Glial hyaluronate-binding protein: a product of metalloproteinase digestion of versican?*

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Glial hyaluronate-binding protein (GHAP) is a 60 kDa glycoprotein with an amino acid sequence identical to that of the hyaluronate-binding region of versican, a large fibroblast aggregating proteoglycan found in the brain. Both GHAP and versican were identified by immunoblot in bovine brain extracts prepared only minutes after death. Human recombinant collagenase, stromelysin, mouse gelatinase and gelatinases iso-

lated from human brain by affinity chromatography digest versican and give rise to a polypeptide with electrophoretic mobility identical to GHAP. Immunoblot analysis, peptide mapping and C-terminal amino acid sequencing indicate that the polypeptide generated by digestion with human brain gelatinases is identical to GHAP. We suggest that GHAP is a naturally occurring versican degradation product.

INTRODUCTION

During the late 1970s, several laboratories started working on the identification of proteoglycans and hyaluronate-binding proteins in the brain [1,2]. Glial hyaluronate-binding protein (GHAP) is a 60 kDa glycoprotein expressed primarily in the extracellular matrix of the white matter [3]. Partial amino acid sequences indicated that GHAP is identical to the hyaluronate-binding region of a large fibroblast proteoglycan, versican [4]. Versican has been isolated recently from human brain [5] and was found in the precartilaginous mesenchyma during early development and post-natally in the brain [6,7]. At least four different alternative spliced forms of versican have been identified [8–10] and several more are predicted [11,12].

Matrix metalloproteinases (MMPs) require both zinc and calcium for activity and digest proteins of the extracellular matrix. Collagenase (MMP1), gelatinases A and B (MMP2 and MMP9) and stromelysin (MMP3) are the main metalloproteinases and have the capacity to digest collagen, gelatin, fibronectin and the components of the basement membrane [13,14]. Collagenase, stromelysin, gelatinases and PUMP (MMP7) have also been reported to cleave aggrecan, the main hyaluronate-binding proteoglycan in cartilage [15–17]. In cartilage, it is likely that the hyaluronate-binding region remains attached to the hyaluronate in the matrix while the large chondroitin sulphate-bearing domain is degraded further and diffuses out into the synovial fluid. It has been shown that during maturation and aging, as well as in osteoarthritis, there is an accumulation of degradation products of aggrecan including the hyaluronate-binding region [18–20].

GHAP is identical, at the amino acid level, to the hyaluronate-binding region of versican [3,4]. It was therefore pertinent to address the question of the relationship between GHAP and versican. The hypothesis was raised that cleavage of versican by

metalloproteinases in the brain would produce GHAP as part of the physiological turnover of the matrix. Here we present evidence that GHAP is present *in vivo* and may be a product of metalloproteinase digestion of versican.

MATERIALS AND METHODS

Materials

High- and low-molecular-mass markers, gelatin–Sepharose and Q-Sepharose fast flow were purchased from Pharmacia (Piscataway, NJ, U.S.A.). Goat anti-(mouse IgG) (heavy and light chain) biotin-conjugated and phosphatase avidin-conjugated were obtained from Vector (Burlingame, CA, U.S.A.). Laminin, Dulbecco's modified Eagle's medium and fetal-calf serum were purchased from Life Technologies (Gaithersburg, MD, U.S.A.). Bovine gelatin and a protein assay kit were obtained from Bio-Rad (Hercules, CA, U.S.A.), bovine testicular hyaluronidase and β -casein from Sigma (St. Louis, MO, U.S.A.). Chondroitinase ABC and carboxypeptidase Y were purchased from Boehringer Mannheim, and poly(vinylidene difluoride) (PVDF) membranes (Immobilon) were from Millipore (Bedford, MA, U.S.A.). All other chemicals were of analytical grade and purchased from Sigma.

Proteins

Monoclonal antibodies (mAbs) 6F7, 12C5 and 12D6 and rabbit antisera raised against GHAP and versican isolated from human brain have been described previously [3,5,21]. Human brain versican and GHAP were prepared as described earlier in the presence of protease inhibitors [3,5]. Human brain versican was

Abbreviations used: CNS, central nervous system; GHAP, glial hyaluronate-binding protein; mAb(s), monoclonal antibody(ies); MMP, matrix metalloproteinase; p-APMA, p-aminophenylmercuric acetate; PVDF, poly(vinylidene difluoride).

* Dedicated to the memory of Amico Bignami, who died on 5 August 1994.

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also prepared after extraction by digestion with testicular hyaluronidase. Briefly, 50 g of human brain was homogenized in 150 ml of 10 mM Tris acetate, pH 7.6, in the presence of 10 mM EDTA, 1 mM PMSF, 1.5 μ M pepstatin (1 μ g/ml) and 1 mM iodoacetic acid. The homogenate was centrifuged at 20000 *g* for 10 min, the pellet was washed once and the final pellet was resuspended in the same buffer containing 25 mg of testicular hyaluronidase (1200 turbidity reducing units per mg). The mixture was incubated for 3 h at 37 °C and centrifuged at 20000 *g* for 10 min. The supernatant was adjusted to 6 M urea and subjected to anion-exchange chromatography followed by gel-filtration chromatography [5].

Recombinant human procollagenase and prostromelysin were expressed as described previously [22,23]. The enzymes were purified using a combination of ion-exchange and dye-matrix chromatography. Human stromelysin [24] from articular cartilage was kindly provided by Dr. H. Nagase (University of Kansas). Mouse gelatinase was prepared from 3T3 mouse fibroblasts cultured in 10% fetal bovine serum in Dulbecco's modified Eagle's medium at 37 °C in an atmosphere of 5% CO₂ and 100% humidity. The culture medium was removed, the flask washed twice with medium without serum and the cells were further cultured in Dulbecco's modified Eagle's medium without serum. After 3 days the medium was removed, centrifuged at 10000 *g* for 10 min at 2 °C to remove any debris and the supernatant was applied to a gelatin-Sepharose column (10 ml) at a 10 ml/h flow rate. The column was washed with 200 ml of 10 mM Tris acetate, pH 7.6, containing 10 mM CaCl₂ and 1 M NaCl and bound gelatinase was eluted with the same buffer containing 5% DMSO.

Human brain gelatinases were prepared from brain removed during autopsy (12–18 h after death); 50 g of human brain was homogenized in 150 ml of 10 mM Tris acetate, pH 7.6, in the presence of 10 mM CaCl₂ to stabilize the metalloproteinases and 1 mM PMSF, 1 μ g/ml pepstatin and 1 mM iodoacetic acid as inhibitors. The homogenate was centrifuged at 20000 *g* for 10 min and the supernatant was applied to a Q-Sepharose fast flow column (2 cm $\times$ 10 cm) at a 50 ml/h flow rate. The column was washed with 100 ml of 10 mM Tris acetate, pH 7.6, followed by 100 ml of 250 mM NaCl/10 mM CaCl₂ in 10 mM Tris acetate, pH 7.6. The 250 mM NaCl eluant was diluted with an equal volume of 10 mM Tris acetate, pH 7.6, 10 mM CaCl₂ and was run through a 10 ml gelatin-Sepharose column. The column was washed with 200 ml of 10 mM Tris acetate, pH 7.6, containing 10 mM CaCl₂ and 1 M NaCl and bound metalloproteinases were eluted with the same buffer containing 5% DMSO.

Reduction and alkylation

Proteins were dissolved at a concentration of 1 mg/ml in 0.4 M Tris/HCl, pH 8.5, containing 6 M guanidine HCl, 2 mM EDTA and 3 mM dithiothreitol. The solution was purged with N₂ and incubated at 37 °C for 60 min. One-tenth volume of 200 mM iodoacetic acid (final concentration 20 mM) was added, the pH was adjusted to 7 with NaOH and further incubated for 1 h at 37 °C in the dark.

Analytical procedures

Protein determination was performed using BSA as a standard, either by measuring the absorption at 280 nm or at 595 nm using the Bio-Rad protein determination kit [25].

SDS/PAGE (4–15% polyacrylamide) was carried out as described previously [26] with the omission of 2-mercaptoethanol unless noted. Staining was performed using 0.25% Coomassie Brilliant Blue R-250 in 10% acetic acid/25% methanol for 1 h.

Destaining was performed overnight in 10% acetic acid/10% methanol. Versican, a chondroitin sulphate proteoglycan, is negatively charged. Therefore staining with Coomassie Brilliant Blue is very weak and difficult to photograph. Versican stains very intensely with silver because of the large amounts of carbohydrate. Versican stains much faster than the other proteins and appears as a large smear which leads to non-comparable results. To be able to photograph versican together with other proteins we stained gels first with Coomassie Brilliant Blue and then with silver for a limited time [27].

Immunoblots were performed as described [28] and reported earlier [5]. Occasionally the biotin-avidin phosphatase system from Vector was also used.

Zymograms employing 1% (w/v) gelatin or 1% (w/v) β -casein in 9% and 11% SDS/PAGE, respectively, were performed by including these proteins in the polyacrylamide solution prior to polymerization [29]. Samples to be tested for their metalloproteinase activity were not heated and were not treated with 2-mercaptoethanol. The electrophoresis was carried out at 2 °C and the gels were allowed to equilibrate for 4–5 h in 2.5% Triton X-100 while shaking at 2 °C (or 1 h at room temperature). The gels were then incubated in 20 mM Tris acetate, pH 7.6, 10 mM CaCl₂ for 16 and 40 h (overnight or 2 days) at 37 °C for detection of gelatinolytic and caseinolytic activity respectively.

Amino acid analysis and sequencing

Amino acid sequencing and *in situ* digestion with trypsin were performed as follows. Proteins separated by SDS/PAGE were transferred to PVDF as described [28]. Protein bands were stained with 0.1% Ponceau S in 1% acetic acid for 60 s followed by destaining with 1% acetic acid. After several washes with water the bands of interest were cut out and subjected to amino acid analysis to determine the amount of protein transferred to the membrane.

For *in situ* digestion the PVDF membrane was cut into 1 mm $\times$ 2 mm pieces and placed in 100 μ l of 10% acetonitrile/1% Triton X-100/100 mM Tris/HCl, pH 8.0/0.5 μ g of trypsin previously reduced and methylated to prevent autolytic digestion and treated with *N*-tosyl-L-phenylalanine chloromethyl ketone to remove any chymotrypsin-like activity (Promega, Madison, WI, U.S.A.). The incubation at 37 °C was allowed to proceed for 24 h. The resulting peptide mixture was separated by narrow-bore HPLC using a 2.1 mm $\times$ 150 mm Vydac C-18 reverse-phase column on a Hewlett-Packard 1090 HPLC with 1040 diode-array detector. Separation of the peptides was achieved using HPLC with a gradient of 5 to 90% acetonitrile in water with 0.06% trifluoroacetic acid over a period of 90 min. Optimum fractions were chosen based on symmetry, resolution and UV absorbance, and submitted to automated Edman degradation on an Applied Biosystems model 477A protein sequencer. Strategies for peak selection, reverse-phase separation and protein microsequencing have been previously described [30]. All sequenator reagents and solvents were from Applied Biosystems, Inc.

For C-terminal sequencing PVDF membrane containing the protein of interest was cut into 1 mm $\times$ 2 mm pieces and incubated at 37 °C for 30 min with 0.5% poly(vinylpyrrolidone) in 100 mM acetic acid and then washed thoroughly (ten times) with water. The PVDF membranes were incubated for 1 h with 50 mM sodium citrate, pH 4.5, containing 5% acetonitrile at room temperature and then for 2 h with 0.5 μ g of carboxypeptidase Y at 37 °C in 5% acetonitrile/50 mM sodium citrate, pH 4.5. The solution was then subjected to amino acid analysis without hydrolysis.

RESULTS

GHAP and versican in bovine and human brain

We have previously reported that the monoclonal antibodies (mAbs) 6F7, 12C5 and 12D6, raised against GHAP [21], do not react with versican on immunoblots [5]. We first determined whether GHAP and versican have the same electrophoretic mobility under reduced and non-reduced conditions. GHAP treated with reducing agents appears as a band with a molecular mass of 60 kDa [3] while GHAP not treated with reducing agents migrates as a band corresponding to a molecular mass of 53 kDa (Figure 1). Versican's electrophoretic mobility was unaffected by the presence or absence of 2-mercaptoethanol. The polyclonal antibody raised against GHAP [3] recognized GHAP and versican in immunoblots regardless of the treatment with reducing agents (Figure 1). The 6F7 and 12C5 mAbs did not recognize versican under reduced conditions while they reacted with non-reduced versican. The mAbs also recognize the non-reduced GHAP better than the reduced GHAP (Figure 1). Identical results were obtained using the 12D6 mAb raised against GHAP (results not shown). The 6F7 (Figure 1), 12C5 and 12D6 mAbs (results not shown) did not recognize at all reduced and alkylated GHAP and versican, suggesting that the antigenic epitopes are disulphide bond-dependent.

The GHAP preparation requires extraction under acidic conditions and three 30 min centrifugations in different pH and salt concentrations [3]. Therefore, there has been the suspicion that GHAP may not exist *in vivo*, but is rather a product of proteolytic digestion occurring during the preparation [31]. To address this question we first investigated whether human brain contains GHAP prior to the acidic extraction. Human brain white matter (200 mg) obtained during autopsy was homogenized in 1 ml of ice-cold 10 mM Tris acetate, pH 7.6, in the presence of

Figure 1 Monoclonal antibodies against GHAP recognize only non-reduced versican

Isolated GHAP (G) (1 μ g) and versican (V) (10 μ g) were separated in the presence (+) or absence (—) of 2-mercaptoethanol using 4–15% SDS/PAGE. Proteins from the gels were stained with Coomassie Brilliant Blue followed by silver staining (C.B.B./Silver) or electrophoretically transferred to PVDF [23] and subjected to immunoblotting with a rabbit antiserum and the 6F7 and the 12C5 mAbs raised against GHAP. Note that non-reduced GHAP has an electrophoretic mobility corresponding to a lower-molecular-mass protein (53 kDa) than reduced GHAP (60 kDa). The mAbs 6F7 and 12C5 do not react with reduced GHAP as well as they react with non-reduced GHAP, while they recognized only the non-reduced versican. The mAb 6F7 did not react with reduced and alkylated GHAP and versican (r/a). M, high-molecular-mass markers from Pharmacia-LKB from top to bottom: myosin (212 kDa), α_2 -microglobulin (170 kDa), β -galactosidase (116 kDa), transferrin (76 kDa) and glutamic dehydrogenase (53 kDa). M', low-molecular-mass standards from Pharmacia-LKB from top to bottom: phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin, (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), α -lactalbumin (14.4 kDa).

Figure 2 GHAP in human and bovine white matter

Human (a) and bovine (b) brain white matter were homogenized in the presence of protease inhibitors with 5 vol. of ice-cold 10 mM Tris acetate, pH 7.5. The homogenate was centrifuged for 2 min at 2 °C at 8000 g. 10 μ l of the human and bovine supernatant (W), isolated human and bovine GHAP (G), and human versican (V) were subjected to 4–15% SDS/PAGE. The proteins were stained with Coomassie Brilliant Blue followed by silver staining (C.B.B./Silver) or electrophoretically transferred to PVDF [23] and subjected to immunoblotting with the 6F7 monoclonal antibody. M, high-molecular-mass markers; M', laminin (400 and 200 kDa) on top and low-molecular-mass markers as in Figure 1.

1 mM PMSF, 1 μ g/ml pepstatin, 1 mM iodoacetic acid, 10 mM EDTA and 1 mM 1,10-phenanthroline to inhibit serine-, aspartic acid-, cysteine-, Ca^{2+} -activated proteinases and metalloproteinases respectively. The homogenization occurred within 30 s and the homogenate was centrifuged in a microfuge at 8000 g for 2 min at 2 °C. The supernatant was used for 4–15% SDS/PAGE in the absence of 2-mercaptoethanol and stained with Coomassie Brilliant Blue followed by silver staining or subjected to immunoblotting with the 6F7 mAb. Both versican and GHAP were observed in the immunoblot (Figure 2a). The length of the centrifugation or the pH of the extraction did not seem to have any effect on the amount of the GHAP present in the extract as long as the pepstatin was freshly prepared. When the extraction was performed at low pH and pepstatin was omitted, GHAP appeared at a lower molecular mass (results not shown). At neutral pH the absence or presence of pepstatin did not affect the electrophoretic mobility of GHAP. The time interval, however, between death and autopsy required to obtain the material is usually between 8 and 16 h. There was therefore concern that versican could be degraded and give rise to GHAP due to autolysis. To reduce the time interval from death to brain removal to a minimum, we used bovine instead of human brain. In SDS/PAGE, under reducing conditions, isolated bovine GHAP appears as four bands at 76, 66, 54 and 52 kDa [32]. Bovine brain was obtained from a local slaughterhouse. Small pieces (0.2–0.5 g) of brain white matter were frozen in liquid nitrogen within 15 min of death and transported to the laboratory. The tissue was subjected to the short extraction and immunoblot as described above. The immunoblots again showed that GHAP exists together with versican (Figure 2b).

To determine whether versican and GHAP are present in other areas of the central nervous system (CNS) we obtained tissues, including cerebral grey and white matter, spinal cord and cerebellum, from human CNS. The tissue was obtained as described above, the proteins were extracted in the presence of

Figure 3 Versican in human and bovine grey and white matter

Human brain white (W) and grey matter (G), cerebellum (C) and spinal cord (SC) were homogenized in the presence of protease inhibitors with 5 vol. of ice-cold 10 mM Tris acetate, pH 7.5. The homogenate was centrifuged for 1 min at 2 °C and 10 µl of the supernatant were run in 4–15% SDS/PAGE. The proteins were stained with Coomassie Brilliant Blue followed by silver staining (C.B.B./Silver) or electrophoretically transferred to PVDF [23] and subjected to immunoblotting with the 6F7 monoclonal antibody.

protease inhibitors, and immunoblot analysis was performed to determine the presence of versican and GHAP in these extracts (Figure 3). Versican and GHAP were present at the highest levels in cerebral white matter and only small amounts of versican were present in the human cerebral grey matter. This is in agreement with our immunohistochemical findings [3,7]. Cerebellum and spinal cord also contained versican and GHAP; comparisons, however, of the amounts between these tissues are not warranted since the tissue from cerebellum and spinal cord contained both white and grey matter.

Metalloproteinase digestion of versican

Metalloproteinases digest aggrecan separating the hyaluronate-binding region from the chondroitin sulphate-rich region of the molecule. Collagenase (MMP1), gelatinases A and B (MMP2 and 9), stromelysin (MMP3) and PUMP (MMP7) have been reported to cleave the large hyaluronate-binding proteoglycan aggrecan [15–17]. We therefore hypothesized that GHAP may be the result of metalloproteinase digestion of versican. To test the hypothesis, versican was incubated with human recombinant stromelysin at 37 °C in the presence of 10 mM CaCl₂. After a 16 h period the proteoglycan was partially digested, shifting to a slightly lower molecular mass. At the same time a new band appeared with electrophoretic mobility identical to that of GHAP (53 kDa under non-reduced conditions) (Figure 4a). The anti-serum raised against versican recognized all newly generated polypeptides. The 6F7 mAb, raised against GHAP, did not react with the large fragment but recognized the newly generated 53 kDa band (Figure 4a). Amino acid sequencing of a polypeptide derived after trypsin digestion of the 53 kDa band revealed the sequence LATVG, which corresponds to the 277–281 amino acid sequence of versican [4]. Human stromelysin from articular

Figure 4 Stromelysin, collagenase and gelatinase digestion of versican

(a) Human brain versican (80 µg) was incubated with 0.216 µg of human recombinant stromelysin in the presence of 1 mM *p*-APMA, 10 mM CaCl₂, 150 mM NaCl and 20 mM Tris acetate, pH 7.5, at 37 °C for 16 h. The reaction was stopped by boiling the mixture in SDS-sample buffer containing 10 mM EDTA, and 32 µg of initial versican were used for 4–15% SDS/PAGE. Proteins were silver-stained or immunoblotted with the rabbit antiserum raised against versican or the 6F7 mAb raised against GHAP. V, control versican; Vs, stromelysin-digested versican; G, human GHAP. (b) Human brain versican isolated after hyaluronidase digestion (117 µg) (V') was incubated with 0.32 µg of human recombinant stromelysin (V's), 0.24 µg human recombinant collagenase (V'c) or 0.12 µg of mouse gelatinase (V'g) in the presence of 10 mM CaCl₂, 2 mM *p*-APMA and 40 mM Tris acetate, pH 7.5, at 37 °C for 40 h. The reaction was stopped by boiling the mixture in SDS-sample buffer and proteins were subjected to 4–15% SDS/PAGE. Proteins were stained with Coomassie Brilliant Blue (58 µg of initial versican) or subjected to immunoblot with the 6F7 mAb (10 µg of initial versican). G, human GHAP; M and M', high- and low-molecular-mass markers as in Figure 1 including laminin (400 and 200 kDa).

cartilage gave similar results as recombinant stromelysin (results not shown).

Versican isolated from human brain extracted by digestion with testicular hyaluronidase contains little or no chondroitin sulphate (versican') and stains better with Coomassie Brilliant Blue than versican not digested with testicular hyaluronidase. Stromelysin digestion of versican' proceeded much faster than digestion of natural versican. In fact, a major band at 53 kDa was apparent by Coomassie Brilliant Blue staining (Figure 4b). It is possible that the removal of the chondroitin sulphate chains allows a better digestion of the versican by stromelysin. Identical results were obtained after stromelysin digestion of chondroitinase-digested versican [4] (results not shown). Limited proteolysis of versican' with human recombinant collagenase and 3T3 mouse gelatinase in the presence of 1 mM *p*-aminophenylmercuric acetate (*p*-APMA) was monitored by immunoblot. Newly generated GHAP was observed with both enzymes (Figure 4b).

Isolation of human brain gelatinases

To determine whether brain contains any metalloproteinase activity which could digest versican, we used bovine and human brain, as well as rat spinal cord extracts. In all three cases, endogenous versican was completely digested after 24 h in the presence of 2 mM *p*-APMA, while no digestion was observed after incubation in the presence of EDTA which inhibits all metalloproteinases and other Ca²⁺-dependent proteinases (results not shown). Gelatinolytic activity in human and rat brain has been reported [33,34]. In an effort to isolate the gelatinases present in the human brain, low-ionic-strength brain extracts

Figure 5 Isolation of human brain gelatinases

(a) Human brain gelatinases (0.1 μ g) (HBG), isolated as described in the Materials and methods section, were separated in 4–15% SDS/PAGE and stained with Coomassie Brilliant Blue followed by silver staining (Silver). Arrows point to the gelatinases at 90, 130 and 230 kDa. Note that the preparation contains two additional bands (arrowheads). M and M', high- and low-molecular-mass standards as described in Figure 1. (b) Human recombinant collagenase (0.3 μ g) (C), mouse gelatinase (0.1 μ g) (G), human recombinant stromelysin (0.5 μ g) (S) and human brain gelatinases (1 μ g) (HBG) were subjected to zymography in 1% gelatin- and casein-containing gels. Gelatin-containing zymograms contained 9% polyacrylamide while casein-containing zymograms contained 11% polyacrylamide to ensure immobilization of gelatin and casein respectively. Arrows point to the gelatinolytic and caseinolytic bands. The difference of the polyacrylamide content in the three gels accounts for the differences in the electrophoretic mobility of the human brain gelatinases.

were applied to a Q-Sepharose fast flow column, followed by affinity chromatography on gelatin-Sepharose as described in the Materials and methods section. The gelatinase activity was followed using gelatin-containing zymography. We observed all four gelatinases previously reported to exist in the human brain (approx. 70, 90, 130 and 250 kDa) [33,35] at similar molecular masses (64, 90, 125 and 230 kDa). Elution of bound proteins from the gelatin-Sepharose column occurs with 5% DMSO. The 64 kDa gelatinase was eluted two fractions later and could be thus separated from the other gelatinases. The isolated gelatinases were subjected to 4–15% SDS/PAGE and stained with Coomassie Brilliant Blue followed by silver staining to determine if any other proteins were present in the preparation (Figure 5). To determine whether this preparation contains enzymes with gelatinolytic and caseinolytic activity zymography was performed with 9 and 11% polyacrylamide respectively. We chose a different polyacrylamide percentage since casein and gelatin are mobile in low percentage polyacrylamide gels and it becomes difficult to demonstrate enzymic activity. The enriched proteinases possess not only gelatinolytic but also caseinolytic activity (arrows in Figure 5b).

Digestion of versican with human brain gelatinases

Versican isolated after extraction by hyaluronidase digestion of human brain was incubated with these gelatinases in the presence of 1 mM *p*-APMA and 10 mM CaCl_2 for 20 h. Several polypeptides were observed by 4–15% SDS/PAGE followed by silver staining and a major band with the same molecular mass

Figure 6 Digestion of versican with human brain gelatinases

Versican isolated after hyaluronidase digestion (117 μ g) (V') was incubated with human brain gelatinases (0.5 μ g) in the presence of 1 mM *p*-APMA, 10 mM CaCl_2 , 40 mM Tris acetate, pH 7.4, at 37 °C for 20 h (V'g). The reaction was stopped by boiling the mixture for 1 min in SDS-sample buffer. The proteins were separated by 4–15% SDS/PAGE and stained with Coomassie Brilliant Blue or subjected to immunoblotting with the 6F7 mAb. V'a, Versican incubated as above with *p*-APMA in the absence of human brain gelatinases; V'c, versican incubated as above in the presence of human brain gelatinases in the absence of *p*-APMA; G, human GHAP; and M', high- and low-molecular-mass standards as described in Figure 1.

as GHAP (Figure 6). The newly generated polypeptide reacted with the 6F7 mAb raised against GHAP (Figure 6). After prolonged incubation (72 h) of versican even the generated polypeptide was digested (results not shown). To determine if the versican preparation contains any (metallo-)proteinase activity, versican was incubated without human brain gelatinases in the presence of *p*-APMA and 10 mM CaCl_2 (Figure 6, V'a). To determine whether the preparation of human brain gelatinases can digest versican due to other proteinolytic activity, versican was incubated with human brain gelatinases without *p*-APMA (Figure 6, V'c). No versican digestion was observed under either of these conditions after 20 h at 37 °C.

Samples (60 μ g) of versican digested with human brain gelatinases and 10 μ g of GHAP were subjected to 4–15% SDS/PAGE and transferred to PVDF membranes. GHAP and the band with electrophoretic mobility identical to GHAP were excised and digested *in situ* with trypsin as described in the Materials and methods section. The chromatograms from the peptide maps obtained at 210 nm (peptide bonds) were virtually identical (Figure 7). The only qualitative difference is an additional peak in the tryptic map from the polypeptide obtained after versican digestion (arrowhead in Figure 7). The peptide was sequenced and found to belong to the hyaluronate-binding region of versican starting at the 171st amino acid (ACLDV). Quantitative differences between the chromatograms obtained at 210 nm are noted with arrows. The peak noted with the large arrow was sequenced and found to start at the 234th amino acid of versican, which is also part of the hyaluronate-binding region. The sequencing revealed that several amino acids were released

Figure 7 Peptide maps of naturally occurring and newly generated GHAP

Samples (10 µg) of naturally occurring GHAP and the polypeptide generated after digestion of 80 µg of versican' with human brain gelatinases (V'g) were electrophoresed in 4–15% SDS/PAGE transferred to PVDF and subjected to digestion with 0.5 µg of trypsin. The mixtures were subjected to reverse-phase HPLC as described in the Materials and methods section. The elution of the various peptides was monitored at 210 nm and aromatic amino acid-containing peptides at 277 nm. Arrows point to quantitative differences between the two chromatograms, the arrowhead indicates a qualitative difference and asterisks point to peaks from both chromatograms that have been subjected to sequencing.

after each cycle of Edman degradation, suggesting that the peak from both chromatograms contained several additional polypeptides, which could account for the difference in absorption. Two peaks were sequenced and found to be identical starting at the 101st (VSVPT) and 289th (NGFDQ) amino acid of versican (Figure 7, asterisks from left to right). The chromatograms obtained at 277 nm (aromatic amino acids) were also similar with the only differences being the ones mentioned above (large arrow and arrowhead).

Edman degradation of the N-terminus of GHAP and the newly generated polypeptide after digestion of versican with the human brain gelatinases did not yield any amino acid sequence data. To determine the C-terminal sequence the following strategy was employed. GHAP (10 µg) and the newly generated polypeptide were electrophoresed on 4–15% SDS/PAGE, transferred to PVDF membranes and stained with Ponceau S. The membranes were incubated with 0.5 µg of carboxypeptidase Y in 50 mM sodium citrate. Carboxypeptidase digests amino acids from the C-terminus. Amino acid analysis of the supernatants

Table 1 Amino acid analysis after carboxypeptidase Y digestion of GHAP and the polypeptide generated by human brain gelatinases digestion of versican

Sixty µg of versican were digested with human brain gelatinases. Ten µg of GHAP and the digested versican were electrophoresed in a 4–15% SDS/PAGE, transferred to PVDF membranes and stained with Ponceau S. GHAP and the band with electrophoretic mobility identical to GHAP were excised and incubated for 1 h with 50 mM sodium citrate, pH 4.5, containing 5% acetonitrile at room temperature and then for 2 h with 0.5 µg of carboxypeptidase Y in the same buffer. The solution was then subjected to amino acid analysis. Control values were obtained by digestion of blank PVDF membrane with carboxypeptidase Y. Values represent picomoles of each amino acid and are the average of four experiments.

Amino acid	GHAP (pmol)	Newly generated polypeptide (pmol)
Asx	27.9	24.8
Glx	15.4	17.0
Ser	6.7	6.4
Gly	18.8	7.8
His	0	0.2
Arg	4.3	0.9
Thr	13.6	15.9
Ala	15.3	16.7
Pro	6.0	4.8
Tyr	4.1	3.9
Val	83.0	40.8
Met	1.8	0
Cys	0.2	0.6
Ile	4.2	7.2
Leu	6.0	6.2
Phe	5.1	4.7
Lys	5.3	4.7

revealed that the main amino acid released was Val, followed by Asx > Glx > Ala > Thr (Table 1). This is consistent with the primary sequence of versican at the position ⁴⁰⁸EEEEECANATDV₄₁₆. Gly is in the transfer buffer and was ignored. These results suggest that both GHAP and the newly generated polypeptide are identical in their respective C-termini, and that the cleavage occurs after the last cysteine of the hyaluronate-binding region of versican.

GHAP and versican in calf and bovine brain and spinal cord

Newborn cartilage contains undetectable amounts of the hyaluronate-binding region, while mature cartilage contains amounts large enough to prevent the formation of high-buoyant-density hyaluronate–proteoglycan aggregates [18]. In an attempt to determine whether this may be the case with versican in the CNS, cerebral white matter and spinal cord from a 1-year-old calf and 5-year-old cow were extracted as described previously.

Proteins corresponding to equal amounts of wet weight material were separated by 4–15% SDS/PAGE and subjected to Coomassie Brilliant Blue staining which showed that it corresponded to identical amounts of protein (Figure 8). The presence of versican and GHAP was monitored using Western blot analysis with the 6F7 mAb. The amount of versican and GHAP are increased in the adult compared with that found in the calf, both in the brain and in the spinal cord. Although versican is expressed in both calf and cow, GHAP is almost undetectable in the calf (except the 74 kDa band; Figure 8). The reactive band corresponding to versican and the four bands corresponding to the isolated bovine GHAP were scanned. Analysis using the NIH Image program indicated that there is an 80% increase of versican in the cerebral white matter and a 1060% increase of

Figure 8 GHAP and versican in young and adult bovine

Material from a 1-year-old calf and 5-year-old cow were obtained at a local slaughterhouse. Small pieces (approx. 0.5 g) of brain white matter and spinal cord from both animals were obtained within 15 min of death, frozen in liquid nitrogen and transported to the laboratory. The tissue was homogenized and 20 μ l (corresponding to 4 μ g of wet weight initial material) was used for 4–15% SDS/PAGE. Proteins were stained with Coomassie Brilliant Blue followed by silver staining (C.B.B./Silver) or stained with the monoclonal antibodies 6F7 raised against GHAP. Note the difference between the young calf and the adult cow on the amount of versican and GHAP.

GHAP. Similarly, in the spinal cord the increase of versican and GHAP is 160% and 550% respectively.

DISCUSSION

We have previously reported that the mAbs raised against GHAP do not recognize versican in immunoblots, while a rabbit antiserum does [5]. We have observed that areas of the nervous system which tested negative for GHAP in immunoblots were positive by immunofluorescence. Here we show that the mAbs fail to recognize versican treated with reducing agents (i.e. 2-mercaptoethanol) but react well with untreated versican. Similarly the antibodies recognize reduced GHAP less efficiently than non-reduced. Versican from human brain (365 kDa), a chondroitin sulphate proteoglycan with a large content of carbohydrate, is a much larger protein than GHAP (60 kDa). The chances of the disulphide bonds forming correctly after treatment with 2-mercaptoethanol are smaller for versican than for GHAP. In fact, the mAbs did not recognize reduced and alkylated GHAP or versican and we conclude that the antigenic determinants are disulphide bond-dependent.

The origin of GHAP has been debated frequently [4,31]. We first investigated whether GHAP is in fact present in brain and is not a result of the homogenization of the tissue at low pH. Human brain GHAP and versican were extracted at neutral pH from material obtained during autopsy. The time interval between death and autopsy, however, is usually rather long (8–16 h) and the presence of GHAP could be due to autolysis. We

therefore tested bovine brain removed and frozen at the slaughterhouse immediately after death (within 15 min). In this case too, immunoblots indicated the presence of both GHAP and versican. Given the time required to generate GHAP by metalloproteinase digestion of versican (24 h) and the need to activate the metalloproteinases with *p*-APMA, it is unlikely that the presence of EDTA and the 30 min it takes for the homogenization at 2 °C allow the digestion of versican and the generation of GHAP. We conclude, thus, that GHAP is present in the CNS *in vivo*.

Matrix metalloproteinases digest aggrecan, the major aggregating proteoglycan in articular cartilage, both *in vitro* and *in vivo* [15–17,19,20]. We hypothesized that versican may be susceptible to digestion by MMPs as well. Digestion with human recombinant collagenase, human recombinant stromelysin and mouse gelatinase A gave rise to a stable polypeptide with a molecular mass identical to GHAP. This polypeptide reacts with the antisera and mAbs raised against versican and GHAP.

There have been several reports in the literature on the secretion of metalloproteinases by brain tumours [35], glioma [36–40] and neuronal [41–43] cell lines. It has also been shown that gelatinases are present in the human [33] and rat [34] brain. We isolated these gelatinases from human brain by means of anionic and affinity chromatography. We obtained highly enriched gelatinases which possess both gelatinolytic and caseinolytic activity. The preparation contains the 90, 130 and 230 kDa proteins reported earlier [33]. Two additional bands at 58 and 190 kDa were present on silver-stained gels but did not possess any caseinolytic or gelatinolytic activity.

The enriched metalloproteinase preparation was used for digestion of versican. Similarly to the other metalloproteinases tested, the human brain gelatinases gave rise to a polypeptide with molecular mass identical to that of GHAP, which reacts with antibodies raised against GHAP (Figures 4 and 6). Digestion of GHAP and the newly generated polypeptide with trypsin revealed identical peptide maps (Figure 7), and C-terminal sequencing suggested that the two polypeptides have identical C-termini. We conclude thus that the newly generated polypeptide is identical to GHAP. We suggest that GHAP is an *in vivo* product of versican degradation in the brain.

The C-terminal sequence of GHAP and the newly generated GHAP indicates that the human brain gelatinases cleave versican at the $_{416}\text{VT}_{417}$ position. Although this position has not been predicted to be a preferred one for metalloproteinase digestion [44], we obtained results suggesting it as the site of cleavage. The physiological degradation of aggrecan has been reported with respect to articular cartilage. The hyaluronate-binding region is found in adult but not in newborn human cartilage and competes for binding to hyaluronate [18], while the large chondroitin sulphate-containing fragment is further digested and diffuses into the synovial-joint space. Similarly, hyaluronate-binding fragments have been found in increased amounts in cartilage of aging individuals [45]. Increased concentrations of metalloproteinases have been found in cartilage from patients with rheumatoid arthritis and osteoarthritis [46]. Versican bound to hyaluronate creates a network surrounding certain neurons and myelinated axons in the CNS. Neurocan in the developing rat [47] and the Cat-301 chondroitin sulphate proteoglycan in the grey matter [48] may also participate in this network, which may serve the following functions. It has been suggested that extracellular matrix specifically binds to growth factors to increase their concentration locally [49]. The proteoglycans with the highly charged chondroitin sulphate chains may regulate ion transport between cells. A mechanical function is also envisaged. Immunofluorescence images of cryostat sections with antibodies

against versican give the impression of barbed wire surrounding the myelinated axons and the large motor neurons providing a scaffold on which the neurons are stabilized. Degradation of the network may cause growth factor depletion, abnormalities in ion and small molecule supply, or destabilization of neuronal positioning. While at the initial stages the degradation of the extracellular matrix does not pose a problem, as it progresses it may cause heavy loss of neurons and have deleterious effects leading to dementia. Here we present evidence that in adult bovine brain there is an increased ratio of GHAP to versican compared with calf brain. In fact, we have recently reported that there is increased immunostaining of GHAP in the cerebral cortex of Alzheimer's patients and we suggested a role for matrix metalloproteinases in senile dementia [50]. We are presently investigating whether there are significant differences in the versican and GHAP content of young, healthy old, and demented individuals.

Versican is a major component of the extracellular matrix of the CNS (approx. 20 mg of versican per 100 g of tissue). GHAP has been found to be non-permissive for neuronal growth [51]. GHAP and versican are expressed very late in development, i.e. post-natally and after the onset of myelination, in dog [52], rat [6] and mouse [7] brain. Gliomas are notorious for their invasiveness. Brain tumours [35] and glioma cell lines secrete stromelysin and gelatinases [36–40]. Thus far no substrate has been determined for these metalloproteinases in the adult CNS except in the connective tissue forming blood vessels and the meninges. Spreading of the C6 glioma cells on CNS myelin or optic nerve explants was inhibited by 1,10-phenanthroline, a metalloproteinase inhibitor [46]. It has been suggested that the myelin-bound inhibitory proteins may be substrates for metalloproteinase activity during glioblastoma infiltration of the CNS [53]. We suggest that gliomas secrete high levels of MMPs which digest versican, paving the way for the invasion of the tissue by the tumour cells.

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