mononuclear inflammatory cells and cells with the appearance of oligodendroglia in patients who survived from 3 to 29 days after stroke (Fig. 4F, i and ii).

RHAMM expression was upregulated in neurons and blood vessels after stroke

We were unable to detect the presence of RHAMM gene expression with the primers listed. RHAMM protein was

### Table 3 Relative protein expression in white matter compared with contralateral tissue (given an arbitrary value of 1.0)

<table>
<thead>
<tr>
<th>S*</th>
<th>HAS1</th>
<th>HAS2</th>
<th>HYAL1</th>
<th>HYAL2</th>
<th>RHAMM</th>
<th>CD44</th>
<th>TSG-6</th>
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<td>10.8</td>
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<td>12.5</td>
<td>5.0</td>
</tr>
<tr>
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<td>1.0</td>
<td>1.0</td>
<td>0.4</td>
<td>3.0</td>
<td>0.5</td>
<td>12.5</td>
</tr>
<tr>
<td>14</td>
<td>6</td>
<td>1.5</td>
<td>1.3</td>
<td>25</td>
<td>0.4</td>
<td>0.4</td>
<td>2.0</td>
</tr>
<tr>
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<td>10</td>
<td>1.0</td>
<td>1.0</td>
<td>4.3</td>
<td>0.6</td>
<td>3.6</td>
<td>11.8</td>
</tr>
<tr>
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<td>1.0</td>
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<td>1.1</td>
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<td>1.5</td>
<td>1.0</td>
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<tr>
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<td>2.0</td>
<td>1.0</td>
<td>0.8</td>
<td>5.5</td>
<td>5.0</td>
</tr>
<tr>
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<td>1.8</td>
<td>11.5</td>
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<tr>
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<tr>
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<td>12.1</td>
<td>0.0</td>
<td>1.2</td>
<td>0.7</td>
</tr>
</tbody>
</table>

**Note:**

*S* Survival time after stroke in days.
upregulated mainly in grey matter (7 out of 9 in peri-infarcted tissue, range: 1.5- to 8.2-fold; and 6 out of 9 in infarcted tissue, range: 3.0- to 5.6-fold). Representative western blots are shown (Fig. 7A). Weak intracellular staining of RHAMM was evident in neurons, and some non-neuronal cells, possibly oligodendrocytes, although astrocytes and blood vessels showed an absence of detectable labelling in normal grey matter.
In peri-infarcted grey matter, there was much stronger intracellular as well as nuclear staining of neurons, and more prominent staining of non-neuronal cells (Fig. 7C and D, Patient 13), whilst both the EC and tunica media of blood vessels were strongly stained in peri-infarcted and infarcted regions in patients surviving from 3 to 29 days after stroke (Fig. 7E and F, respectively, Patient 21; arrows).

(Fig. 7B, Patient 20). In peri-infarcted grey matter, there was much stronger intracellular as well as nuclear staining of neurons, and more prominent staining of non-neuronal cells (Fig. 7C and D, Patient 13), whilst both the EC and tunica media of blood vessels were strongly stained in peri-infarcted and infarcted regions in patients surviving from 3 to 29 days after stroke (Fig. 7E and F, respectively, Patient 21; arrows).
**Fig. 6** HAS2 expression in stroke tissue. (A) RT-PCR showing increased gene expression of HAS2 in peri-infarcted and infarcted samples. Example shows the grey matter from Patient 12. Lane 1, contralateral hemisphere; Lane 2, peri-infarcted region; Lane 3, infarcted region; Lane 4, positive control; Lane 5, external control; and Lane 6, negative control (-cDNA). (B) Western blots of HAS2 expression in Patients 12 and 20. (i) Antibody specificity was demonstrated using T98G cell lysate. (ii) Increased expression was seen in peri-infarcted and infarcted regions. Lanes 1–3 are grey matter and Lanes 4–6 are white matter; C, control; PI, peri-infarcted; and I, infarcted region. α-Actin loading gels are also shown. Each experiment was carried out at least twice and a representative example is shown. (iii) Bar charts show relative protein expression compared with the control, given an arbitrary value of 1.0. (C–F) Immunohistochemical analysis of HAS2 distribution in stroke tissue. (C) Normal-looking grey matter showing negative staining in neurons (×100; insert: ×200, arrows). (D and E) Strong staining of neuronal nuclei from the peri-infarcted regions of damaged tissue (Patient 20 and 24, respectively; ×100; insert: ×200). (F (i and ii)] Shows HAS2 positive (brown) peri-infarcted neurons identified by co-labelling with β-tubulin (Vector blue; ×200).
Fig. 7 RHAMM expression in stroke tissue. (A) Western blots of RHAMM expression in Patients 22 and 24. (i) Antibody specificity was demonstrated using Jurkat cell lysate. (ii) Increased expression was seen in peri-infarcted and infarcted regions. Lanes 1–3 are grey matter and Lanes 4–6 are white matter; C, control; PI, peri-infarcted; and I, infarcted region. α-Actin loading gels are also shown. Each experiment was carried out at least twice and a representative example is shown. (iii) Bar charts show relative protein expression compared with the control, given an arbitrary value of 1.0. (B–F) Immunohistochemical analysis of RHAMM distribution in stroke tissue. (B) Normal-looking grey matter showing weak intracellular staining in neurons (insert i, arrow) and possibly oligodendroglia (insert i, broken arrow). There was an absence of detectable labelling in astrocytes. Insert (ii) shows a GFAP positive (brown) astrocyte with no detectable labelling of RHAMM (arrow) and a weakly positive neuron (Vector blue; broken arrow) (×100; inserts: ×200, arrows). (C) Strong intracellular staining of peri-infarcted neurons from Patient 20 and (D) in the nucleus (×100; insert: ×200, arrows). (E) Increased RHAMM expression in peri-infarcted microvessels (×100; insert: ×200), and (F) in the tunica media of larger blood vessels from stroke-affected regions (×100) from Patient 24.
Hyaluronan production following stroke

CD44 was upregulated in stroke tissue
CD44 was upregulated in the majority of samples from infarcted tissue (6 out of 9 in both grey and white matter, range: 3.5- to 10.0-fold and 2.5- to 8.0-fold, respectively) but only a minority of samples from peri-infarcted regions (3 out of 9 from both grey and white matter, range: 5- to 8.1-fold and 2- and 10-fold, respectively). Representative RT–PCR and western blots are shown in Fig. 8A and B. CD44 staining was not evident in normal contralateral white matter (data not shown), and sporadic weak CD44 staining of neurons occurred in normal grey matter (Fig. 8C). Strong surface staining for CD44 was evident in dead or dying neurons from peri-infarcted/infarcted tissue as well as in inflammatory mononuclear cells 3–17 days after stroke (Fig. 8D and E; arrows).

TSG-6 expression in stroke tissue
Expression of TSG-6 was increased in the majority of stroke-affected tissue. In white matter, 7 out of 9 samples were increased in both peri-infarcted and infarcted tissue (range: 1.6- to 8.8-fold and 1.5- to 4.8-fold, respectively). In grey matter, TSG-6 increased in 6 out of 9 samples in peri-infarcted tissue (range: 1.5- to 8.0-fold) and 5 out of 9 in infarcted tissue (range: 2.4- to 9.0-fold). Representative RT–PCR and western blots are shown for TSG-6 (Fig. 9A and B). There was little TSG-6 staining in normal-looking grey or white matter (Fig. 9C); however, increased TSG-6 staining was associated with inflammatory mononuclear cells and damaged neurons from infarcted regions in patients surviving from 3 to 29 days after stroke (Fig. 9D; arrows).

Discussion
Using a HA-specific biotinylated probe we have demonstrated increased staining for HA in stroke-affected tissue of human brain. HA accumulation in tissue and serum was confirmed using biochemical assays up to 37 days after stroke. Although HA has been demonstrated to form coats around neurons, especially in the cerebral cortex and hypothalamus in normal human and rat brain (Yasahara et al., 1994), the pathological brain has not been studied in detail. The HA concentration in cerebral tissues decreases from the foetus to the adult. In the rat embryo, HA forms an extracellular component of the migration and proliferation areas of the cerebral cortex, suggesting an important role in the modelling process (Delpech et al., 1989). The concentration of HA was increased in the CSF of patients with head injury and cerebral infarction, concomitant with accumulation in the superficial layer of the cerebral cortex (Laurent et al., 1996). HA was consistently increased in tissue and serum over the period of our study, suggesting continuous HA synthesis.

Rapid increases in circulating levels of HA have been demonstrated in patients with burns, septicemia and shock, and are also increased in those with rheumatoid arthritis and vasculitis (Mio et al., 2000). The increase in HA serum production may have been at least partly as a result of the systemic inflammatory effects of the acute phase response; however, our evidence for increased expression in the damaged brain tissue, together with a lack of correlation with markers of inflammation including leucocyte concentration and fibrinogen, and a strong correlation with increased FGF-2 expression, which was also increased in brain tissue (Issa et al., 2005), suggests a relationship to ECM changes in the brain.

The synthesis of HA by stromal cells usually accompanies the re-establishment of an ECM during wound repair followed at a later stage by cell migration and cell division (Rauch, 2004). It has been suggested that HAS1 maintains a basal level of HA synthesis, whereas HAS2 stimulates morphogenesis and cell migration and invasion during embryonic development (Adamia et al., 2005). In this study, immunohistochemistry demonstrated increased HAS1 and HAS2 staining in inflammatory cells from peri-infarcted regions. Of particular interest was the nuclear staining of peri-infarcted neurons by HAS2, in combination with HA nuclear staining. HA is normally synthesized by HASs on the inner surface of the plasma membrane and is extruded across the cell membrane into the ECM (Itano and Kimata, 2002). In vitro data have suggested that HAS1 and HAS2 produce high molecular weight HA (RMM $3.9 \times 10^6$ Da), and HAS3 a lower molecular weight form (RMM $0.12-1 \times 10^8$ Da) (Jonas and Paraskevi, 1999). Although antibodies to HAS3 are not available, analysis of gene expression by RT–PCR showed no changes in stroke-affected areas (data not included).

To our knowledge, no previous studies have identified HAS expression in cell nuclei; however, HA has been observed in rat neuronal nuclei and nucleoli as well as in other cells (Ripollino et al., 1988), and appears to be involved in chromatin condensation and cell mitosis (Hascall et al., 2004). Our results suggest a preferential synthesis of high molecular weight HA in stroke tissue, perhaps in an attempt to re-establish the ECM that accompanies tissue remodelling after IS. HA from the matrix may be taken up by receptor-mediated mechanisms in neurons or may represent newly synthesized material (Stern, 2003). Increased HA expression enhanced peripheral nerve regeneration and axon myelination in sciatic nerve-transsected rats, suggesting a protective role after stroke (Wang et al., 1998). Recently, the structural and cytochemical characteristics as well as the molecular composition of ECM perineuronal nets, rich in proteoglycans including HA, and surrounding the soma and proximal parts of dendrites have been described (Bruckner et al., 2006). These may have the ability to stabilize the neuronal micro-environment maintaining synaptic plasticity and the generation of action potentials. Net-bearing neurons in the cerebellar cortex of rats expressed HAS, which might be important in retaining HA on the cell surface (Carulli et al., 2006). Therefore, reduction in HA within these perineuronal nets after stroke...
Fig. 8 CD44 expression after IS. (A) RT–PCR showing increased gene expression of CD44 in peri-infarcted and infarcted samples. Example shows the grey matter from Patient 20 who survived for 3 days after stroke. Lane 1, contralateral hemisphere; Lane 2, peri-infarcted region; Lane 3, infarcted region; Lane 4, positive control; Lane 5, external control; and Lane 6, negative control (-cDNA). (B) Western blots of CD44 expression in Patients 20 and 22. (i) Antibody specificity was demonstrated using Jurkat cell lysate. (ii) Increased expression was seen in peri-infarcted and infarcted regions. Lanes 1–3 are grey matter and Lanes 4–6 are white matter; C, control; PI, peri-infarcted; and I, infarcted region. α-Actin loading gels are also shown. Each experiment was carried out at least twice and a representative example is shown. (iii) Bar charts show relative protein expression compared with the control, given an arbitrary value of 1.0. (C–E) Immunohistochemical analysis of CD44 distribution in stroke tissue. (C) Normal-looking grey matter showing weak surface staining of neurons (insert, arrow) and some blood vessels (×100; insert: ×200). Staining of inflammatory mononuclear cells (D) and dead or dying neurons (E) in peri-infarcted regions from Patient 22 (×100; insert: ×200, arrows).
might reduce the electrical activity and stability of neurons in these regions.

HYALs are involved in tissue remodelling during embryonic development, promotion of tumour invasion and wound healing. We have demonstrated increased expression of HYAL in the serum of patients with ICH and IS. HYAL1 and 2 were also upregulated in stroke-affected tissue, suggesting that breakdown of high molecular weight HA is a feature of stroke pathophysiology. HYAL2, a GPI-anchored membrane protein, generates a 20-kDa HA fragment internalized by receptor-mediated endocytosis and delivered to lysosomes, where further degradation by HYAL1 to potentially angiogenic oligosaccharides of HA occurs (Slevin et al., 2002; Stern, 2003). HYAL1 is a universally distributed lysosomal enzyme, but although HYAL2 is also present in many tissues it is not normally found in the adult brain where the gene is inactivated by hypermethylation (Lepperdinger et al., 2001). Mouse astrocytoma cells over-expressing HYAL2 become invasive and form highly vascularized tumours (Novak et al., 1999). Our results suggest that the HYAL2 gene may be re-activated after stroke.

HYAL1 was expressed by non-neuronal cells, possibly oligodendrocytes in normal-looking grey matter tissue. Degradation of HA following spinal cord injury in rats

Fig. 9  TSG-6 expression after IS. (A) RT–PCR showing increased gene expression of TSG-6 in peri-infarcted and infarcted samples. Example shows the grey matter from Patient 24. Lane 1, contralateral hemisphere; Lane 2, peri-infarcted region; Lane 3, infarcted region; Lane 4, positive control; Lane 5, external control; and Lane 6, negative control (-cDNA). (B) Western blots of TSG-6 expression in Patients 13 and 24. (i) Antibody specificity was demonstrated using Jurkat cell lysate. (ii) Increased expression was seen in peri-infarcted and infarcted regions. Lanes 1–3 are grey matter and Lanes 4–6 are white matter; C, control; PI, peri-infarcted; and I, infarcted region. α-Actin loading gels are also shown. Each experiment was carried out at least twice and a representative example is shown. (iii) Bar charts show relative protein expression compared with the control, given an arbitrary value of 1.0. (C and D) Immunohistochemical analysis of TSG-6 distribution in stroke tissue. (C) Normal-looking grey matter with no TSG-6 detectable labelling of neurons (×100; insert: ×200, arrow). (D) Increased staining of inflammatory mononuclear cells and abnormal-looking neurons (arrow) in infarcted regions from Patient 13 (×100; insert: ×200, arrow).
increased astrocyte proliferation, and addition of HYAL to quiescent astrocyte cultures induced proliferation, suggesting potential involvement in gliosis (Struve et al., 2005). Oligodendrocytes could be a potential source of HYAL secretion after stroke. Increased expression of HYAL1 and 2 was observed in infiltrating mononuclear inflammatory cells, whilst HYAL1 was also seen intracellularly in peri-infarcted neurons, perhaps indicating the possibility of intracellular signalling pathway activation via o-HA in ischaemic cells.

HA signalling through RHAMM and CD44 receptors mediates EC and neuronal migration and angiogenesis (Savani et al., 2001; Slevin et al., 2002). RHAMM is expressed by normal neurons, where it regulates neurite migration and axon growth, and also by oligodendrocytes and astrocytes, where it affects motility and proliferation (Nagy et al., 1995; Lynne et al., 2001). Binding of o-HA to RHAMM in human EC cell lines in vitro activated MAP kinase (ERK1/2) and initiated mitogenesis (Savani et al., 2001). We were unable to demonstrate RHAMM gene expression in any of our post-mortem samples using several different primer designs. Other tissues and positive controls showed RHAMM gene product using the same primers; therefore, we presume that the gene product must have been substantially degraded in our tissues. The general measurement of gene expression in post-mortem samples has been demonstrated to be representative and proportional to that of fresh samples (Mitsios et al., 2006 and references therein), and so this result was surprising. RHAMM protein was expressed by some non-neuronal cells, possibly oligodendrocytes in normal-looking grey matter tissue. RHAMM expression was increased in peri-infarcted and infarcted tissue of stroke patients and was expressed both intracellularly and in the nuclei of peri-infarcted neurons, as well as in the intima and EC of microvessels. Increased RHAMM expression might enhance calmodulin-mediated signalling to cytoskeletal elements in neurons (Lynn et al., 2001), but has also been observed in the mitotic spindles of smooth muscle cell nuclei, suggesting a role in mitosis (Hascall et al., 2004). It could also be involved in stimulation of angiogenesis, improving collateral circulation in ischaemic regions after stroke.

CD44 is involved in multiple responses to inflammation including leucocyte recruitment, cell–matrix interactions and matrix remodelling (Pure and Cuff, 2001). CD44 expression is regulated by HA fragments, interleukin-1β and tumour necrosis factor-alpha (TNF-α), all of which are increased in stroke (Slevin et al., 2005). Previous studies have shown that CD44-deficient mice were protected against cerebral ischaemia injury, in association with reduced expression of interleukin-1-beta (Wang et al., 2002). In this study, CD44 was upregulated in stroke tissue, mainly associated with inflammatory cells. The concurrent induction of CD44 and HA in the ischaemic area may potentiate the inflammatory effects of hypoxia. We have shown increased neuronal expression in larger neurons, whilst increased CD44 has been previously demonstrated in growth factor-treated ischaemic rat cortical cultures, which might impact upon survival via modulation of growth factor-induced signalling pathways (Yoshida et al., 2004).

TSG-6 expression is also associated with inflammation and tissue remodelling (Milner et al., 2003; Mahoney et al., 2005), and modulates the interaction between CD44 and HA (Lesley et al., 2004). We have shown increased expression of TSG-6 in the majority of tissue samples and localized it mainly to inflammatory cells. Since tissue remodelling is a known feature of most sites of TSG-6 expression, it may have the same function in stroke tissue (Mahoney et al., 2005).

In summary, the results presented here show a significant change in the enzymes responsible for HA synthesis and degradation together with upregulation of HA receptors. This may reflect tissue remodelling after stroke and be partially responsible for increased angiogenesis and neuronal migration. One limitation of this study was that we were unable to determine the exact size of the HA molecules expressed in the infarcted regions of stroke tissue and in the serum. Antibodies recognizing HA of specific molecular weight are not available and other methods of isolation and analysis are not yet sensitive enough to detect such small quantities. Owing to the small numbers of patient tissue samples examined, it was not possible to carry out statistical analysis of protein concentration in relation to clinical parameters, and so these data are mostly qualitative rather than quantitative analysis. Within the nine samples studied, there was a notable variation in expression of investigated proteins, partly because the original dissected material, for example, peri-infarcted region, was subsequently shown to contain some areas of lesser damaged tissue at the perimeters, as well as infarcted zones closest to the point of stroke origin. However, the greater sensitivity and ability of IHC to cellular sub-type has allowed us to identify the particular zonal expression of our test proteins.

One study of recovery from cortical injury used HA- and laminin-impregnated gels to demonstrate HA scaffolding into which collateral blood vessels grew, resulting in inhibition of gial scarring, suggesting that its manipulation could be beneficial to the recovery after stroke (Hou et al., 2005). It will be important to identify the mechanisms through which this occurs and the effects on cell survival and growth. Further studies should attempt to identify potential therapeutic benefits of HA/receptor manipulation after stroke.

References
Hyaluronan production following stroke


