Research Report

Distribution of collagen XVII in the human brain

Allan Seppänen, Tiina Suuronen, Silke C. Hofmann, Kari Majamaa, Irina Alafuzoff

Abbreviations: ALS, amyotrophic lateral sclerosis; BP, bullous pemphigoid; CA, cornu ammonis (Ammon's horn); coll XVII, collagen XVII; HE, hematoxylin–eosin; IHC, immunohistochemistry; IR, immunoreactivity

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ABSTRACT

We have recently discovered collagen XVII to be present in neurons of the human central nervous system. Collagen XVII has previously been primarily studied in the field of dermatopathology since it is abundantly expressed in the skin, which, like the nervous system, is ectodermal in origin. A link between dermatopathological and neurological entities has been implied due to clinical case studies revealing an association between bullous pemphigoid and age-related neurodegenerative disorders. The objective of this study was to assess the distribution of collagen XVII in the human brain in relation to normal ageing. Post-mortem brain tissue was obtained from 11 neurologically unimpaired subjects who had died from cardiovascular causes at the age of 17 to 78 years. Collagen XVII expression in various neuroanatomical regions, representing essentially the entire human brain, was then assessed using immunohistochemistry. We found collagen XVII to be expressed widely in the brain and to be located primarily in the soma and proximal axons of neurons. In contrast, glial cells did not express collagen XVII. The expression varied strikingly between different neuroanatomical regions, being most notable in motor nuclei and Betz cells followed by pyramidal neurons. There was no correlation between collagen XVII expression and variables such as gender, age at death, post-mortem delay and fixation time whereas a mode of death leading to notable neuronal ischemia depleted the protein expression. Many neurodegenerative disorders display a specific pattern of neuroanatomical involvement, thus the regionally variable expression of collagen XVII offers new prospects for research.

1. Introduction

Recently, we discovered collagen XVII (coll XVII) to be present in neurons of the cerebral cortex, hippocampus and amygdala of the human brain (Seppänen et al., 2006). Furthermore, in animal studies Claudepierre et al. (2005) demonstrated the presence of coll XVII in the retina, cerebellum and olfactory bulb.

Previously, coll XVII has primarily been studied in the field of dermatopathology since it is abundantly expressed in the skin, a tissue which, like the nervous system, is ectodermal in origin. Coll XVII is known to be a structural component of...
hemidesmosomes, which mediate the adhesion of epithelial cells to the underlying dermis. The ectodomain of this transmembrane collagen is constitutively shed from the cell surface by proteases, thus allowing the anchored cell to detach and migrate. The lack of coll XVII or the loss of its function leads to diminished epidermal adhesion and skin blistering, such as occurs in the autoimmune skin disease, bullous pemphigoid (BP) (Franzke et al., 2005).

The normal distribution and the possible functions of coll XVII in the central nervous system are currently unknown. However, the role of coll XVII may be related to anchoring neurons to their substratum and thus it may play a role in neuronal migration during morphogenesis (Marin and Rubenstein, 2003). Furthermore, Claudepierre et al. (2005) suggested that coll XVII might function as a synaptic stabilizer in the central nervous system, thus influencing synaptic plasticity. Interestingly, clinical case studies have pointed to an association between bullous pemphigoid (BP) (Franzke et al., 2005).

The expression of coll XVII was similar in the youngest and the oldest subject and no gender influence was seen. Furthermore, factors known to influence the expression of proteins in post-mortem tissue, such as age-related neurodegenerative disorders (Chosidow et al., 2000; Foureur et al., 2001) and multiple sclerosis (Kirtschig et al., 1995; Stinco et al., 2005). This proposed association between specific dermatopathological and neuropathological entities raises the question of whether or not coll XVII might be a common ethiopathogenetic denominator. However, before one can assess the expression of coll XVII in diseased brains, it is necessary to establish its normal expression both in relation to brain region and age. Furthermore, factors known to influence the expression of proteins in post-mortem tissue, such as age, post-mortem delay and fixation time, have to be accounted for in order to avoid erroneous results (Hynd et al., 2003). In this study, we have used standardized post-mortem brain tissue samples and an immunohistochemical detection method using a non-commercial monoclonal antibody directed against the NC16a epitope of coll XVII. The objective was to assess in detail the distribution of collagen XVII in the human brain in relation to normal ageing.

Table 1 – Description of the antibodies

<table>
<thead>
<tr>
<th>Detection of</th>
<th>Code</th>
<th>Species/clone</th>
<th>Epitope</th>
<th>Dilution</th>
<th>Pre-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen XVII</td>
<td>NC16a-3 (in house*)</td>
<td>Mouse/SA9</td>
<td>AA 545-557 (NC16a)</td>
<td>None</td>
<td>Acl in CB</td>
</tr>
<tr>
<td>Collagen XVII</td>
<td>NC16a-3 (in house*)</td>
<td>Rabbit/polyclonal</td>
<td>AA 490-566 (NC16a)</td>
<td>1:1500</td>
<td>MW in CB</td>
</tr>
<tr>
<td>β-Amyloid</td>
<td>M0872 (Dako)</td>
<td>Mouse/6F/3D</td>
<td>AA 9-14</td>
<td>1:100</td>
<td>FA 6 h</td>
</tr>
<tr>
<td>α-Synuclein</td>
<td>NCL-ASYN (Novocastra)</td>
<td>Mouse/KM51</td>
<td>AA 1-140</td>
<td>1:1000</td>
<td>Acl in CB+ FA 5 min</td>
</tr>
<tr>
<td>PHF-Tau</td>
<td>BR-03 (Innogenetics)</td>
<td>Mouse/AT8</td>
<td>Ser202</td>
<td>1:500</td>
<td>None</td>
</tr>
</tbody>
</table>

*Hofmann et al. (manuscript in preparation); Schumann et al. (2000). AA=amino acid; Acl=autoclave 120 °C 10 min; CB=citrate buffer pH6; FA=formic acid 80%; MW=microwave.

All stainings were performed manually and all antibodies were incubated overnight at 5 °C.

The staining results achieved by the polyclonal antibody used previously (Seppänen et al., 2006) and the presently utilized monoclonal antibody were congruent: the neuronal labelling/immunoreactivity (IR) was similar whereas the neuropil IR with the monoclonal antibody was weaker than that with the polyclonal antibody. Stainings where the primary antibody had been omitted were used as a negative control and did not display any neuronal or neuropil IR.

Coll XVII expressing neurons were seen in nearly all of the brain areas studied (Table 2). The staining was primarily localized to the cell body, specifically to the cytoplasm, in either a diffuse or a granular pattern (Fig. 1). In the latter case, the localization resembled the pattern seen in HE staining of the rough endoplasmic reticulum, i.e. Nissl substance. In addition to the staining of the neuronal cell body, there was a light staining of the neuropil throughout the samples. In contrast, no staining of white matter was noted, i.e. there was a strict demarcation between grey and white matter. Neither glial cells nor endothelial cells were labelled, whereas labelling of the ependymal lining was noted.

The coll XVII-IR varied markedly between neuroanatomical regions. The intensity of the coll XVII-IR was consistently prominent in the hypoglossal nucleus (XII), olivocerebellar nucleus (III), nucleus basalis of Meynert, supraoptic nucleus, subthalamic nuclei and pyramidal cells of the hippocampal regions of cornu ammonis (CA) 4-2.

Interestingly, there were also regions in which the coll XVII-IR seemed to vary markedly from case to case. These regions were the cerebral cortex, the pallidum, putamen and caudatum in the basal ganglia, and the vermis, dentate and Purkinje cells of the cerebellum. In the cerebral cortex, staining was consistently strongest in the deeper layers, i.e. layer V (ganglionic layer). Expression also varied from one cortical area to another, being strongest in the motor cortex, which is mainly accounted for by strong coll XVII-IR of Betz cells, and weakest in the temporal lobe.

A consistent lack of coll XVII-IR was noted in the granular cells of hippocampal CA, granular cells of the cerebellar cortex, the neurons of the mamillary body and caudatus.

The expression of coll XVII was similar in the youngest and the oldest subject and no gender influence was seen. Furthermore, no difference in coll XVII-IR was seen in relation to post-mortem delay, which ranged from 48 to 124 h, or fixation time, ranging from 23 to 65 days. In line with this, statistical analysis did not reveal any correlation between coll XVII and the studied variables, i.e. age at death, gender, post-mortem delay and fixation time. However, an almost total lack of coll XVII-IR was noted in the one case that suffered pre-mortem cerebral

2. Results

First, the basic histology, neuroanatomy and possible pathological features were determined in consecutive paraffin embedded sections by using hematoxilin–eosin (HE) stains and three different immunohistochemical assays (Table 1). Inspection of HE stained sections did not reveal any diagnostic pathological alterations and no age-related degenerative changes were noted applying β-amyloid, α-synuclein or PHF-Tau immunohistochemistry.
**Table 2 – Distribution of collagen XVII immunoreactivity in the human brain**

<table>
<thead>
<tr>
<th>Case</th>
<th>Cortical structures</th>
<th>Subcortical structures</th>
<th>Subtentorial structures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cerebrum</td>
<td>Hippocampus</td>
<td>Basal forebrain</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>F cortex d</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>2</td>
<td>F cortex d</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>3</td>
<td>F cortex d</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>4</td>
<td>F cortex d</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>5</td>
<td>F cortex d</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>6</td>
<td>F corpus callosum</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>7</td>
<td>F corpus callosum</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>8</td>
<td>F corpus callosum</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>9</td>
<td>F corpus callosum</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>10</td>
<td>F corpus callosum</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>11</td>
<td>F corpus callosum</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

Acc=accessorius; bas=basalis; CA=cornu ammonis; cereb=cerebellar; d=deep; F=frontal; gr=granular cells; inf=inferior; M=motor; nucl=nucleus; O=occipital; P=parietal; Pu=Purkinje cells; s=superficial; T=temporal.

Within the given region, the predominantly noted collagen XVII immunoreactivity, as assessed by immunohistochemical stainings and light microscopy, is classified as none=white, mild to moderate=grey and strong=black. Section not available = n.a.
hypoxia and that displayed numerous ischemic neurons in the brain samples (case no 11, rapidity of death Aiii). No differences in coll XVII-IR were noted between subjects dying instantaneously (Aii) or within 24 h after the onset of symptoms but lacking signs of cerebral ischemia (Aii). Thus, the only noted influential variable was the prolonged mode of death, causing irreversible ischemic damage and depletion of coll XVII-IR.

Coll XVII immunoblotting revealed a single band of ca. 150 kDa in the post-mortem human brain extract (Fig. 2) as compared to the 180-kDa sized coll XVII found in keratinocytes (Nishizawa et al., 1993).

3. Discussion

This study provides the first systematic assessment of the neuroanatomical distribution of coll XVII in the human brain.
We found coll XVII to be widely expressed in neurons of the brain but the level of expression varied strikingly between different neuroanatomical regions. The most prominent expression was noted in the motor nuclei (hypoglossal nucleus, oculomotor nucleus), Betz cells, pyramidal neurons in hippocampal CA2-CA4 regions, subthalamic nuclei and various nuclei of the basal forebrain.

In keratinocytes, biochemical and ultrastructural studies have shown the expression of coll XVII to be localized to the cell membrane (Nishizawa et al., 1993). Surprisingly, in neurons the expression was seen either diffusely in the cytoplasm or in some cell types strongly related to the rough endoplasmic reticulum. This might suggest significant post-transcriptional variability of the protein, leading to different isoforms in neuronal and epithelial cells. The divergent cellular localization might also indicate functional differences of the protein in different cell types. The differing size of the protein in the post-mortem human brain extract (ca. 150 kDa) as compared to keratinocytes (180 kDa), however, may be due to partial degradation of the protein during the post-mortem delay. The influence of post-mortem delay and post-mortem storage temperature on protein preservation has recently been studied in detail by Ferrer et al. (2007). Hence, further studies are required in order to conclusively characterize neuronal coll XVII.

Previously, we have shown that coll XVII is indeed expressed in the human brain and is localized to neurons by employing in situ hybridization, immunohistochemistry using a polyclonal antibody and RT-PCR followed by sequencing (Seppänen et al., 2006). Furthermore, in support of the presence of coll XVII in the central nervous system, an earlier study reported coll XVII to be expressed in the murine and bovine retina and cerebellum and murine olfactory bulb (Claudepierre et al., 2005).

Protein expression and enzyme activity in post-mortem human brain are known to be influenced by many factors, such as agonal state, post-mortem delay and fixation time (Laitinen et al., 2001; Hynd et al., 2003; Ferrer et al., 2007). Our results indicate that standardized brain samples that were fixed for up to 2 months and that had been obtained at autopsy carried out within 5 days of death, the deceased having been placed in 4 °C until autopsy, could readily be used when assessing coll XVII expression immunohistochemically. However, one should be very cautious if HE stained brain samples display ischemic neuronal changes, as the coll XVII expression seems to be depleted in the damaged cells. Whether the depleted expression of coll XVII is associated with general ischemic damage of organelles or whether coll XVII is particularly susceptible to ischemia remains to be elucidated. Also, the concentrations of certain proteins are age- or gender-dependent and thus the effect of age and gender on the proteins to be studied should always be analyzed (Adolfsson et al., 1979; Castensson et al., 2000; Hynd et al., 2003). Coll XVII expression seems to be age- and gender-independent at least within the range of 17 to 78 years.

Collagen XVII expression in specific neuroanatomical regions offers new prospects for research on diseases involving them. Indeed, Stinco et al. (2005) and Foureur et al. (2001) have already suggested an association between BP and neurological disorders such as Parkinson’s disease and Alzheimer’s disease. In Alzheimer’s disease, neurons in the nucleus basalis of Meynert and in Parkinson’s disease, neurons in the substantia nigra are severely affected. Here, both these regions were shown to display moderate to strong coll XVII-IR. Interestingly, several motor nuclei as well as Betz cells displayed a strong coll XVII-IR and they are known to be affected in motor neuron disease. In line with this, an earlier clinical report suggested an association between amyotrophic lateral sclerosis (ALS) and BP (Chosidow et al., 2000) and another study reported collagen abnormalities in the spinal chord of ALS-patients (Kolde et al., 1996). Also, the proposed anchoring function of coll XVII may be impaired in migrational abnormalities leading to clinical phenotypes such as epilepsy and mental retardation. Indeed, the lack of integrin α6, an extracellular ligand of collagen XVII, has already been implicated in abnormalities in the laminar organization of the developing cerebral cortex and retina in mice (Georges-Labouesse et al., 1998).

In conclusion, we report that collagen XVII is widely expressed by neurons of the human brain. Expression varies in distribution and intensity and, in contrast with keratinocytes, the cellular localization is cytoplasmic. Together with the previously reported clinical associations between BP and certain brain disorders, our findings advocate new research approaches to major neurological diseases.

4. Experimental procedures

4.1. Subject ascertainment

Informed consent was obtained from the next of kin for necropsy and neuropathological investigation. The research program was approved by the ethical committee of Kuopio University Hospital.
4.2. Case selection

All subjects, aged from 17 to 78 years at death, lacked any signs of neurological dysfunction and routine gross- and microscopical neuropathological assessment did not reveal any disease-related neuropathological changes. Each subject had died from cardiovascular causes (Table 3). The deaths had been virtually instantaneous (Ai) in seven subjects; three subjects had died within 24 h with no evidence of cerebral hypoxia (Aii) and one subject displayed evidence of final cerebral hypoxia (Aiii). The mode of death was categorized as previously described by Hynd et al. (2003).

4.3. Brain sampling

The brains were fixed at autopsy with 10% buffered formalin by in situ perfusion via the carotid artery for 1 h in order to achieve consistent fixation throughout the entire brain. The brains were then removed, weighed and fixed in 10% buffered formalin for 23 to 65 days. After fixation, the brains were grossly evaluated, cut into coronal slices and examined for macroscopically detectable lesions. The same neuropathologist dissected all the brains, using a standardized sampling protocol. Brain specimens were taken from cortical, subcortical and subtentorial gray matter structures as follows.

Cortical: frontal (Brodmann 9), temporal (Brodmann 22), parietal (Brodmann 39), occipital and motor, or precentral, and insular cerebral cortices and gyrus cinguli and hippocampus, including the subicum and entorhinal cortex. Subcortical: basal forebrain, including amygdala, basal ganglia and thalamus. Subtentorial: midbrain, including substantia nigra,pons, medulla, cerebellar cortex, vermis and dentatus (Table 2). All specimens were embedded in paraffin.

4.4. Immunohistochemistry

For generation of the NC16a-3 antibody, Balb/c mice were immunized three times with 30 μg of GST-NC16a fusion protein at weekly intervals. Fusion of splenocytes with SP2/0 myeloma cells was performed as previously described (Sorkin et al., 1992). Initial screening for antibody production was achieved by in situ perfusion via the carotid artery for 1 h in order to achieve consistent fixation throughout the entire brain. The brains were then removed, weighed and fixed in 10% buffered formalin for 23 to 65 days. After fixation, the brains were grossly evaluated, cut into coronal slices and examined for macroscopically detectable lesions. The same neuropathologist dissected all the brains, using a standardized sampling protocol. Brain specimens were taken from cortical, subcortical and subtentorial gray matter structures as follows.

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4.5. Quantitative analysis

Each stain and structure was first scanned at 100× magnification and then detailed assessment of the IR was carried out at up to 400× magnification. The quantitative assessment was repeated at least twice for each sample and the given intensity of IR is the predominant intensity noted. The strength of the coll XVII-IR was assessed on a three-grade scale (none=white, mild to moderate=grey and strong=black) (Table 2).

Table 3 – Demographics

<table>
<thead>
<tr>
<th>Case</th>
<th>Gender</th>
<th>Rapidity of death</th>
<th>Age at death (years)</th>
<th>Cause of death</th>
<th>Post-mortem delay (hours)</th>
<th>Fixation time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>Ai</td>
<td>17</td>
<td>Cardiac infarction</td>
<td>48</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>Ai</td>
<td>38</td>
<td>Cardiac infarction</td>
<td>96</td>
<td>28</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>Ai</td>
<td>44</td>
<td>Cardiac infarction</td>
<td>124</td>
<td>65</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>Ai</td>
<td>54</td>
<td>Cardiac infarction</td>
<td>48</td>
<td>44</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>Ai</td>
<td>67</td>
<td>Cardiac infarction</td>
<td>120</td>
<td>64</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>Ai</td>
<td>68</td>
<td>Pulmonary embolism</td>
<td>120</td>
<td>64</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>Ai</td>
<td>76</td>
<td>Cardiac infarction</td>
<td>96</td>
<td>62</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>Aii</td>
<td>60</td>
<td>Cardiac insufficiency</td>
<td>96</td>
<td>50</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>Aii</td>
<td>65</td>
<td>Cardiac insufficiency</td>
<td>94</td>
<td>44</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>Aii</td>
<td>78</td>
<td>Cardiac insufficiency</td>
<td>96</td>
<td>32</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>Aiii</td>
<td>56</td>
<td>Cardiac insufficiency</td>
<td>72</td>
<td>65</td>
</tr>
</tbody>
</table>

M=male; F=female. The post-mortem delay consists of a maximum of 8 h at room temperature after which the deceased were placed in 4 °C until autopsy. The classification for the rapidity of death is Ai, virtually instantaneous death; Aii, death within 24 h with no evidence of cerebral hypoxia; and Aiii, death within 24 h with evidence of cerebral hypoxia (Hynd et al., 2003).
4.6. Statistical analysis

For statistics SPSS 11.5 for Windows was used, employing Pearson and Spearman tests and t-test analysis.

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