

# Determination of the role of HA, HYAL and HA-receptor expression in a rat model of Middle Cerebral Artery Occlusion

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## Abstract

Hyaluronan (HA), a component of the extracellular matrix, has been implicated in regulating angiogenesis and cell proliferation, migration, and signaling. We used the rat MCAO to show hyaluronan accumulation in stroke-affected areas. Using RT-PCR and Western blotting we showed up-regulation of hyaluronidase-1 and 2 between 1h and 21 days after stroke. Hyaluronidase-1 was up regulated earlier than hyaluronidase-2. RHAMM and CD44 receptors were also increased after stroke. Immunohistochemistry results, showed an association of hyaluronidases1/2 and hyaladherins with neurons in the infarcted and peri-infarcted regions and hyaluronidase-1 with microvessels. HA synthesis and degradation in the stroke hemisphere might have an impact on neuronal survival, angiogenesis and general tissue remodeling after stroke.

Keywords: MCAO; rat stroke model; hyaladherins; hyaluronidase1 and 2; RHAMM, CD44

## Introduction

Stroke is the third leading cause of death and is a major source of chronic disability (1-3). Recent evidence suggests that angiogenesis in the penumbra region is correlated with patient survival and protection from the later consequences of stroke such as dementia (4-6). Angiogenesis involves growth of new endothelial cells (EC) and is initiated by angiogenic molecules produced by stroke and penumbra tissue. One of these molecules, hyaluronan (HA) has differing roles in angiogenesis depending on its size. High molecular weight HA (n-HA) can promote wound healing in damaged tissue whereas oligosaccharides of specific size (3-10 disaccharides; o-HA) stimulate EC proliferation and migration (7-9), and operates by stimulation of a

receptor-mediated process (10,11). CD44 binds both n-hyaluronan and o-hyaluronan, leading to the organization and turnover of the extracellular matrix at the cell surface. The receptor for hyaluronan-mediated motility (RHAMM) is found in intracellular compartments and in the cell membrane and on binding hyaluronan initiates cellular signaling resulting in motility [12,13].

Hyaluronan turnover may increase in stroke tissue as a result of tissue remodeling and, therefore, we have investigated the expression of hyaluronan, hyaluronan metabolizing enzymes and hyaladherins in a rat middle cerebral artery occlusion (MCAO) model of stroke.

## **Material and methods**

### **Animals and anaesthesia**

Stroke was induced in non-pregnant female Sprague–Dawley rats weighing 230–270 g that suffer less during ischaemia/traumatic insult owing to higher endogenous steroid levels reducing free radical damage [14]. The animals were housed at 21°C with ad libitum access to food and water. Animal welfare was conducted according to regulations of the Real Decreto 223:1988 in accordance with the National Institutes of Health, Public Health Service Policy of the Care and Handling of Laboratory Animals and the experimental protocol approved by the Ethical Committee of the Hospital Universitari de Bellvitge, Barcelona.

### **Middle cerebral artery occlusion**

Cerebral ischaemia was induced by distal, permanent occlusion of the middle cerebral artery by electrocautery [15]. Sets of six animals (three each for morphological and biochemical studies) were killed at 1, 4, 12 and 24 h, and 3, 4, 7 and 21 days, and at 1 and 2 months for immunohistochemistry. These times were chosen to reflect the major pathophysiological events, inflammation, angiogenesis and wound recovery occurring after stroke. Animals were guillotined and brain samples snap frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$ . Infarcted and peri-infarcted tissue from the grey and white matter, and control areas in the contralateral hemisphere, were removed. The peri-infarcted area was identified as in our previous report [16].

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

Total cellular RNA was extracted and first strand cDNA generated from animals at the above time points using a standard kit (Promega, Southampton, UK). Sequences for polymerase chain reaction primers for hyaluronidase-1, forward (F) 5'-CCT CTG GGG CTT CTA CCT CT and reverse (R) 5'-CCA CAG GAC TTG CTC TAG-3'; hyaluronidase-2, F 5'-CCT CTG GGG CTT CTA CCT CT-3' and R 5'-CTG AAC ACG GAA GCT CAC AA-3'; CD44, F 5'-GTG GGC CAA CAA AGA ACA CT-3' and R 5'-TGG GAG CAG GCC CAA ATT A-3'; receptor for hyaluronan-mediated motility, F 5'-GAA AGG GAA GAA GGG TGA AC-3' and R 5'-TGC CAA AAT CTG ATG CTG AA-3'; S16, F 5'-GAT GCA AGA AGA CAG CCA CA-3' and R 5'-GGA CAG CCG GAT AGC ATA AA-3'. cDNA samples were amplified (35

cycles) under standard conditions [17]. All polymerase chain reaction experiments were repeated at least twice and representative results shown. The S16 housekeeping gene was used as a control to show equality of amplification.

### **Extraction of protein from tissue samples and Western blotting**

The method is described in detail elsewhere [11]. After separation, proteins were stained with donated antibodies to hyaluronidase-1 (mouse monoclonal [18]), hyaluronidase- 2 (rabbit polyclonal [19]), receptor for hyaluronan-mediated motility (rabbit polyclonal [20]) (the accompanying reference describes the characterization of the antibody and the donor) and CD44 (mouse monoclonal, Santa Cruz Biotechnologies, Santa Cruz, California, USA). Antibodies were used at a dilution of 1:1000 except anti-CD44 (1:500). Alphaactin control (Calbiochem, San Diego, California, USA) was run with each set of samples.

After staining with peroxidase-conjugated secondary antibody (anti-rabbit or anti-mouse, 1:1000) for 1 h at room temperature, blots were developed using the ECL detection system (Amersham, Aylesbury, UK). The intensity of staining relative to a-actin was measured by densitometry. Results are semi-quantitative and given as a numerical change compared with the control (1/41.0). Experiments were performed at least twice and a representative example is shown. The specificity of antibodies was confirmed using human positive control Jurkat cell lysate and bands of the expected mass (hyaluronidase-1 57 kDa, hyaluronidase-2 60 kDa, receptor for hyaluronan-mediated motility 55 kDa and CD44 80 kDa) were seen.

### **Affinity: histochemical localization of hyaluronan**

Sections were incubated overnight with hyaluronan-specific biotinylated probe (a gift of the Seikagaku Corporation, Chuo-ku, Tokyo, Japan) as described elsewhere [9]. Specificity of staining was tested by digesting control sections with *Streptomyces hyaluronidase* in the presence of protease inhibitors.

### **Immunohistochemical localization of proteins**

The avidin–biotin–peroxidase method was used for immunohistochemical localization of hyaluronidase 1/2, receptor for hyaluronan-mediated motility and CD44 as described elsewhere [17]. Specificity of the immunoreaction was tested by replacement with pre-immune serum.

## **Results**

### **Expression of hyaluronidase**

Diffuse staining of hyaluronan was seen in the extracellular matrix. Increased staining was seen in infarcted and peri-infarcted tissues especially at the boundary zone of the stroke (Fig. 1a and b). Increased hyaluronan was visible 48 h after stroke and persisted for 21 days. Control sections pre-digested with hyaluronidase were negatively stained (data not shown).

### **Expression of hyaluronidase-1**

Hyaluronidase-1 mRNA was up-regulated 1 h after stroke and remained increased after 21 days (range of increase 15- to 18-fold) in infarcted and peri-infarcted areas. Hyaluronidase-1 protein expression was increased between 24 h and 7 days in both grey and white matter. Expression was greater in infarcted tissue at earlier time points and in peri-infarcted tissue at later time points (range 2- to 4-fold).

Little staining was noted for hyaluronidase-1 in normal tissue but increased nuclear staining at early time points after stroke in micro-vessels in infarcted and peri-infarcted tissue (Fig. 1c). Staining persisted up to 21 days after stroke.

### **Expression of hyaluronidase-2**

Hyaluronidase-2 mRNA was up-regulated 24 h after stroke in peri-infarcted and infarcted tissue and reached a maximum (15-fold) at 3 days. It remained elevated in stroke tissue at 21 days but returned to control levels in the peri-infarcted area. Hyaluronidase-2 protein expression was increased between 24 h and 7 days in grey and white matter, and was greatest in peri-infarcted areas (range 1.5 to 5-fold). Increased intracellular staining of neurons distributed throughout the peri-infarcted area was seen up to 4 days after stroke (Fig. 1d).

### **Expression of receptor for hyaluronan-mediated motility**

mRNA was up-regulated in peri-infarcted and infarcted tissue 1 h after stroke and was still elevated after 21 days (approximately 20-fold). Receptor for hyaluronan-mediated motility protein expression was increased between 12 h and 7 days after stroke in both peri-infarcted and infarcted grey matter and between 1 h 7 days in white matter (range 1- to 8-fold). Weak cytoplasmic staining for receptor for hyaluronan-mediated motility in neurons in the corpus callosum was seen in control sections. Increased intracellular staining was seen in infarcted and peri-infarcted neurons (Fig. 1e).

### **Expression of CD44**

CD44 mRNA was up-regulated 1 h after stroke and remained increased 21 days later in both peri-infarcted and infarcted tissue (range 20-fold). CD44 protein was upregulated between 1 h and 7 days in both grey and white matter after stroke. Expression was greater in peri-infarcted tissue (range 2- to 6-fold). In control tissue there was weak staining for CD44 in neurons in grey matter. In periinfarcted tissue there was staining for CD44 in blood vessels and some microglia after 12 h and in neurons after 24 h in the V and VI cortical layers (Fig. 1f). After 48 h, staining was observed in dying neurons and all staining disappeared after 96 h. We also observed increased expression of hyaluronan synthase-1 (between 24 h and 21 days) and hyaluronan synthase-2 (between 3 and 21 days) and staining in inflammatory cells and in glia in peri-infarcted regions (data not shown).

## Discussion

Increased staining for hyaluronan throughout the infarcted area in the rat brain was seen, indicating up-regulated synthesis after stroke. In the rat embryo, hyaluronan directs migration and proliferation in the cerebral cortex, suggesting a role in the establishment of neuronal pathways. Hyaluronan was increased in the cerebrospinal fluid of patients with head injury and cerebral infarction together with accumulation in the superficial layer of the cerebral cortex [21]. Our results suggest increased synthesis of hyaluronan in infarcted tissue, perhaps due to the reestablishment of the extracellular matrix during tissue remodelling after ischaemic stroke. This study used female rats that undergo a cyclic variation in oestrogen levels known to affect glycosaminoglycan synthesis in the endometrium [22] but as we compared stroke and contralateral tissues from the same rats, this should not have had a bearing on our findings. No studies show such a hormonal effect in the brain.

Hyaluronidases are involved in tissue remodelling during embryonic development, tumour invasion and wound healing. Hyaluronidase-1 mRNA was up-regulated after 1 h and remained increased up to 21 days in stroke-affected tissue. Hyaluronidase-2 was up-regulated 24 h after stroke and reached maximum levels after 3 days. Hyaluronidase 1/2 protein expression was elevated in the grey and white matter of all stroke-affected tissue. This suggests that breakdown of high molecular weight hyaluronan is a feature of stroke pathophysiology. Immunohistochemistry demonstrated increased hyaluronidase expression in neurons in infarcted tissue. The results suggest a rapid degradation of hyaluronan at the site of tissue injury after stroke, which could enhance neuronal plasticity of nerve cell bodies or possibly stimulate angiogenesis in peri-infarcted regions. Hyaluronidase-2 generates a 20 kDa fragment internalized by receptor-mediated endocytosis and delivered to lysosomes where further degradation by hyaluronidase-1 occurs [7]. Although hyaluronidase-2 is also present in many tissues, it is not normally found in the brain owing to gene inactivation by hypermethylation [23]. Hyaluronidase-2 may have undergone gene re-activation after stroke. As hyaluronan levels did not appear to decrease over the time of the study there must have been resynthesis through the action of hyaluronan synthase 1/2 (detected after 24 h and 3 days, respectively, unpublished data) thus generating high molecular weight hyaluronan (RMM 3.9\_106).

Cell signalling through receptor for hyaluronan-mediated motility and CD44 mediates neuronal migration, astrocyte motility and angiogenesis [11,24]. Binding of o-hyaluronan to receptor for hyaluronan-mediated motility and CD44 in human endothelial cell lines in vitro activated mitogen activated protein kinase (ERK1/2) and initiated mitogenesis [11]. Receptor for hyaluronan-mediated motility mRNA was up-regulated after 1 h in both peri-infarcted and infarcted tissue and remained increased 21 days after stroke compared with normal contralateral hemisphere. Increased expression was seen in infarcted tissue, associated with neuronal cytoplasm. Receptor for hyaluronan-mediated motility expression might enhance calmodulin-mediated signalling to cytoskeletal elements in neurons [25]. CD44 is involved in multiple responses to inflammation including leukocyte recruitment, cell-matrix interactions and matrix remodelling. CD44 mRNA was increased after 12 h in stroke-affected areas and remained increased up to 21 days after stroke. CD44 protein associated with

neurons and micro-vessels in the stroke hemisphere. The concurrent induction of CD44 and hyaluronan in the ischaemic area may potentiate the inflammatory effect of hypoxia. CD44 expression is regulated by hyaluronan fragments, interleukin-1b and tumour necrosis factor-a, which are increased after stroke [6]. Previous studies have shown that CD44-deficient mice were protected against cerebral ischaemia injury, in association with reduced expression of interleukin-1b. Increased CD44 expression in micro-vessels together with hyaluronidase-1 up-regulation could activate angiogenesis.

## Conclusion

The results presented here show hyaluronan accumulation, increased expression of the enzymes responsible for hyaluronan degradation and up-regulation of hyaluronan receptors after induced stroke in the rat. This may reflect tissue remodelling after stroke and may have an important role in modulating angiogenesis and neuronal survival.

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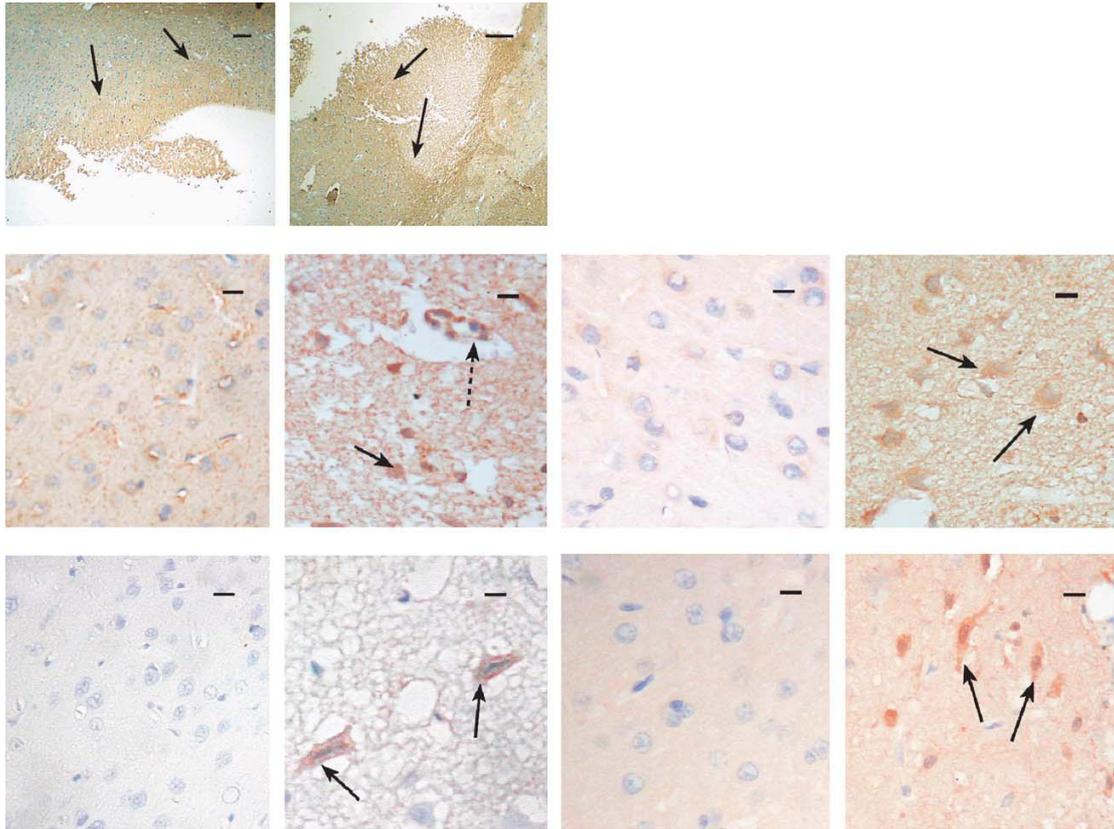
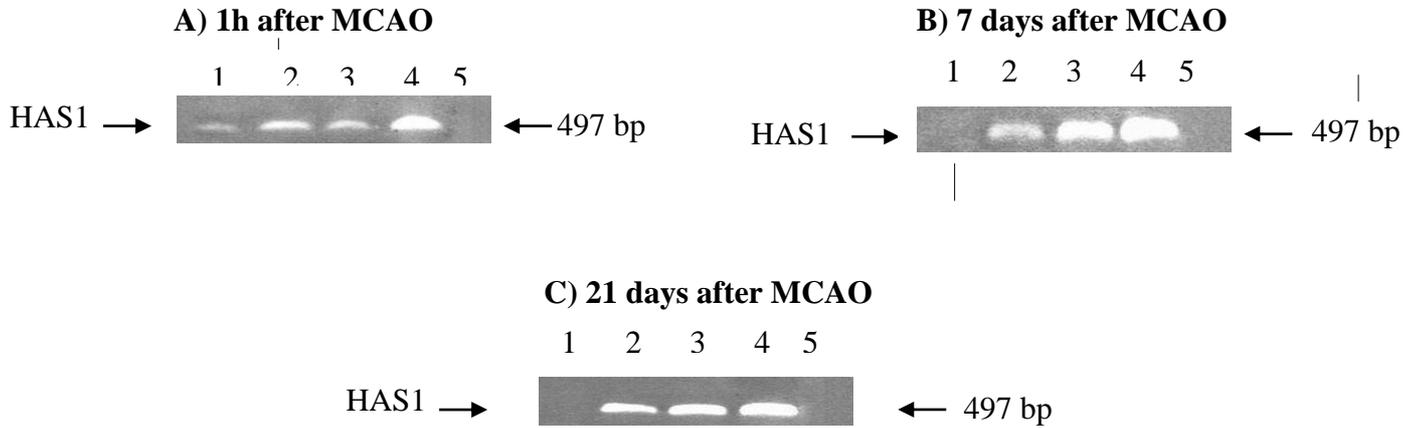


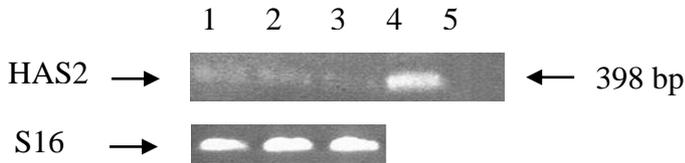
Fig. 1 Hyaluronan and associated proteins: distribution in the brain after stroke; hyaluronan expression at the boundary zone (arrows) between infarcted and peri-infarcted grey matter 4 h (a) and 24 h (b) after stroke. (c) (i) Weak staining for hyaluronidase-1 in neurons in normal looking grey matter; (ii) strong nuclear staining in a neuron from infarcted tissue (arrow) and a blood vessel (broken arrow) after 1h. (d) (i) Weak neuronal staining for hyaluronidase-2 in normal looking grey matter; (ii) strong intracellular staining in neurons from infarcted tissue (arrows) after 7 days. (e) (i) Weak neuronal staining for receptor for hyaluronan-mediated motility in normal looking grey matter; (ii) strong intracellular staining in neurons from peri-infarcted tissue (arrows) after 24 h. (f) (i) Weak neuronal staining for CD44 in neurons in normal looking grey matter; (ii) strong staining in neurons from infarcted tissue (arrows) after 7 days.



**Figure. 2:** RT-PCR showing increased gene expression of HAS1 and peaked at 7 days after MCAO. RT-PCR products (10 $\mu$ l/lane) were separated on 1.5% agarose gels and stained with ethidium bromide. Top gel: Lane 1: Contralateral hemisphere; Lane 2: Penumbra; Lane 3: Stroke; Lane 4: Positive control of total RNAs from mixed rat tissues; Lane 5: Negative control (without cDNA).

Results showed up-regulation of HAS2 mRNA 12 h after MCAO in both penumbra and infarct. HAS2 remained increased after 21 days, in penumbra and infarct compared to contralateral hemisphere (Fig 4.5).

**A) 1h after MCAO**



**F) 7 days after MCAO**

1 2 3 4 5

**G) 21 days after MCAO**

