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Immunoblot Investigation of Aggrecan Protein in Post-mortem Brain Tissue

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A B S T R A C T

The MS tissue banks estimated that more than fifty millions patient in the world, each patient cost more than one millions for treatment, has social and economic effects. The breakdown of blood brain barrier considered as autoimmune disease and early events in MS parthenogenesis. This study include investigation the modification of aggrecan glycoprotein in MS (30 samples) postmortem compared to NAWM (20 samples) via using western blotting. Fifty blocks of postmortem brain tissue were extracted their proteins for protein electrophoresis. Results indicated that human brain tissue express aggrecan proteins and its fragments were increased in MS active lesion compared to the normal control. The presence of aggrecan fragments considered as evidence of activation of different matrix mattaloproeinase enzymes to help neuron outgrowth axons..

1.0 Inroduction

Failure of remyelination is a critical impediment to recovery in demyelinating diseases such as multiple sclerosis MS (Albert et al., 2007; Patrikios et al., 2006). WithinMS lesions, oligodendrocyte precursor cells (OPCs) can be found in areaswith demyelinated axons, yet many of these do not go on to form compact myelin (Chang et al., 2002). Chondroitin sulphate proteoglycan (CSPGs), in particularly aggrecan have been reported to accumulate also in MS lesions and inhibit remyelination by impairing OPCs (Back et al., 2005; Sloan et al, 2010). Perineuronal net (PNNs) are also lost around neurons in demyelinated regions, and many of the affected neurons appear to be compromised, as indicated by accumulation of phosphorylated neurofilament protein in their cell bodies. It is unknown whether these changes in PNNs affect synaptic plasticity and function (Grey et al, 2008, Gregg et al., 2014). There is now considerable evidence indicating that the aggrecan affect oligodendrocyte lineage cells, including their proliferation, survival, migration, differentiation, process extension and myelination (Lin et al., 2011; Sherman et al, 2008). Cell culture studies have emphasized that aggrecan inhibit the spreading and survival of oligodendrocytes by interacting with β1 integrin to trigger signalingcascades. Treatment of human neuronal cells with chondroitinase ABC increased the number of OPCs in the surrounding lesion, and the addition of growth factors promoted the differentiation of transplanted OPCs into mature oligodendrocytes (Karimi et al., 2012). Another group at Sheffield hallam university in UK provided that matrix metalloproteinase enzymes, a disintegrin and metalloproteinase with thrombospondin motif 1, 4, 5 breakdown chondrotinsulphate proteoglycan and promote neurite outgrowth (Gibrel et al., 2012). The main aggrecanase cleavage site is Glu373-Ala374, and there are four additional cleavages sites in the GAG attachment region at Glu1480-Gly1481, Glu1667-Gly1668, Glu1771-Ala1772 and Glu1871-Leu1872 (Jones et al, 2005, Porter et al., 2005; Treobeg et al., 2012). Westling and colleagues indicated that ADAMTS-4 could cleaveaggrecan within GAGs (Glu405-GLu406) and (Glu441-Ala442) in neural tissue, which produced 70KDa and 64KDa fragments respectively known aggrecanneoepitopes (Westling et al., 2004). Aggrecanbiosynthesis is catalysed by a series of intracellular enzymes including xylosyltransferase-1, which is required during aggrecan synthesis to form xylose-galactose-galactose-glucuronic acid which initiates GAG polymerisation on the core protein. Notably, targeting of xylosyltransferase-1 mRNA with locally applied mRNA

knockdown disrupted CSPG production in the ECM, resulting in increased axonal regeneration around lesion in animal models (Grimpe et al., 2004; Lau et al, 2013).

2.0 Materials and Methods

Human brain tissue: Human brain was removed from the deceased donor; specimens were prepared by cutting a whole brain into anterior and posterior halves by a single cut through the mammillary bodies. Each block was one centimeter thick coronal slices. Cutting slices were numbered according to their position, as anterior (frontal pole) or posterior (occipital pole) to the mammillary bodies. They were numbered A1, A2 etc and the latter were numbered P1, P2 etc respectively. The coronal slices were laid on a gride, cut into 2cm brain tissue blocks stored at -80°C. Furthermore, each patient already given an ID number related to the coronal section. For instance, MS087 A2D3, the patient number is 087, and A2 meaning coronal slice was taken from the second section anterior to the mammillary bodies, and the another brain tissue block derived from gride co-ordinate D3

During this study fifty clinically snap-brain tissue blocks and confirmed MS cases, together with twenty normal control cases were received from the UK Multiple Sclerosis Tissue Bank. Multiple sclerosis post-mortem specimens were included seventeen females, mean ages 54.52 years (ranges 39-77) and thirteen males with a mean age of 60.54years (range 38-75), while control cases include eight males, mean ages of 67.8 (range 35-88) and twelve females, mean age of 68.3 (range 50-77).

Protein Extraction of postmortem brain tissue blocks: The region of post-mortem brain tissue blocks of interest in this paper was cut according to immunohistochemistry results for multiple sclerosis lesions as , NAWM and normal control white matter. The tissues were then weighted mentioned in previous publish 2014, (Abuneeza *et al*., 2014), and an appropriate amount of celLytic™ Mammalian Tissue Lysis/Extraction Reagent (Sigma-Aldrich, UK) and protease inhibitor cocktail (Sigma-Aldrich, UK) used. For this procedure, a ratio of tissue to celLytic™ reagent of 1:10 (1gram of tissue/ 10 ml of reagent) was used. Each specimen of postmortem tissue block was transferred to a pre-chilled microhomogenizer, where it was homogenized at 4°C. The pellet was discarded and the supernatant was centrifuged at 20,000 g for 10mins at 4°C,the supernatant was stored at-20°C for another experiment as following.

Estimation of total protein concentration by using Bradford assay: Total protein concentration was determined using a quick start Bradford dye ™ reagent kit (Bio-Rad, UK) according to the manufacturer's instructions. 5µL of bovine serum albumin (BSA, Sigma Aldrich, UK) protein standard in PBS (ranging from 0.1 to 200mg/ml) alongside 5µl of extracted protein sample in triplicate were pipette into a 96 well plate. 200µL of Bradford dye reagent was added to each well. The plate was incubated for 30 mins at RT and the absorbance was read with a Victor Wallac[2] plate reader at 570nm. A standard curve was drawn in excel and protein concentration was determined using the trend line of the equation of the standard curve (Figure 1).

Sodium Dodecyl Sulphate PAGE (SDS PAGE): The extracted proteins from tissue blocks were fractionated on pre-cast 7% Tris acetate gels, 1mm thick with 10 wells, in the presence of NupageTris Acetate running buffer. 15-30µg of each protein sample was loaded per well alongside 10µl of multicolor high molecular weight markers standard (range 40-300kDa, Thermo Scientific, UK) and electrophoresis carried out at 130 voltages and 200 mA for approximately 1h, until the dye front reached the bottom of the gel.

Protein electro-blotting: Separated proteins were transferred onto Hybond-C nitrocellulose membrane (GE Healthcare, UK) in order to make the proteins accessible to antibody. A piece of nitrocellulose membrane was soaked for 5 mins in cold transfer buffer along with two pieces of blotting paper and two pieces of sponge per mini-gel. One sponge was placed on the open transfer cassette, followed by a piece of blotting paper and the gel

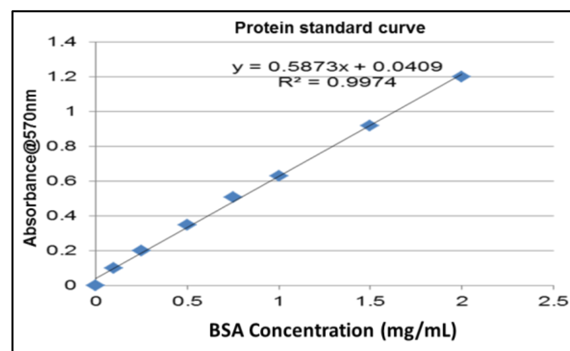

Figure 1. Bradford assay standard curve.

Standard curve illustrate determination of protein concentration of an unknown tissue extract sample using bovine serum albumin (BSA) as standard. Specimen absorbance at 570nm were read and plotted as illustrated in the graph above. Equation of chart line and R2 value of the chart are given. Y= aX + b where(Y) corresponds to absorbance, (a)corresponds to the slope of the trend line;(X) corresponds to the concentration of protein in the sample and (b) corresponds to the intercept point with the X-axis.

Nitrocellulose membrane (NCM), a second piece of blotting paper and another sponge:

Proteins were transferred from the SDS-PAGE gel to the NCM at 100 V for 1h To verify the protein had transferred successfully onto the membrane, the gel was immersed in instant blue (Sigma Aldrich, UK) and the NCM in Ponceau red (Sigma Aldrich, UK) in order to visualize the protein.

Immunoprobing the membrane: Following transfer of protein to the NCM, it was immediately placed in blocking solution[5% non-fat milk in PBS-T (phosphate buffered saline containing 0.02% Tween)]. Following this, the membrane was washed three times for 5 mins each in PBS, with gentle agitation on a platform shaker. The primary antibodies of β actin, aggrecan and its neoepitope were then diluted in 10mL of PBS and added to the membrane and incubated overnight at 4°C. For negative controls, primary antibody was omitted and the membrane was incubated with PBS alone. Following three washes of the membrane with PBS for 5 mins each, labelled secondary antibody was then diluted in PBS and incubated for 1h with gentle agitation at RT. The membrane was then washed twice for 5 mins each in PBS on the shaker followed by one final wash for 5 mins in PBS only with gentle agitation.

Determination of molecular weight of proteins migrated on SDS-PAGE: Molecular weight markers (spectra multi-color high range protein ladder (40-300kDa), Thermo, UK) were used to identify the approximate mass of a protein run on a gel; using the principle that molecular weight is inversely proportion to migration distance through a gel matrix. Consequently, once used in gel electrophoresis, markers effectively provide a logarithmic scale by which to estimate the size of the protein (Figure 2). In order to determine the mass of a protein band on western blotting, a marker lane was always included. The distance migrated by the protein markers was measured and a standard curve constructed using a plot of log10 mass versus relative mobility (distance migrated by band/distance migrated by dye). The molecular weight (MW) of the unknown protein band was then determined by using the equation of the line using Microsoft Excel.

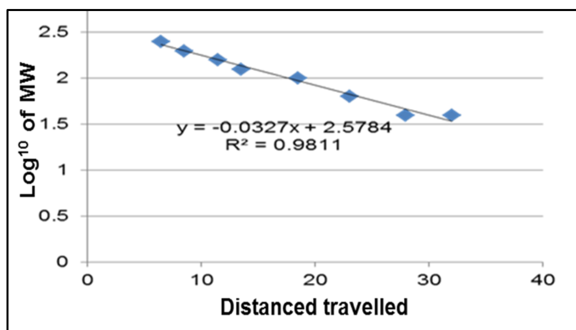

Figure 2. Measurement of the molecular weight of sample protein.

Determination of the MW of the protein sample on SDS-PAGE using a known molecular weight marker(spectra multicolour high range protein ladder, Thermo Scientific, UK).A standard curve generated by blotting log molecular weight (MW) versus migration distance (Rf) of each band of the protein ladder marker through an SDS-PAGE gel.

3.0 Results

Following initial optimisation, SDS-PAGE was performed with 20µg protein/well before transferring separated proteins onto nitrocellulose membrane. Western blotting demonstrated that aggrecan and its neoepitope were present in all samples of MS active, NAWM and normal control brain tissue studied. Immunoblotting for β-actin (42kDa)

was used as a reference protein. Two bands of 250kDa and 70 kDa were observed for aggrecan and aggrecanneoepitope respectively, with the relevant antibodies. Figure-3shows the variation in aggrecan and its neoepitope expression for all MSand normal brain samples examined, as determined by densitometry. Although expression was variable, overall there was a significant increase in aggrecan and aggrecan-neoepitope expression in active lesions compared to NAWM and normal brain samples (***P<0.001, n=30). Increase in levels of these proteins was due to increases in both the 250kDa and 70kDa bands. Densitometric analysis values are expressed as the density ratio of target (aggrecan or aggrecan-neoepitope) to loading β-actin in arbitrary units for aggrecan and its fragment in control white matter, MS NAWM and MS active lesional brain tissue. As shown in figure 3, there was much greater expression of aggrecan and its neoepitope in active lesions compared to NAWM and normal control brain. Data was represented as the mean ± SEM using Kruskal-Wallis analysis.

4.0 Discussion

This paperillustrate that aggrecanneo-epitopescould be generated by characteristic ADAMTS(aggrecanase, a disintegrin matrix metalloproteinase with thrombospondin motif) cleavage, another group at Sheffield hallam university also showed that ADAMTS-9 protein was expressed by astrocytes, endothelium, neurons and activated microglia with up-regulationin active lesionscompared to the normal control in *ex vivo*CNS tissue, indicating that ADAMTS-9 secreted from these cells could play a part in ECM remodelling, particularly of aggrecan and versican (Abuneeza *et al*., 2014). However, It has been demonstrated that expression of a number of ADAMTS enzymes can be modulated by a number of mediators (e.g. cytokines) in different CNS cell types (Gross *et al*, 2006). ADAMTS-1 and -4 expressions in astrocytes and neurons has been reported following physical or toxic injury to the CNS and it was modulated by pro-inflammatory cytokines, suggesting that this expression at the site of injury may support neurite outgrowth (Cau *et al*., 2013). In view of the abundance of ADAMTS enzymes and other ECM molecule-degrading enzymes in demyelinating areas, loss of aggrecan and versican in MS plaques is in linewith previous observations. We did not, however, anticipate identifying large granules of versican and its neoepitope within cells in active lesions [Abuneeza *et al*, 2014]. These granules likely are aggregates of the versican or products of its extracellular degradation by ADAMTS that is phagocytosed either by activated microglia or macrophages. Alternately, this staining pattern may represent synthesisedversican prior to secretion by the cells (Troebeg *et al*, 2012; Westling *et al*., 2004).

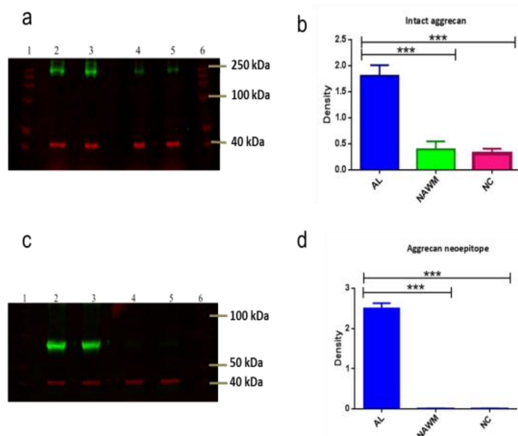

Figure 3. Western blotting analysis of aggrecan and its neoepitope expression in MS and normal post-mortem brain tissue

Immunoblot of aggrecan (green), its neoepitope (green) and β-actin (red) from an active MS lesion (MS090 P3D3)(lanes 2, 3); control white matter (NC022 P3D3)(lane 4) and MS NAWM (MS103 P3D3)(lane 5). (Lane 1 and 6) represent standard molecular weight markers (40-300KDa) (Thermo Scientific, UK). (a) and (c) demonstrate expression of aggrecan (MW 250kDa) and aggrecanneoepitope (MW 70kDa) respectively in an active lesion, normal control and normal appearing white matter. Densitometric quantification shows much greater expression of aggrecan and its neoepitope in AL compared to NC and MS NAWM using Kruskal-Wallis (***P<0.001, n=7 blocks)(b) and (d). (n=50).

Studies have emphasized that CSPGs can interact with adhesion molecules expressed on various cell types (Afshari *et al*, 2010; Properzi *et al*., 2010). When axonal growth cones come into contact with CSPGs, they retract and collapse. This might be the reason for the abortive regenerative sprouting observed in spinal cord injury lesions (Fleming *et al*., 2006; Tan *et al*., 2011). The interaction of CSPGs and neurons stimulates the Rho-associated protein kinase Rho-ROCK and/or protein kinase C (PKC) intracellular signaling cascades, which inhibit process extension. Also by blocking activation of the Rho-ROCK and PKC signalling pathways, the inhibitory effect of CSPGs could be observed (Lim *et al*., 2013). While aggrecan are widely accepted to be inhibitory to axonal regeneration, the inhibitory nature of individual lectican molecules varies amongst the CSPGs. *In vitro,* purified brevican, another type of CSPGs has been shown to be inhibitory to both axonal attachment and growth, while neurocan has been shown to interact with neuronal cell adhesion molecules (N-CAM) similarly inhibiting neurite outgrowth (Jones *et al*., 2003; Inatani *et al*., 2001; Yuan *et al*., 2013).

At the molecular level, the inhibitory activity of aggrecan on neurite outgrowth and axon re-growth is mediated mainly by GAGs and their structural motifs. Treatment with chondroitinase ABC, an enzyme able to cleave the sugar side chains without altering the core protein structure, promotes axon re-growth both *in vivo* and *in vitro* (Properzi *et al*, 2003; Cau *et al*., 2013). The ECM of the glial scar contains high level of CSPGs, Yiu *et al.,* (2006) demonstrated that neurons cannot cross the glial scar, in which CSPG levels are increased. Similarly, the increased expression of intact aggrecan and versican around sites of injury in MS lesions in this study might be involved in the inhibition of neuite re-growth and axon elongation (Yiu *et al*., 2006). The precise relationship of aggrecan protein deposition to glial scarring and axon re-growth inhibition, binding to other ECM components and cell migration in the mature CNS are currently poorly understood. Previous studies performed in UK laboratory demonstrated evidence for expression of ADAMTS-1, 4 and 5 in both normal and MS lesion with up-regulation of ADAMTS-4 at the protein level in MS lesions [15, 29]. Current knowledge suggests that these alterations may be beneficial, enabling neurite outgrowth and neuronal repair,since CSPGs inhibit neurite outgrowth, axonal regeneration and promote neural cell death. However, ADAMTS mediated degradation of aggrecan may increase access of inflammatory cells to sites of tissue destruction leading to detrimental outcomes (Cross *et al*., 2006; Haddock *et al*., 2006).

5.0 Conclusion

This paper provides evidence to suggest that aggrecan breakdown is involved in MS pathogenesis; high levels express within active lesion was observed, they likely contribute to the failure of axon regeneration and neurite outgrowth. ECM lectican abnormalities in the CNS of MS patients should be considered in the design of future interventional therapies and their breakdown by glutamylendopeptidase ADAMTSs may be beneficial, enabling neurite outgrowth and neuronal repair.

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