Histol Histopathol (2003) 18: 1067-1080

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Immunohistochemical evaluation of versican, in relation to chondroitin sulphate, in canine mammary tumours

I. Erdélyi[1], D.H.M. Nieskens[1], J.E. van Dijk[1], L. Vass[2] and H. Nederbragt[1]

[1]Department of Pathobiology, Faculty of Veterinary Medicine, Utrecht University,Utrecht, The Netherlands and [2]P.M.Flór Ferenc Hospital, Division of Pathology-Histopathology-Cytology, Kistarcsa, Hungary

Summary. The expression of increased amounts of versican, a chondroitin sulphate proteoglycan, in neoplastic tissues may play a role in promoting tumour cell proliferation and migration. This study investigated the immunolocalization of versican in normal and neoplastic canine mammary tissues, using antibodies 12C5 and 2B1, against different epitopes of the protein core of versican. Antibody CS56, recognising chondroitin sulphate (CS), was used to investigate the relation between versican and CS, which accumulates in canine mammary tumours. We found enhanced versican expression in both benign and malignant tumours, appearing in three main patterns: in periductal tissues, probably in association with basement membranes of ducts; in peripheral invasive areas of malignant tumours; and in spindle cell proliferations and myxoid areas of complex and mixed tumours. The 12C5 and 2B1 immunoreactivities co-localised in all types of tumours, and could be improved by chondroitinase digestion. The only exception was the abundant extracellular matrix (ECM) of spindle cell proliferations, particularly in myxoid areas of complex and mixed tumours, which displayed intense and diffuse 12C5 immunoreactivity and patchy or absent 2B1 and CS56 immunoreactivities; versican immunoreactivity could not be enhanced by chondroitinase digestion. The results indicate that versican is one of the extracellular matrix components characteristic of canine mammary tumours. It appears likely that in complex and mixed tumours versican exists in at least two forms, one of them lacking the CS attachment domain and the 2B1 epitope. Furthermore, the enhanced versican expression in the invasive areas of malignant tumours indicates the involvement of this proteoglycan in tumour cell invasion.

Key words: Versican, Chondroitin sulphate, Proteoglycan, Extracellular matrix, Canine mammary tumour

Offprint requests to: I. Erdélyi, Department of Pathobiology, Faculty of Veterinary Medicien, Utretcht University, P.B. 80.158, 3508 TD Utrecht, The Netherlands. e-mail: I.Erdelyi@vet.uu.ul

Introduction

Among the multiple components of the extracellular matrix (ECM) in neoplastic tissues, the proteoglycans (PGs), such as versican, are in a central position because of their role in cell adhesion and proliferation (Ruoslahti, 1989).

Versican, also called PG-M, belongs to the family of large aggregating chondroitin sulphate (CS)-containing PGs (Ruoslahti, 1989; Kjellen and Lindahl, 1991) which also includes the cartilage-derived aggrecan, and two smaller PGs expressed in nervous tissues, namely neurocan and brevican (Iozzo, 1998). These PGs have common features such as the tridomain structure of the protein core: a highly similar hyaluronan-binding domain (G1) at the N-terminal region; a central domain skip (G2) bearing the glycosaminoglycan (GAG) attachment sites; and a selectin-like domain (G3) composed of epidermal growth factor (EGF)-like, lectin-like, and complement regulatory protein (CRP)-like elements at the C-terminal globular region (Margolis and Margolis, 1994). Versican has a strong hyaluronan (HA) binding affinity via its G1 domain (LeBaron et al., 1992) and its G3 domain has been shown to bind tenascin-R (Aspberg et al., 1997), fibulin-1 (Aspberg et al., 1999), fibrillin-1 (Isogai et al., 2002), ß1-integrin (Wu et al., 2002) and several carbohydates (Ujita et al., 1994). The central domain of versican is subdivided into two large subdomains, designated GAG-α and GAG-ß that are encoded by two alternatively spliced exons (Shinomura et al., 1995). As a result of alternative splicing the mammalian versican exists at least in four isoforms, namely V0, V1, V2 and V3, which may express different functions. The largest V0 isoform is known to contain both αGAG and ßGAG exons, V1 containing the ßGAG exon, V2 having the ßGAG, and V3 lacking any GAG subdomain. Thus far, protein products have been demonstrated for the V0, V1, and V2 forms which differ in length and in number of attached CS chains (Dours-Zimmermann and Zimmermann, 1994; Ito et al., 1995).

Versican localises in a variety of human and animal tissues (Bignami et al., 1993; Yamauchi et al., 1997). It

has been identified in the loose connective tissue of various organs, often associated with the elastic fiber network, in smooth muscle, cartilage, veins and elastic arteries, and in the central and peripheral nervous system (Yao et al., 1994; Bode-Lesniewska et al., 1996). It has also been detected in the proliferative zone of the epidermis and has been proven to be involved in hair follicle development (Zimmermann et al., 1994; du Cros et al., 1995). The exact function of versican remains elusive, although the molecule appears to have several functions. It may be involved in intercellular signaling through its lectin- and EGF-like sequences and may take part in cell recognition via interaction with other ECM components and cell surface molecules.

Abnormal versican deposition has been observed in a number of tumour types, including human colon (Adany and Iozzo, 1990), prostate (Ricciardelli et al., 1998) and breast cancer (Nara et al., 1997; Ricciardelli et al., 2002), and the pleomorphic adenoma of the salivary glands (Zhao et al., 1999). Furthermore, it has been described in brain tumours (Paulus et al., 1996) and in malignant melanoma (Touab et al., 2002), as well as in some other non-epithelial neoplasms (Ohiwa et al., 1991). It has been suggested that the versican-rich extracellular matrices exert an anti-adhesive effect on cells, thus facilitating tumour-cell migration and invasion (Yamagata et al., 1989).

Mammary tumours are the most frequent tumours in bitches and are notorious for their complexity in histological pattern and biological behaviour. On the basis of the cell types and ECM components present in the tumour, they are classified histologically as simple, complex and mixed tumours. Simple tumours are composed of only one type of proliferating epithelial cell. In complex tumours, the luminar epithelium and the spindle-shaped cells of possible myoepithelial origin largely contribute to tumour formation. The stroma of mixed tumours is characterized by the presence of cartilage and bone, and tumourous epithelial and mesenchymal cells can proliferate simultaneously. There is only scarce literature concerning the PG content of canine tumours. In mammary gland tumours, Palmer and Monlux (1979) have reported the presence of a tumour stroma rich in GAGs in complex carcinomas and Hinrichs et al. (1999) demonstrated an enhanced expression of CS, in particular in complex and mixed tumours. However, which CS-containing PGs are present in canine mammary tumours and the biological significance of these ECM compounds still remains to be elucidated. Therefore, in the present work we have performed immunohistochemical labelling to identify the large interstitial PG, versican, as a possible candidate for containing CS in mammary tumours (Nara et al., 1997), with two anti-versican monoclonal antibodies in a large number of mammary tumours. The relation between the distribution of versican and CS was also studied.

Table 1. Histological diagnosis of the 81 canine mammary tissues examined.

HISTOLOGY	NUMBER OF TUMOURS
Normal mammary tissue (n=10)	
Inactive	3
Lactating*	7
Benign tumour (n=37)	
Simple adenoma	
Tubulopapillary	12
Complex adenoma	15
Mixed tumour	10
Malignant tumour (n=34)	
Simple carcinoma	12
Tubulopapillary	6
Solid	4
Anaplastic	7
Complex carcinoma	
Mixed tumour with malignant epithelial component	5

*: 6 out of the 7 lactating mammary tissues also showed some regression. Classification according to WHO criteria (Misdorp et al., 1999).

Materials and methods

Antibodies

The antibodies used in the present study were: (1) monoclonal antibody 12C5 raised against versican isolated from human brain (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) and known to recognise the HA-binding domain of versican (Asher et al., 1991); (2) monoclonal antibody 2B1 against versican, purified from human yolk sac tumour (Seikagaku Co., Tokyo, Japan) (Sobue et al., 1989); and (3) monoclonal antibody CS56 (Sigma Chemical Co., St Louis, MO, USA), which reacts with chondroitin sulphate but not with dermatan sulphate (Avnur and Geiger, 1984), was used to confirm the presence and distribution of CS.

Tissues

The eighty-one mammary tissues examined in the present study were derived from 75 dogs and were resected surgically or obtained from the archives of necropsies of the Department of Veterinary Pathology, Utrecht University. The samples included inactive and lactating (secretory) normal mammary tissues, benign and malignant tumours. One out of the 7 lactating mammary tissues was derived from a pregnant dog and 4 were derived from pseudopregnant dogs with clinical lactation. There was no clinical information available concerning the ovarian cycle of the remaining two dogs with lactating (secretory) mammary tissues. The histological type and number of the examined tissues are

summarized in Table 1. Tissues were fixed immediately after surgery in 10% neutral-buffered formalin and embedded in paraffin, after which 5 mm sections were cut and stained with haematoxylin and eosin (H&E). Tumours were classified from the H&E-stained sections according to the recent WHO classification (Misdorp et al., 1999).

Follow-up of most animals was not available in this retrospective study. Malignancy was based on the evaluation of histologic features, predominantly on the growth pattern and cellular morphology. The term of infiltrating carcinoma was used in case of clear signs of stromal invasion of tumour cells regardless of the presence or absence of lymph/vascular invasion or lymph node metastasis.

Immunohistochemistry

Immunohistochemistry was performed using an indirect immunoperoxidase staining procedure, applying the avidin-biotin based technique (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA). After deparaffinisation and rehydration, the sections were washed in Tris-HCl buffer (0.25 M, pH 8.0). All incubation steps, except where stated, were performed at room temperature. Proteolytic demasking of antigens by trypsin increased the intensity of immunostaining for versican with antibody 2B1, but not with antibody 12C5 and did not improve CS immunolabelling with antibody CS56. Thus sections were incubated with 0.1% trypsin (Dako A/S, Glostrup, Denmark) in 0.01 M PBS with 0.1% CaCl2 (pH 7.8) at 37 ºC for 20 minutes only before the use of 2B1 antibody. We also used chondroitinase ABC (from Proteus vulgaris; Sigma Chemicals) digestion before the application of the primary antibodies. Digestion with chondroitinase ABC was performed at 37 °C for 2 hours with 0.5 U/ml of the enzyme in 0.25 M Tris buffer (pH 8.0) containing 0.18 M sodium chloride, 0.05% bovin serum albumin (BSA), 0.1 M 6-amino-n-caproic-acid and 5 mM benzamidine hydrochloride to inhibit protease activity. To control the specificity of chondroitinase pretreatment control slides were treated at the same time with the same protease inhibiting buffer without enzymes. All sections were rinsed with S-PBS (PBS with 0.03 % saponine), soaked in 0.3% H_2O_2 in S-PBS for 30 minutes to inhibit the activity of endogenous peroxidase, rinsed again, and then allowed to react with 10% normal horse-serum in 1% BSA-containing S-PBS, followed by overnight incubation with diluted primary monoclonal antibodies, 2B1 (1:1000), 12C5 (1:50) and CS56 (1:200), at 4 ºC. After incubation and washing, the sections were treated with biotinylated horse anti-mouse immunoglobulin diluted 1:125 in S-PBS, followed by incubation with avidin-biotin complex according to the manufacturer's instructions (Vector Laboratories). Reactions were visualised using 0.5% 3,3-diaminobenzidine tetrahydrochloride (Sigma Chemicals) and 0.3% H_2O_2 diluted in Tris/HCl buffer (0.05 M, pH 7.4), during a 10-minute incubation step. Sections were counter-stained with Mayer's hematoxylin and mounted. As negative controls, normal mouse serum was employed for the reaction instead of primary antibodies.

Slides were assessed for staining intensity and location of immunoreactivity within the tissues (Tables 2 and 3). The intensity and pattern of the staining were graded using an empirical semi-quantitative system: (–), no reaction; (+), weak reaction; (++), moderate reaction; (+++), intense reaction; (+++d), intense diffuse reaction.

Results

The results of immunohistochemical analyses of versican and CS expression in all types of mammary tissues examined are summarized in Table 2.

In skin covering mammary tissues, versican immunoreactivity with antibodies 12C5 and 2B1 was frequently noted at the dermoepidermal junction as a moderate to strong discontinuous line along the epidermal basement membrane and as a weak fibrillar staining in the immediate superficial dermis. Furthermore, reactivity could be seen in connective tissue sheets, and in necks and dermal papillae of hair follicles in accordance with the observation in human

Table 2. Imunohistochemical labelling with antibodies.

SITE	12C5	2B1	CS56
Normal mammary tissues			
Inactive	-	-	-
Lactating			
Intralobular connective tissue	-	-	++
Periductal tissues	-	-	+++
Cytoplasm of secretory epithelial cells	-	-	+++
Benign Tumours			
Fibrous connective tissue	++/-	++/-	+++
Periductal tissues	++/+++	++/+++	+++
Cytoplasm of neoplastic epithelium	-	-	++/-
Malignant Tumours			
Fibrous connective tissue	+/-	+/-	+++
Periductal tissues	++/-	++/-	+++
Peripheral invasive area	+++/-	+++/-	+++
Cytoplasm of neoplastic epithelium	-	-	++/-
Benign and Malignant Complex and Mixed Tumours			
Myoepithelium-like spindle cells proliferations			
Poor ECM	+++	+++	+++
Abundant, myxoid ECM	+++d	++/-	++/-
Cytoplasm of spindle cells	+++	+++	+++
Cartilage matrix	-	-	+/++
Perichondral tissues	++	++	++
Bone	-	-	-

–: no reaction; +: weak reaction; ++: moderate reaction; +++: intense reaction; +++d: intense, diffuse reaction

skin (du Cros et al., 1995). The same structures were stained for CS, but CS immunoreactivity was often more intense and widespread compared to versican. In general, large blood vessels showed immunoreactivity with all three antibodies in all three layers of their muscular walls, but in a number of cases staining for versican was observed only in perivascular fibrous elements. The adventitia of small blood and lymph vessels was also immunostained. These positively-stained structures in skin and also blood vessels served as internal positive controls in our samples.

Normal mammary tissues

The histological characteristics of the normal mammary gland during the ovarian cycle are comparable in women and female dogs (Nelson and Kelly, 1974; Vogel et al., 1981). In our study none of the normal

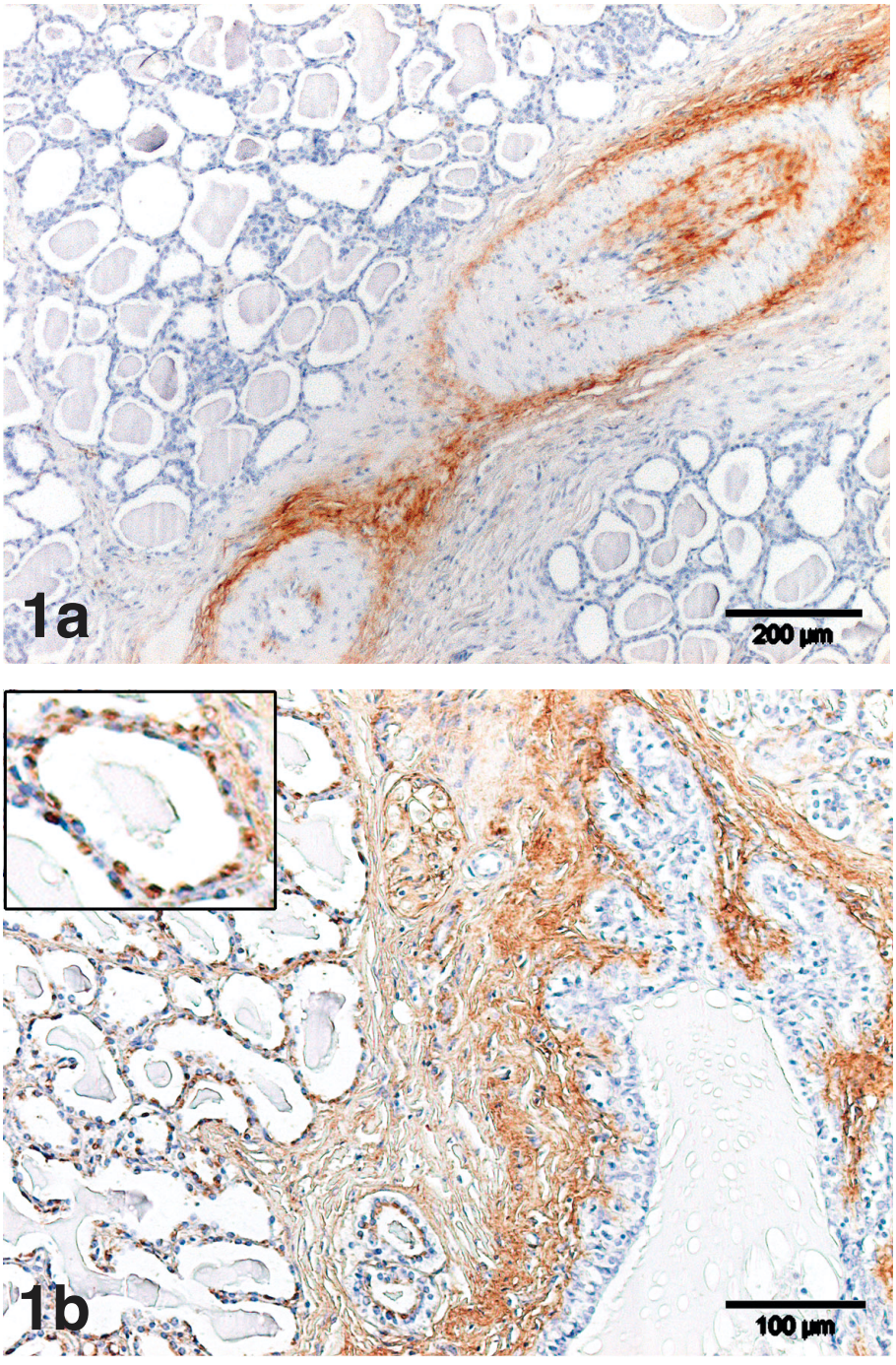

Fig. 1. Immunolabelling of normal mammary tissues**. a.** Lack of versican expression in a normal mammary tissue. Reactivity in blood vessels served as internal positive control (antibody 12C5). **b.** CS expression surrounding an extralobular duct that blends with the staining in the adjacent connective tissue and intense cytoplasmic reactivity of the luminal epithelial cells (inset) in secretory alveoli (antibody CS56).

mammary tissues showed appreciable versican immunoreactivity with antibodies 12C5 and 2B1 (Fig. 1a). CS was widely present in most of the lactating (lobuloalveolar secretory tissue) and regressing (alveolar involution), but not in the inactive mammary tissues (branched ductal system with quiescent epithelium). Consistently noted patterns of CS immunoreactivity were the following: moderate to intense cytoplasmic staining of luminal epithelial cells almost exclusively in secretory differentiated lobules; reactivity of the intralobular connective tissue with more intense staining in the immediate periacinar and periductal structural elements; and prominent periductal reactivity surrounding extralobular ducts that blended with staining in the adjacent connective tissue (Fig. 1b). In the immunoreactive connective tissue the cytoplasm of interstitial fibroblasts was also positively stained.

Benign tumours

In general, fibrous connective tissues within benign tumours displayed moderate to intense immunoreactivity with antibodies 12C5 and 2B1 in the immediate periductal tissues. The most intense reactivity often appeared as a concentric layer surrounding large extralobular ducts (Fig. 2a). In most of the tubulopapillary adenomas the periductal reactivity extended into the loose connective tissue of papillary projections. CS showed a more widespread and homogenous distribution throughout the fibrous connective tissue (Fig. 2b). In the immunoreactive areas of fibrous connective tissue in all types of benign tumours the cytoplasm of interstitial fibroblasts was often immunostained with all three antibodies. Furthermore, apical cytoplasmic staining for CS, but not for versican, was noted in the lining epithelium of several papillary projections. Removal of CS side chains from the protein core of versican by chondroitinase ABC treatment significantly improved the intensity of staining with antibodies 12C5 and 2B1 in the above mentioned areas, but completely demolished CS labelling with CS56 antibody, as was expected.

In complex adenomas immunoreactivity to versican was most prominent in the ECM and in the cytoplasm of a large portion of spindle cells within the areas of myoepithelium-like spindle cell proliferation. In those areas where myoepithelium-like spindle cells formed cell-rich bundles accompanied by a relatively small amount of ECM, there was no considerable difference observed in the intense immunoreactivity to versican with antibodies 12C5 and 2B1 (Fig. 3a,b); the intensity of staining could be enhanced after chondroitinase ABC digestion. In these areas immunoreactivity to CS was rather similar to that to versican. In ECM-rich myxoid areas with few characteristic polygonal-cells (Fig. 4a), the abundant ECM showed not only intense but completely diffuse and homogeneous staining with antibody 12C5, whereas it was only partially stained with antibody 2B1, often displaying a distinct halo around cells (Fig. 4b,c). Moreover, 2B1 immunoreactivity was completely absent in some of the myxoid matrices. Immunoreactivity to CS was patchy or absent in these areas (Fig. 4d). Interestingly, in abundant myxoid ECM the 12C5 immunostaining invariably appeared intense and diffuse even without chondroitinase ABC treatment. Morover, the intensity of 12C5 and 2B1 immunostainings in myxoid ECM could not be improved further by chondroitinase ABC digestion. Cytoplasmic staining of spindle and polygonal shaped cells, both in ECM-poor and ECM-rich areas, was detected with all three antibodies. Summarizing, in spindle cell proliferations the intensity and extension of staining with all three antibodies showed some variation, the only exception being 12C5 immunostaining in myxoid ECM.

In benign mixed tumours the myoepithelium-like spindle cell proliferations and myxoid areas showed immunoreactivity with all three antibodies, with the same distribution and intensity as was decribed in complex adenomas. Dense matrices of cartilage tissues were slightly to moderately reactive to CS with more intense staining in perilacunar regions, whereas appreciable versican immunoreactivity with antibodies 12C5 and 2B1 was mostly restricted to perichondral tissues. In perichondral tissues versican reactivity was improved after chondroitinase ABC digestion. No

Table 3. Immunohistochemical labeling of infiltrative tumours.

HISTOLOGY	INTRATUMORAL CONNECTIVE TISSUE				PERIPHERAL INVASIVE AREAS			
	Versican		CS		Versican		CS	
	staining intensity	R (%)	staining intensity	R (%)	staining intensity	R (%)	staining intensity	R (%)
Tubulopapillary carcinoma (n =8)	+	3/8 (37.5%)	++/+++	8/8 (100%)	+/++	7/8 (87.5%)	++/+++	8/8 (100%)
Solid carcinoma (n = 6)	–	0/6 (0%)	++/+++	6/6 (100%)	++/+++	6/6 (100%)	++/+++	6/6 (100%)
Anaplastic carcinoma (n = 4)	–	0/4 (0%)	++/+++	4/4 (100%)	++/+++	4/4 (100%)	++/+++	4/4 (100%)
Complex carcinoma (n = 5)	+	2/5 (40%)	++/+++	5/5 (100%)	+/++	4/5 (80%)	++/+++	5/5 (100%)
Mixed tumour with carcinoma (n = 5)	+	1/5 (20%)	++/+++	5/5 (100%)	+/+++	5/5 (100%)	+/+++	5/5 (100%)

R (%): Ratio and percentages of infiltrative tumours with reactivity to versican and CS within the intratumoural connective tissues and peripheral invasive zone.

immunoreactivity to versican or to CS was seen in bone tissues.

In summary, fibrous connective tissues within benign tumours displayed a more widespread CS distribution compared to versican. However, in periductal and perichondral tissues and in cell-rich spindle cell proliferations, versican immunostaining was also intense with both 12C5 and 2B1 antibodies. Interestingly, the myxoid ECM showed intense and diffuse immunoreactivity solely with antibody 12C5.

Malignant tumours

Immunohistochemical labelling of malignant tumours demonstrated heterogenous immunoreactivity to versican both in terms of distribution patterns and

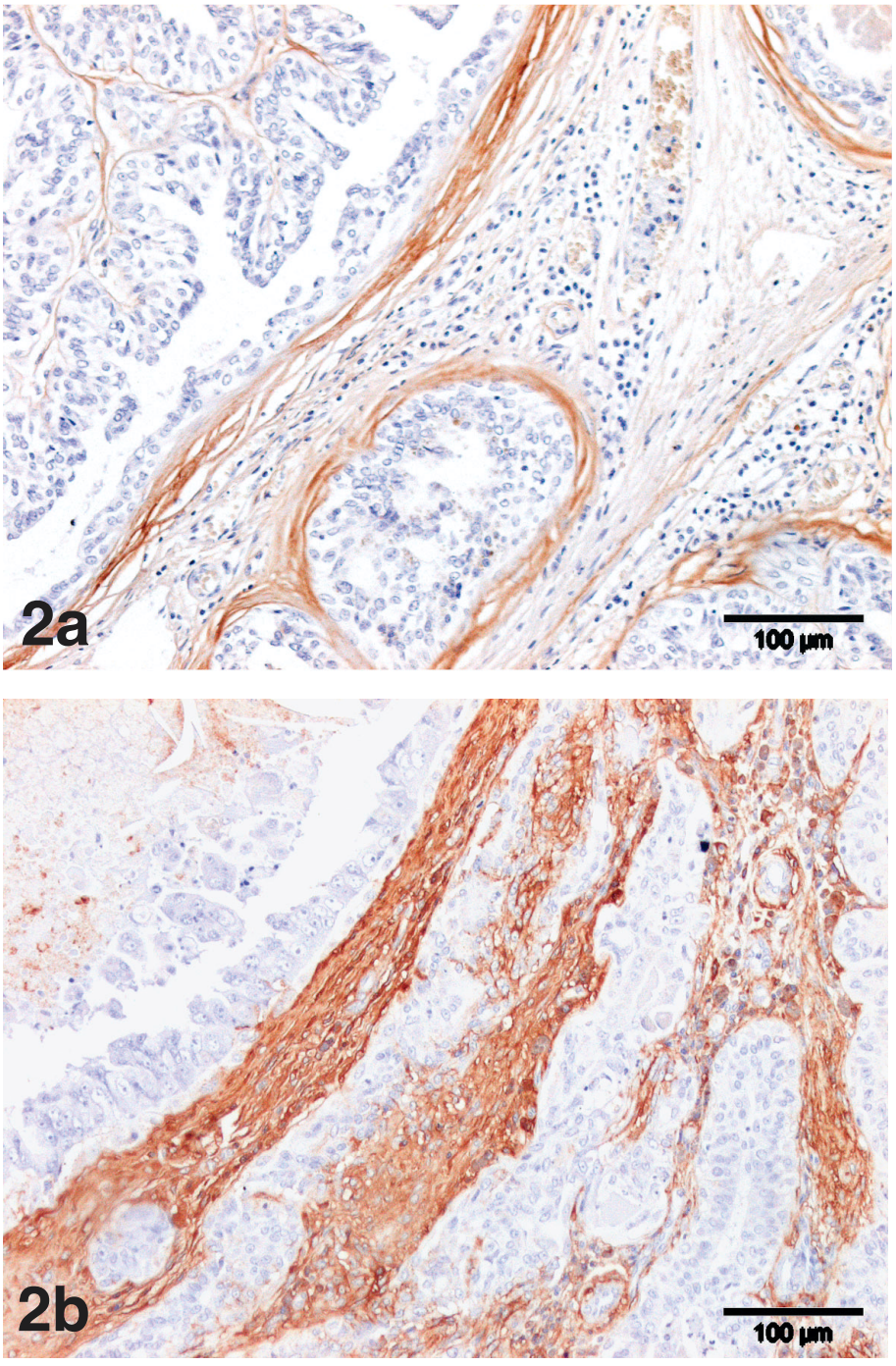

Fig. 2. Immunolabelling of periductal tissues in benign tumours. **a.** Intense versican expression in concentric layers surrounding ducts in a tubulopapillary adenoma. Note also the staining in the loose connective tissue of papillary projections (antibody 2B1). **b.** CS reactivity is not restricted to the periductal tissues, but is intense and widespread throughout the intratumoural fibrous tissue (antibody CS56).

staining intensity between different types of tumours. Benign spindle cell proliferations, myxoid and chondroid tissues within malignant complex and mixed tumours displayed the same intense immunoreactivity to versican as in their benign counterparts. The chondroitinase ABC pretreatment convincingly improved the staining for versican with both anti-versican antibodies in all the stained tissue elements, with the exception of myxoid ECM in complex and mixed tumours, such as in benign tumours.

In general, fibrous connective tissues within malignant tumours showed low or no expression of versican (Fig. 5a; Table 3). However, in the peripheral invasive areas 2B1- and 12C5-reactive elements were seen in the mesenchymal tissue closely around nests and clusters of tumour cells, although not all interfaces

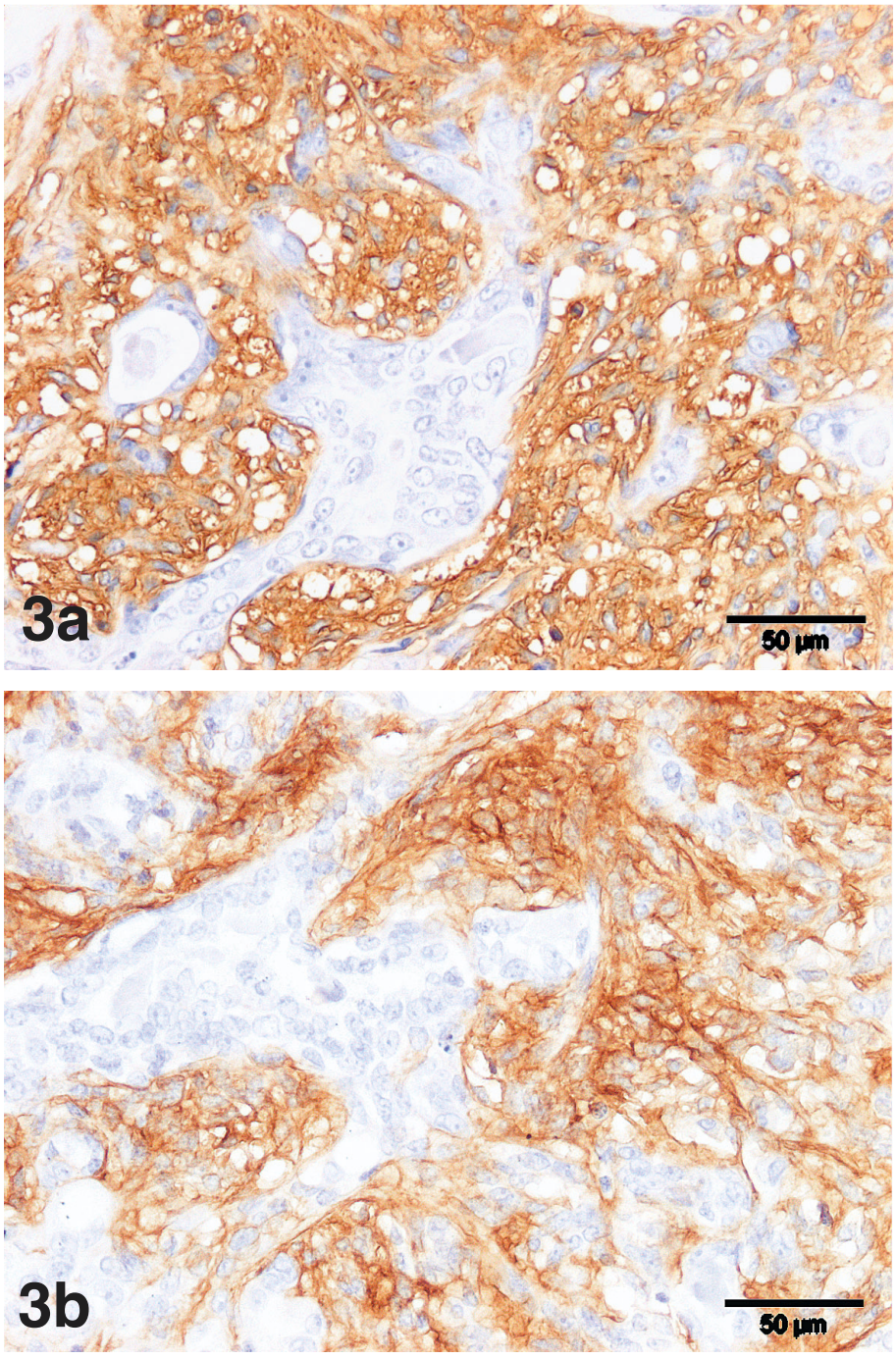

Fig. 3. Immunolabelling of complex and mixed tumours. Intense versican immunoreactivity in cell-rich myoepithelium-like spindle cell proliferations of a complex adenoma. There is no significant difference in the labelling with antibody 12C5 **(a)** and antibody 2B1 **(b)**.

between invading neoplastic epithelium and mesenchymal tissues showed appreciable immunoreactivity to versican (Fig. 6a,b; Table 3). In highly invasive carcinomatous tissues and anaplastic carcinomas, both 2B1 and 12C5 labellings were most prominently positive in this area and around lymph vessels filled with metastatic carcinoma cells, but no appreciable staining of the peri- or intratumoural fibrous connective tissue was found. Furthermore, immunoreactivity with both anti-versican antibodies closely approximated the contours of large ducts, comprising irregular concentric rings with several discontinuities. This periductal staining contrasted with the immunoreactivity located in the loose connective tissue of intraductal papillary projections and in the fibrous septa within tumours, where no or only moderate and scattered immunoreactivity was found. No cytoplasmic staining of the neoplastic epithelial cells with antibody 12C5 and 2B1 could be detected in any types of carcinomas examined. In contrast to the distribution of versican, homogeneous and intense CS immunoreactivity was constantly detected throughout the fibrous stroma of all types of malignant tumours (Fig. 5b). The most intense CS staining was observed in mesenchymal tissues closely interwoven with clusters of carcinoma cells, in periductal fibrous elements, and in sclerotic stromal tissues. The cytoplasm of neoplastic cells was often immunostained for CS. Similar to benign tumours, cytoplasmic staining of interstitial fibroblasts was often seen with all three antibodies.

Complex carcinomas, a tumour type that arises from the transformation of both luminal epithelial and myoepithelial components, displayed the most widespread versican expression of all malignant tumours. As was seen in complex adenoma, myoepithelium-like spindle cell proliferations reacted

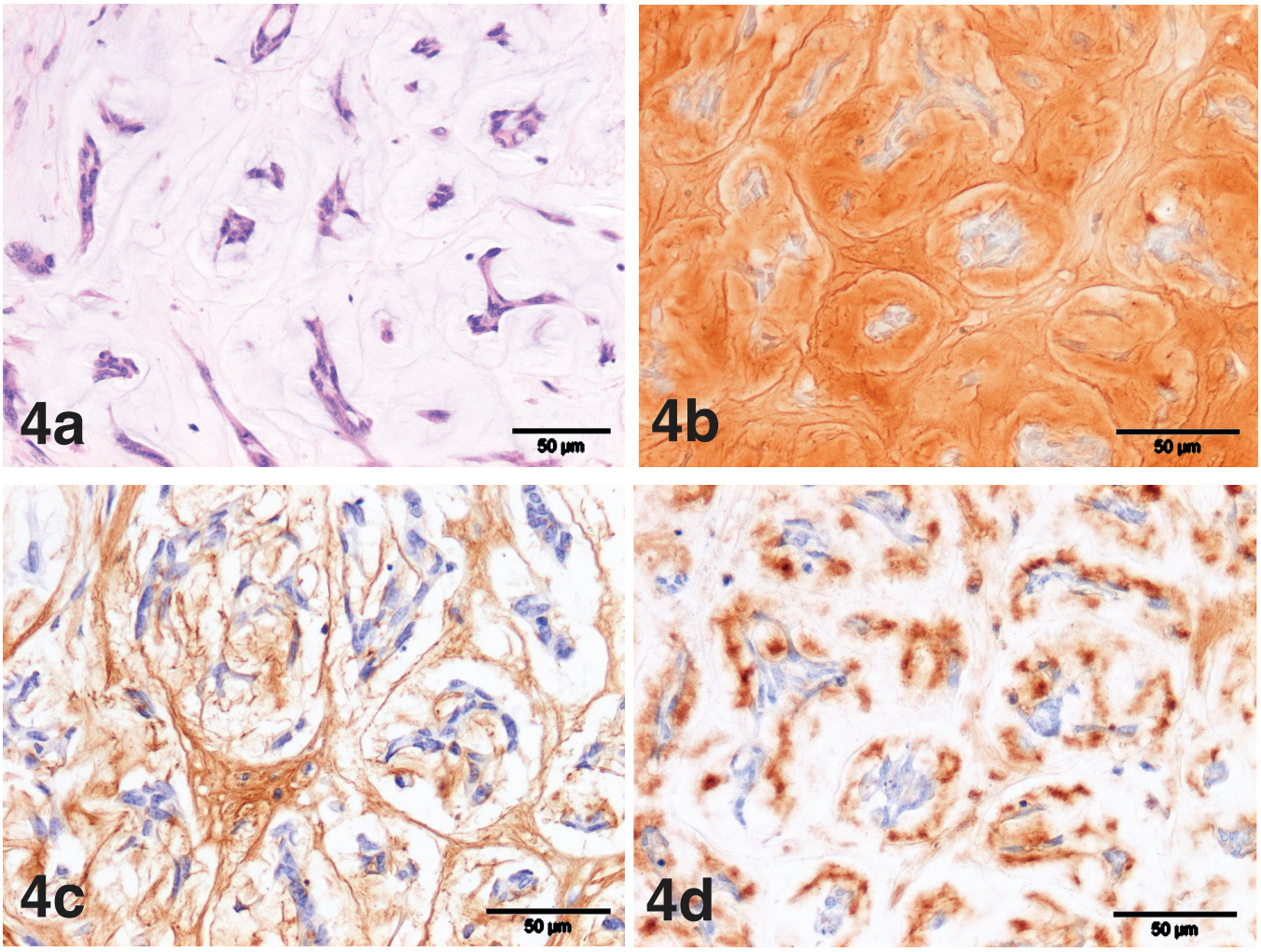

Fig. 4. Immunolabeling of myxoid areas in complex and mixed tumours. ECM-rich myxoid tissue characterized by spindle- and polygonal-shaped cells in serial slides of a benign mixed tumour. **a.** H&E staining. The abundant ECM is stained intensely and diffusely with antibody 12C5 **(b)** and only partially with antibodies **(c)** 2B1 and CS56 **(d)**.

intensely with both anti-versican antibodies and also with CS56 antibody; however, reactivity with antibody 12C5 was more diffuse and homogeneous in the abundant ECM, particularly in myxoid areas, than with antibodies 2B1 and CS56. The chondroid and myxoid areas of mixed tumours with malignant epithelial component, expressed both versican and CS in the same pattern as in benign mixed tumours. The carcinomatous component of mixed tumours displayed similar reactivity to versican and to CS as in simple and complex carcinomas.

Staining intensity and percentages of all types of infiltrative tumours with reactivity to versican and CS within the peripheral invasive zone and/or the intratumoural connective tissue are summarized in Table 3.

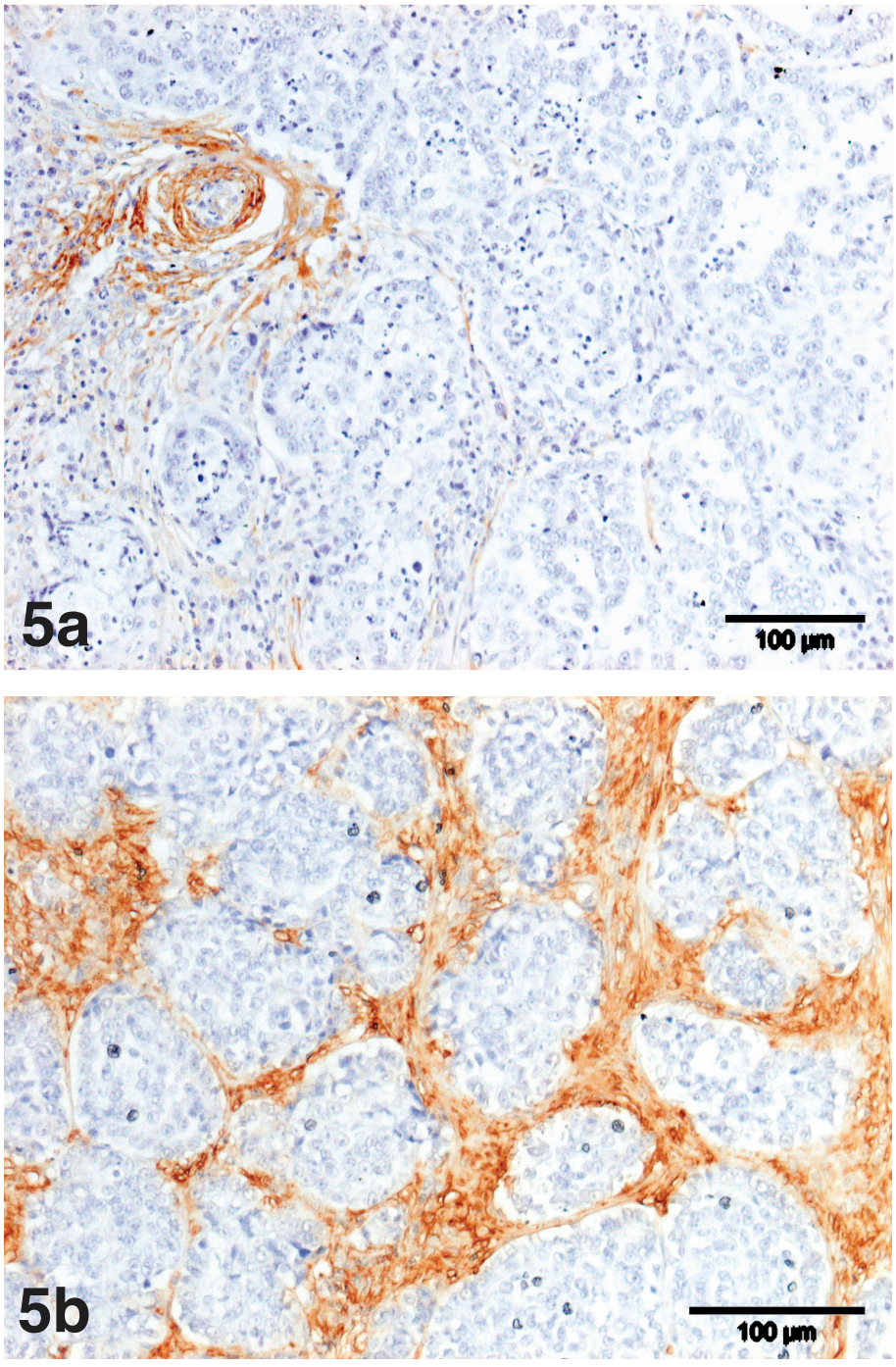

Fig. 5. Immunoreactivity in the fibrous connective tissue within malignant tumours. In a simple carcinoma **(a)** versican reactvity is mostly restricted to the vessel wall and perivascular tissues (antibody 2B1), whereas **(b)** CS reactivity is present throughout the intratumoural fibrous tissue (antibody CS56).

In summary, malignant mammary tumours expressed versican in the periepithelial mesenchymal tissues, in particular surrounding large ducts and in the peripheral invasive areas. Furthermore, benign spindle cell proliferations, and benign myxoid and chondroid tissues within malignant complex and mixed tumours expressed versican similar to their benign counterparts.

Discussion

An increased stromal accumulation of chondroitin sulphate in canine mammary tumours compared to the normal tissues has been previously reported (Hinrichs et al., 1999). The focus of this study was to determine whether versican, a large CS-containing PG, is present in

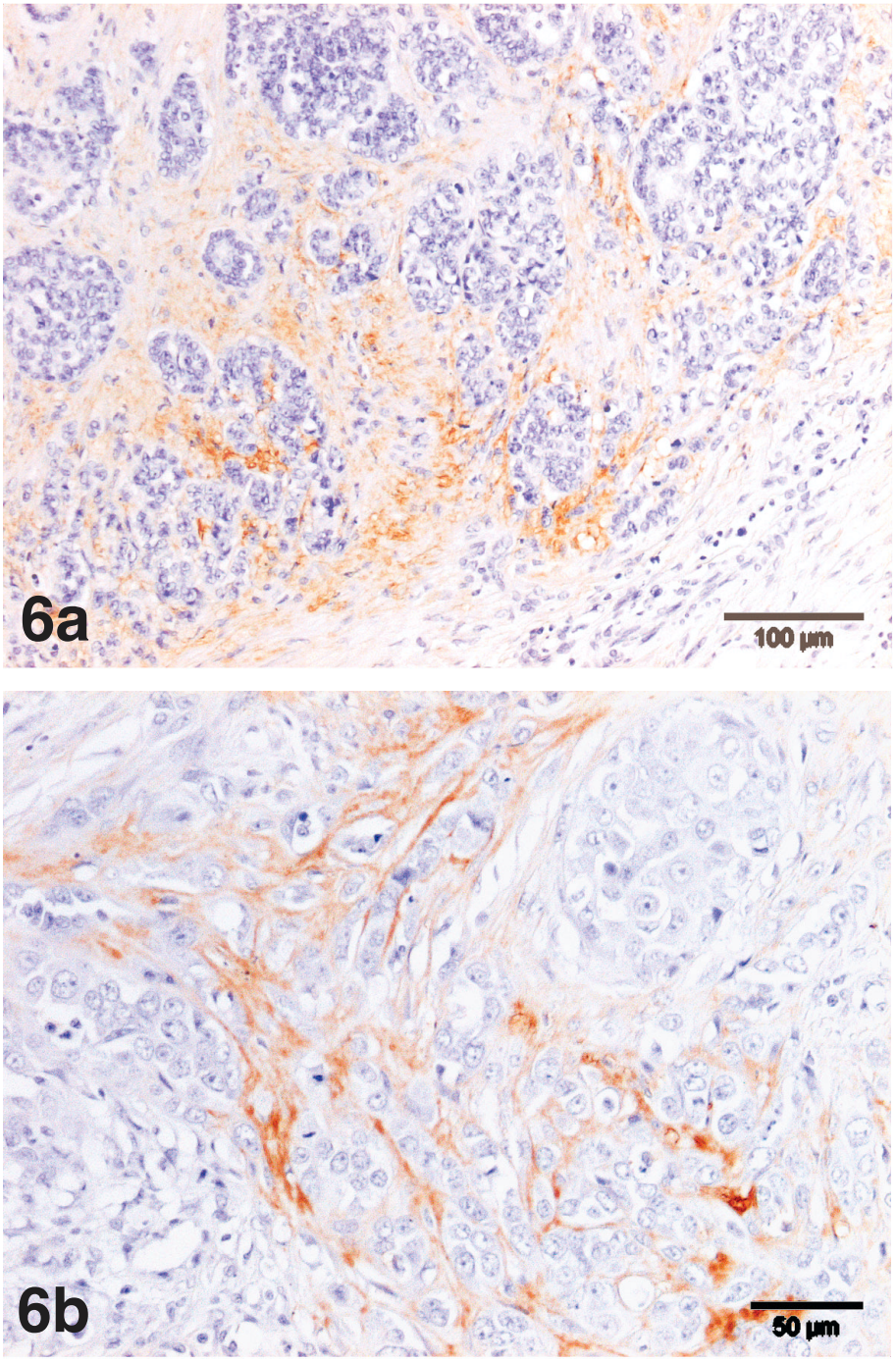

Fig. 6. Versican immunoreactivity in peripheral invasive areas. Enhanced versican expression in the mesenchymal tissue around invading nests and clusters of tumour cells. Not all interfaces between neoplastic epithelium and mesenchymal tissue show appreciable immunoreactivity to versican. **a.** Antibody 2B1. **b.** Antibody 12C5.

mammary tumours of dogs, thus partly or entirely being responsible for the elevated levels of CS. We also tackled the question as to whether in canine mammary tumours, as in a number of human cancers (Ricciardelli et al., 1998; Touab et al., 2002), the presence of versican relates to malignant behaviour.

Our results showed enhanced versican deposition in the ECM of both benign and malignant mammary tumours, especially in complex and mixed tumours, compared to the non-neoplastic mammary tissues. In fact, no immunoreactivity to versican could be detected in inactive or lactating (pregnancy or pseudopregnancy) normal mammary tissues. CS was not present in inactive mammary tissues, but appeared in lactating and regressing tissues; apical cytoplasmic CS staining of luminar epithelial cells could be detected almost exclusively in secretory differentiated lobules. These results may reflect changes in CS expression in the normal mammary tissues of dogs induced by ovarian hormones. Involvement of sGAGs and PGs in mammary tissue morphogenesis and differentiation of different species is well known from the literature (Silberstein and Daniel, 1982; Umbreit, 1996). Changes in the ECM of the normal human breast, such as altered concentration and distribution of CS during the menstrual cycle have also been reported (Ferguson et al., 1992), suggesting that the ECM profile of the normal breast tissue is under hormonal regulation. The lack of versican from non-neoplastic mammary tissue indicates the involvement of other CS-containing PGs than versican in the morphological changes of the normal mammary gland of dogs, but identification of these ECM compounds awaits further studies.

This study has revealed an increased versican expression in neoplastic mammary tissue; however, we found variations in patterns of immunoreactive versican between certain histological types of tumours. The fibrous connective tissue within all types of tumours displayed a more widespread CS distribution compared to versican, indicating that the CS accumulation cannot be entirely attributed to the presence of versican.

The three main patterns of versican accumulation, in periductal tissues, in peripheral invasive areas of malignant tumours and in spindle cell and myxoid areas, will be further discussed.

Periductal staining pattern of versican

All types of mammary tumours showed versican immunoreactivity in a periductal pattern, in particular surrounding large pre-existing extralobular ducts, probably in association with their basement membranes. In these areas versican co-localised with CS. Similar findings have been described in human benign breast tumours where the basement membrane component of the duct epithelium was reactive with antibody 2B1 (Nara et al., 1997). In human yolk sac tumours basement membrane components were also stained positively to 2B1 (Nakashima et al., 1990). As versican is associated with basement membranes, the co-localisation of versican and CS in the immediate periductal tissues in our study strengthens the basement membrane origin of positively- stained tissue elements for CS surrounding epithelial structure in canine mammary tumours as described by Hinrichs et al. (1999). Versican has been shown to be associated with components of the elastic tissues (Zimmermann et al., 1994; Bode-Lesniewska et al., 1996; Merrilees et al., 2002). In the study of Nara et al. (1997), focal elastosis in breast carcinomas was noted in periductal spaces and was found to be reactive to versican, as well as to collagen IV and laminin, indicating that the elastosis masses were composed of basement membrane components. Our finding that 12C5 and 2B1 immunoreactivities often appeared as a concentric layer, with some variability in thickness, encompassing large ducts, may also be explained by the expression of versican in elastic tissues surrounding these ducts. It appears likely that versican is a component of tumour basement membrane in mammary tumours of dogs, although it is uncertain how it contributes to the basement membrane formation.

Versican immunoreactivity in peripheral invasive areas of malignant tumours

In the peripheral invasive areas the remarkable staining of the periepithelial mesenchymal tissue by the two anti-versican antibodies contrasted with the lack of immunoreactivity of fibrous connective tissues within these tumours. The localization of versican in the invasion front may be related to its anti-adhesive effects on cells, a property that has been shown to reside both in the CS side-chains (Yamagata et al., 1989) and in the core protein (Yang et al., 1999). It has been reported that versican can stimulate cell proliferation via two mechanisms: through two EGF-like motifs in the G3 domain which play a role in stimulating cell growth (Zhang et al., 1998), and through the G1 domain, which destabilises cell adhesion and facilitates cell growth. It has been proposed that the effects of the G1 domain on cell proliferation can be explained by its strong HA-binding affinity, resulting in a local increase in HA concentration, which will destabilize cell adhesion and increase cell proliferation (Yang et al., 1999). Preliminary data suggest an overlapping distribution of HA with versican and CS in the peripheral invasive areas of canine mammary tumours (our data, not shown), consistent with the findings in human breast cancer (Nara et al., 1997). The co-existence of HA and versican in the peripheral invasive areas indicates that versican may perform its function by binding to HA in these areas. Consequently, the formation of an ECM rich in versican and HA provides a highly hydrated environment, which may facilitate local cancer cell proliferation and migration by decreasing cell-matrix adhesion. The promotion of tumour cell migration through the ECM may also facilitate formation of distant metastases. This is supported by the findings of

Ricciardelli et al. (1998, 2002), where patients with prostate and breast tumours containing versican-rich peritumoural stroma experienced shorter relapse-free intervals than patients with versican-poor tumours. They suggested that the peritumoural location of versican and its implied involvement in tumour spread is indicative of the production of soluble mediators by more aggressive cancer cells which regulate deposition of versican into the ECM by fibroblastic cells. In accordance with the findings in human breast and prostate cancer (Nara et al., 1997; Ricciardelli et al., 1998, 2002) the cytoplasm of neoplastic luminal epithelium did not contain immunoreactive versican in canine mammary tumours, confirming that versican is a product of stromal cells. These cells might possibly be interstitial fibroblasts, but we showed that myoepithelium-like spindle cells were also able to produce versican since they were immunostained for versican in complex and mixed tumours (see below).

Versican immunoreactivity in myoepithelium-like spindle cell proliferations and myxoid areas

In both benign and malignant complex and mixed tumours, immunoreactive versican was most abundantly present in the areas of myoepithelium-like spindle cell proliferation. 12C5 and 2B1 immunoreactivities substantially co-localised in all types of mammary tumours with the exception of ECM-rich spindle cell proliferations and myxoid areas. Interestingly, the abundant ECM of these areas was only partially stained or not stained at all with antibodies 2B1 and CS56, whereas 12C5 immunoreactivity was intense, completely diffuse and homogeneous. Furthermore, the intensity of versican staining could not be enhanced after chondroitinase ABC digestion in these areas. Taken as a whole these observations suggest that in canine complex and mixed mammary tumours versican carries little or no CS side chains and lacks the 2B1 epitope in the abundant ECM of spindle cell proliferations and myxoid areas. The lack of CS could also explain that chondroitinase ABC digestion has no effect on the intensity of versican immunoreactivity in these tissues. The form of versican carrying little or no CS-side chains and lacking the 2B1 epitope may be generated by enzymatic cleavage, separating the HA-binding region from the 2B1 epitope- and CS-bearing domain of the molecule. It has been shown that members of the large CS-containing PG family are susceptible to partial proteolytic cleavage by matrix metalloproteinases (MMPs) occurring endogenously under pathological conditions (Lark et al., 1997; Zhang et al., 1998; Sandy et al., 2001). MMP digestion of these proteins often results in separating the HA-binding region from the chondroitin sulphate-rich region of the molecules (Vilim and Fosang, 1994). It is likely that the HA-binding region remains attached to the HA in the matrix while the rest of the molecule, including the large CS-bearing domain, is degraded further and removed from the tissues.

The unique pattern of 12C5 immunoreactivity in ECM-rich spindle cell masses and myxoid areas may also be explained by alternative splicing of versican, yielding a short form of the core protein without CS attachment domain and lacking also the 2B1 epitope. Several extracellular matrix proteins have a diversity of molecular forms as a result of alternative splicing (Ito et al., 1995). Isoforms of these proteins have been shown to differ in function and to be regulated developmentally and/or in tissue-dependent manners (Yamagata et al., 1993; du Cros et al., 1995). Alternative splicing of CS attachment domains (GAGα and GAGß) yields multiforms of the versican core protein in several species (Dours-Zimmermann and Zimmermann, 1994; Ito et al., 1995). A transcript encoding a short form of the core protein, V3 was detected in various mouse and human tissues including brain, stomach and liver, using polymerase chain reaction (PCR) (Zako et al., 1995). DNA sequences of these PCR products suggested that V3 had no CS attachment domain; therefore it may be classified as a glycoprotein and not a proteoglycan. V3 has no CS attachment region, and thus lacks the large size and high charge density, but retains the HA-binding domain of the large isoforms; this form may therefore have a unique function. In fact, in vitro studies have recently shown that V3 overexpression in arterial smooth muscle cells, retrovirally transduced with rat V3, alters their smooth muscle cell phenotype and results in increased adhesion and inhibition of migration and proliferation of these cells. It has been suggested that V3 may exert these effects through changes in pericellular coat formation, either by competing with larger isoforms for HA-binding, or by altering other components of the pericellular matrix (Lemire et al., 1999).

In summary, the present findings showed that versican is one of the extracellular matrix PG characteristics of canine mammary tumours. The enhanced versican expression both in benign and malignant canine mammary tumours indicates that it cannot be considered as a general marker for malignancy. However, the high versican expression in the invasive zones of infiltrating malignant tumours suggests a possible involvement in tumour invasion. Furthermore, it appears likely that in complex and mixed tumours versican exists in different forms, one of them lacking the CS attachment domain and the 2B1 epitope. The latter form of versican may be a splice variant or may be a result of proteolytic cleavage by MMPs.

Acknowledgements. This research was supported by a European Community Marie Curie Fellowship and by the Hungarian State Eötvös Scholarship to Ildikó Erdélyi. The technical assistance of Anne Marie van Ederen and Frans van Mil is highly appreciated. We are grateful to Hans de Vos (Pathology Department of Groningen University) for testing the 2B1 antibody on canine mammary tissues. We also thank Evert van Garderen for his help in the histological classification of the examined canine mammary tumours and Professor Erik Gruys for his useful comments on the manuscript.

References

Adany R. and Iozzo R.V. (1990). Altered methylation of versican proteoglycan gene in human colon carcinoma. Biochem. Biophys. Res. Commun. 171, 1402-1413.

Asher R., Perides G., Vanderhaeghen J.J. and Bignami A. (1991). Extracellular matrix of central nervous system white matter: Demonstration of an hyaluronate-protein complex. J. Neurosci. Res. 28, 410-421.

Aspberg A., Adam S., Kostka G., Timpl R. and Heinegard D. (1999). Fibulin-1 is a ligand for the C-type lectin domains of aggrecan and versican. J. Biol. Chem. 274, 20444-20449.

Aspberg A., Miura R., Bourdoulous S., Shimonaka M., Heinegard D., Schachner M., Ruoslahti E. and Yamaguchi Y. (1997). The C-type lectin domains of lecticans, a family of aggregating chondroitin sulfate proteoglycans, bind tenascin-R by protein-protein interactions independent of carbohydrate moiety. Proc. Natl. Acad. Sci. USA. 94, 10116-10121.

Avnur Z. and Geiger B. (1984). Immunocytochemical localization of native chondroitin-sulfate in tissues and cultured cells using specific monoclonal antibody. Cell 38, 811-822.

Bignami A., Hosley M. and Dahl D. (1993). Hyaluronic acid and hyaluronic acid-binding proteins in brain extracellular matrix. Anat. Embryol. (Berl.) 188, 419-433.

Bode-Lesniewska B., Dours-Zimmermann M.T., Odermatt B.F., Briner J., Heitz P.U. and Zimmermann D.R. (1996). Distribution of the large aggregating proteoglycan versican in adult human tissues. J. Histochem. Cytochem. 44, 303-312.

Dours-Zimmermann M.T. and Zimmermann D.R. (1994). A novel glycosaminoglycan attachment domain identified in two alternative splice variants of human versican. J. Biol. Chem. 269, 32992-32998.

du Cros D.L., LeBaron R.G. and Couchman J.R. (1995). Association of versican with dermal matrices and its potential role in hair follicle development and cycling. J. Invest. Dermatol. 105, 426-431.

Ferguson J.E., Schor A.M., Howell A. and Ferguson M.W. (1992). Changes in the extracellular matrix of the normal human breast during the menstrual cycle. Cell Tissue Res. 268, 167-177.

Hinrichs U., Rutteman G.R. and Nederbragt H. (1999). Stromal accumulation of chondroitin sulphate in mammary tumours of dogs. Br. J. Cancer 80, 1359-1365.

Iozzo R.V. (1998). Matrix proteoglycans: From molecular design to cellular function. Annu. Rev. Biochem. 67, 609-652.

Isogai Z., Aspberg A., Keene D.R., Ono R.N., Reinhardt D.P. and Sakai L.Y. (2002). Versican interacts with fibrillin-1 and links extracellular microfibrils to other connective tissue networks. J. Biol. Chem. 277, 4565-4572.

Ito K., Shinomura T., Zako M., Ujita M. and Kimata K. (1995). Multiple forms of mouse PG-M, a large chondroitin sulfate proteoglycan generated by alternative splicing. J. Biol. Chem. 270, 958-965.

Kjellen L. and Lindahl U. (1991). Proteoglycans: structures and interactions. Annu. Rev. Biochem. 60, 443-475.

Lark M.W., Bayne E.K., Flanagan J., Harper C.F., Hoerrner L.A., Hutchinson M.I., Singer I.I., Donatelli S.A., Weidner J.R., Williams H.R., Mumford R.A. and Lohmander L.S. (1997). Aggrecan degradation in human cartilage. Evidence for both matrix metalloproteinase and aggrecanase activity in normal, osteoarthritic, and rheumatoid joints. J. Clin. Invest. 100, 93-106.

LeBaron R.G., Zimmermann D.R. and Ruoslahti E. (1992). Hyaluronate binding properties of versican. J. Biol. Chem. 267, 10003-10010.

Lemire J.M., Braun K.R., Maurel P., Kaplan E.D., Schwartz S.M. and Wight T.N. (1999). Versican/PG-M isoforms in vascular smooth muscle cells. Arterioscler. Thromb. Vasc. Biol. 19, 1630-1639.

Margolis R.U. and Margolis R.K. (1994). Aggrecan-versican-neurocan family proteoglycans. Methods Enzymol. 245, 105-126.

Merrilees M.J., Lemire J.M., Fischer J.W., Kinsella M.G., Braun K.R., Clowes A.W. and Wight T.N. (2002). Retrovirally mediated overexpression of versican V3 by arterial smooth muscle cells induces tropoelastin synthesis and elastic fiber formation in vitro and in neointima after vascular injury. Circ. Res. 90, 481-487.

Misdorp W., Else R.W., Hellmén E. and Lipscomb T.P. (1999). Histological classification of mammary tumours of the dog and the cat. WHO International Histological classification of tumors of domestic animals. 2nd ed. Shulmann F.Y. (ed). Armed Forced Institute of Pathology. Washington, D.C. pp 11-48.

Nakashima N., Sobue M., Fukata S., Fukatsu T., Nagasaka T., Ohiwa N., Nara Y., Lai S. and Takeuchi J. (1990). Immunohistochemical characterization of extracellular matrix components of yolk sac tumors. Virchows Arch. B Cell Pathol. Incl. Mol. Pathol. 58, 309-315.

Nara Y., Kato Y., Torii Y., Tsuji Y., Nakagaki S., Goto S., Isobe H., Nakashima N. and Takeuchi J. (1997). Immunohistochemical localization of extracellular matrix components in human breast tumours with special reference to PG-M/versican. Histochem. J. 29, 21-30.

Nelson L.W. and Kelly W.A. (1974). Changes in canine mammary gland histology during the estrous cycle. Toxicol. Appl. Pharmacol. 27, 113-122.

Ohiwa N., Fukata S., Fukatsu T., Nagasaka T., Nara Y., Lai S., Nakashima N. and Takeuchi J. (1991). Immunohistochemical localization of proteoglycans in non-epithelial tumor tissues. Connect. Tissue. Res. 22, 11-19.

Palmer T.E. and Monlux A.W. (1979). Acid mucopolysaccharides in mammary tumors of dogs. Vet. Pathol. 16, 493-509.

Paulus W., Baur I., Dours-Zimmermann M.T. and Zimmermann D.R. (1996). Differential expression of versican isoforms in brain tumors. J. Neuropathol. Exp. Neurol. 55, 528-533.

Ricciardelli C., Brooks J.H., Suwiwat S., Sakko A.J., Mayne K., Raymond W.A., Seshadri R., LeBaron R.G. and Horsfall D.J. (2002). Regulation of stromal versican expression by breast cancer cells and importance to relapse-free survival in patients with node-negative primary breast cancer. Clin. Cancer Res. 8, 1054-1060.

Ricciardelli C., Mayne K., Sykes P.J., Raymond W.A., McCaul K., Marshall V.R. and Horsfall D.J. (1998). Elevated levels of versican but not decorin predict disease progression in early-stage prostate cancer. Clin. Cancer Res. 4, 963-971.

Ruoslahti E. (1989). Proteoglycans in cell regulation. J. Biol. Chem. 264, 13369-13372.

Sandy J.D., Westling J., Kenagy R.D., Iruela-Arispe M.L., Verscharen C., Rodriguez-Mazaneque J.C., Zimmermann D.R., Lemire J.M., Fischer J.W., Wight T.N. and Clowes A.W. (2001). Versican V1 proteolysis in human aorta in vivo occurs at the Glu441-Ala442 bond, a site that is cleaved by recombinant ADAMTS-1 and ADAMTS-4. J. Biol. Chem. 276, 13372-13378.

Shinomura T., Zako M., Ito K., Ujita M. and Kimata K. (1995). The gene structure and organization of mouse PG-M, a large chondroitin sulfate proteoglycan - genomic background for the generation of multiple PG-M transcripts. J. Biol. Chem. 270, 10328-10333.

Silberstein G.B. and Daniel C.W. (1982). Glycosaminoglycans in the basal lamina and extracellular matrix of the developing mouse

mammary duct. Dev. Biol. 90, 215-222.

Sobue M., Nakashima N., Fukatsu T., Nagasaka T., Fukata S., Ohiwa N., Nara Y., Ogura T., Katoh T. and Takeuchi J. (1989). Production and immunohistochemical characterization of a monoclonal antibody raised to proteoglycan purified from a human yolk sac tumour. Histochem. J. 21, 455-460.

Touab M., Villena J., Barranco C., Arumi-Uria M. and Bassols A. (2002). Versican is differentially expressed in human melanoma and may play a role in tumor development. Am. J. Pathol. 160, 549-557.

Ujita M., Shinomura T., Ito K., Kitagawa Y. and Kimata K. (1994). Expression and binding-activity of the carboxyl-terminal portion of the core protein of PG-M, a large chondroitin sulfate proteoglycan. J. Biol. Chem. 269, 27603-27609.

Umbreit J.N. (1996). Proteoglycans and glycosaminoglycans during maturation of the mouse mammary gland. Anticancer Res. 16, 3013-3029.

Vilim V. and Fosang A.J. (1994). Proteoglycans isolated from dissociative extracts of differently aged human articular cartilage: characterization of naturally occurring hyaluronan-binding fragments of aggrecan. Biochem. J. 304, 887-894.

Vogel P.M., Georgiade N.G., Fetter B.F., Vogel F.S. and McCarty K.S. (1981). The correlation of histologic changes in the human breast with the menstrual cycle. Am. J. Pathol. 104, 23-34.

Wu Y., Chen L., Zheng P.S. and Yang B.B. (2002). beta 1-Integrin-mediated glioma cell adhesion and free radical-induced apoptosis are regulated by binding to a C-terminal domain of PG-M/versican. J. Biol. Chem. 277, 12294-12301.

Yamagata M., Suzuki M., Akiyama S.K., Yamada K.M. and Kimata K. (1989). Regulation of cell-substrate adhesion by proteoglycans immobilized on extracellular substrates. J. Biol. Chem. 264, 8012-8018.

Yamagata M., Shinomura T. and Kimata K. (1993). Tissue variation of two large chondroitin sulfate proteoglycans (PG-M/versican and PG-H/aggrecan) in chick embryos. Anat. Embryol. (Berl.) 187, 433-444.

Yamauchi S., Cheng H., Neame P., Caterson B. and Yamauchi M. (1997). Identification, partial characterization, and distribution of versican and link protein in bovine dental pulp. J. Dent. Res. 76, 1730-1736.

Yang B.L., Zhang Y., Cao L. and Yang B.B. (1999). Cell adhesion and proliferation mediated through the G1 domain of versican. J. Cell Biochem. 72, 210-220.

Yao L.Y., Moody C., Schonherr E., Wight T.N. and Sandell L.J. (1994). Identification of the proteoglycan versican in aorta and smooth muscle cells by DNA sequence analysis, in situ hybridization and immunohistochemistry. Matrix Biol. 14, 213-225.

Zako M., Shinomura T., Ujita M., Ito K. and Kimata K. (1995). Expression of PG-M(V3), an alternatively spliced form of PG-M without a chondroitin sulfate attachment region in mouse and human tissues. J. Biol. Chem. 270, 3914-3918.

Zhang H., Kelly G., Zerillo C., Jaworski D.M. and Hockfield S. (1998). Expression of a cleaved brain-specific extracellular matrix protein mediates glioma cell invasion in vivo. J. Neurosci. 18, 2370-2376.

Zhang Y., Cao L., Yang B.L. and Yang B.B. (1998). The G3 domain of versican enhances cell proliferation via epidermial growth factor-like motifs. J. Biol. Chem. 273, 21342-21351.

Zhao M., Takata T., Ogawa I., Yada T., Kimata K. and Nikai H. (1999). Immunohistochemical evaluation of the small and large proteoglycans in pleomorphic adenoma of salivary glands. J. Oral. Pathol. Med. 28, 37-42.

Zimmermann D.R., Dours-Zimmermann M.T., Schubert M. and Bruckner-Tuderman L. (1994). Versican is expressed in the proliferating zone in the epidermis and in association with the elastic network of the dermis. J. Cell Biol. 124, 817-825.

Accepted May 14, 2003