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Stromal cells and extracellular matrix components in spontaneous canine transmissible venereal tumour at different stages of growth

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Summary. Stromal cells and extracellular matrix (ECM) components are important for tumour cell behaviour. Little is known about the role of stromal cells and ECM components in the progression and regression of spontaneous canine transmissible venereal tumour (CTVT). In this study, the stromal cell type was determined by immunohistochemical labelling with antibodies to desmin, vimentin and α-smooth muscle actin (α-SMA) during the progressive and regressive stages of spontaneous CTVT. The distribution of ECM components tenascin-C, chondroitin sulphate and versican were determined immunohistochemically, and hyaluronan distribution was determined using a biotinylated protein complex with specific affinity for hyaluronan. Stromal cells of tumours in both the progressive and regressive stage were positive for vimentin and negative for desmin. The number of stromal cells expressing α-SMA was significantly higher (P=0.001) in regressing tumours, than progressing tumours. These results suggest that the modulation of stromal cells that occurs during the regression of CTVT is similar to that occurring during wound healing. Tenascin-C was weakly expressed in the stroma of tumours in the progressive stage and in regions of the regressing tumours with tumour infiltrating lymphocytes (TILs), but intensely expressed in the stroma of tumours in late regressive stage. In addition, tenascin-C was also expressed in the cytoplasm of some tumour cells in the late regressive stage. A strong stromal tenascin-C intensity was significantly associated with regressing tumours (P=0.001). Strong stromal hyaluronan intensity and a high proportion of hyaluronan-positive tumour cells were significantly associated with progressing tumours (P=0.001). This suggests that hyaluronan is involved in the growth of the tumour. There was no significant difference in the expression of chondroitin sulphate and versican in progressing and regressing tumours.

Key words: Canine transmissible venereal tumour, Stroma, Tenascin, Hyaluronan

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Introduction

Canine transmissible venereal tumour (CTVT) is a naturally occurring contagious round cell neoplasm of dogs of both sexes which can be transplanted with intact viable cells across major histocompatibility complex barriers within species, and even to other canids such as foxes, coyotes and jackals (Higgins, 1966). It is the only known transplantable tumour. In both natural and experimentally induced cases, the tumour exhibits a predictable growth pattern – progressive growth, stasis and regression. The spontaneous regression of CTVT may be regulated by a variety of factors, including products of neoplastic cells, stromal cells and tumour infiltrating lymphocytes (TILs). In addition, extracellular matrix (ECM) components and cytokines regulate cell-microenvironment interaction, which, in turn, modulate tumour progression and tumour cell activities (Hynes, 1992; Albelda, 1993). The tumour is frequently characterized by the presence of numerous TILs during the early regressive stage, and by the replacement of tumour parenchyma by fibrovascular stroma during the late regressive stage (Gonzalez et al., 2000). Therefore, this tumour represents a neoplastic microenvironment useful in the investigation of the role of stromal cells, ECM components and host immune response in tumour progression.

The fibroblast is the most abundant cell type in normal connective tissue. Some fibroblasts express features of smooth muscle differentiation, and these are called myofibroblasts, and are identified by expression of α-smooth muscle actin (α-SMA) (Sappino et al., 1990). Myofibroblasts play an important role in wound repair and tumour progression through their production

of growth factors and cytokines. Myofibroblasts also repair or remodel the ECM through synthesis of proteoglycans and glycoproteins. During the regression of CTVT, there is an increase in the volume of the stromal tissue and replacement of the tumour parenchyma. The phenotype of the stromal cells in the progressing and regressing CTVT, to our knowledge, has not been studied.

Tenascin-C is a large glycoprotein that is transiently expressed during embryogenesis, wound healing and in neoplastic conditions (Chiquet-Ehrismann et al., 1986; Erickson, 1993). It is thought to be involved in many cellular functions including adhesion, proliferation and tumour metastasis (Erickson and Bourdon, 1989; Orend and Chiquet-Erhismann, 2000). Tenascin-C expression is altered in many solid tumours (Mackie et al., 1987), suggesting that it might be associated with tumour progression.

Hyaluronan is a linear unsulphated glycosaminoglycan synthesized on the cytoplasmic side in the plasma membrane. Hyaluronan has been associated with many different cellular processes including cell adhesion, aggregation, proliferation, inflammation, locomotion and migration (Knudson and Knudson, 1993). Hyaluronan overproduction is a diagnostic or prognostic factor for several types of human cancer, including breast (Auvinen et al., 2000), thyroid (Böhm et al., 2002), ovarian (Anttila et al., 2000), and colorectal (Ropponen et al., 1998) cancers.

Changes in the level and structure of the glycosaminoglycans side chains of proteoglycans, such as elevated expression of chondroitin sulphate, have been associated with the progression of malignancy in various human tumours (Adany et al., 1990; Yeo et al., 1990, Cohen et al., 1994). Measurement of chondroitin sulphate levels in the pertumoral stroma of human prostate cancer can predict tumour progression (Ricciardelli et al., 1999). Versican, a member of the large aggregating chondroitin sulphate proteoglycan family, is a recognized anti-cell adhesive molecule involved in regulating cell motility on ECM components (Yamagata et al., 1989; Evanko et al., 1999). Versican interacts with tenascin (Aspberg et al., 1997) and hyaluronan (LeBaron et al., 1992), and may play an important role in CTVT progression.

Up to now, research on CTVT biology has focused only on the neoplastic cells and TILs, while the stromal cells and the ECM components are thought to lack major biological activities and clinical significance. The objectives of the present study were 1) to assess the phenotype of stromal cells, and 2) to assess the expression of ECM components (tenascin-C, hyaluronan, chondroitin sulphate and versican) during the progression and regression stages of CTVT.

Materials and methods

Case material

Dogs (13 males, 16 females) with natural CTVT restricted to the external genital membranes were observed for 2 weeks, and the size of the tumours were measured. The tumours were classified into two groups according their stage of clinical growth. The first group included 13 tumours, which showed an increase in size when the tumours were surgically excised, and these were classified as progressing tumours. The second group included 16 tumours, which showed a constant or decreasing size when the tumours were excised, and these were classified as regressing tumours. Tissue samples were fixed in 10% buffered formalin for 24 h and embedded in paraffin. Three to 6 sections, representative of each tumour were stained by haematoxylin and eosin for histopathological study.

Immunohistochemical and histochemical labelling

In each case, 4-µm sections of formalin-fixed, paraffin wax-embedded tissues were mounted on poly-L-lysine coated slides. After de-waxing and rehydration, the sections were treated with H_2O_2 0.5% in methanol for 30 min to inactivate the intrinsic peroxidase activity. For labelling of antigens of tenascin-C and versican, deparaffinized sections were pre-treated with chondroitinase 0.2 U/ml ABC (Seikagaku, Kogyo, Tokyo, Japan) in 0.2 M Tris-HCL buffer (pH 8.0) at 37 °C for 1 h. The slides were preincubated with normal horse serum 10% for 30 min before incubation with primary antibody (anti-vimentin, desmin, α-SMA, tenascin-C, chondroitin sulphate and versican) or biotinylated hyaluronan binding complex at 4 °C overnight. Table 1 lists the antibodies and binding

Table 1. Antibodies used in immunohistochemistry.

ANTIBODY	SOURCE	DILUTION
Anti-vimentin (V9)	Biogenex, San Ranon, Ca, USA	1:150
Anti-desmin	RD 301 Euro Diagnostica B.V., Arnhem, The Netherlands	Undiluted
Anti-α-SMA (1A4)	Biogenex	1:1200
Anti-tenascin-C (TN2)	Dako, Glostrup, Denmark	1:50
Anti-chondroitin sulphate (C56)	Sigma, St Louis, MO, USA	1:100
Anti-versican (2B1)	Seikagaku, Kogyo, Tokyo, Japan	1:1000
Hyaluronan binding protein complex	Seikagaku, Kogyo, Tokyo, Japan	2.5 µg/ml

protein complex used in this study, their source and the specific dilutions. All sections were then incubated with horse anti-mouse biotin and avidin-biotin peroxidase complex (Vector, Burlingame, CA, USA). Colour reactions were developed in 3,3'-diaminobenzidine containing 0.01% hydrogen peroxide, and the sections were counterstained with haematoxylin for light microscopic examination. Negative controls consisted of serial sections incubated with buffer alone or purified mouse immunoglobulin instead of primary antibody. Each run also included a positive control slide of tissue previously demonstrated to be strongly positive for the above components.

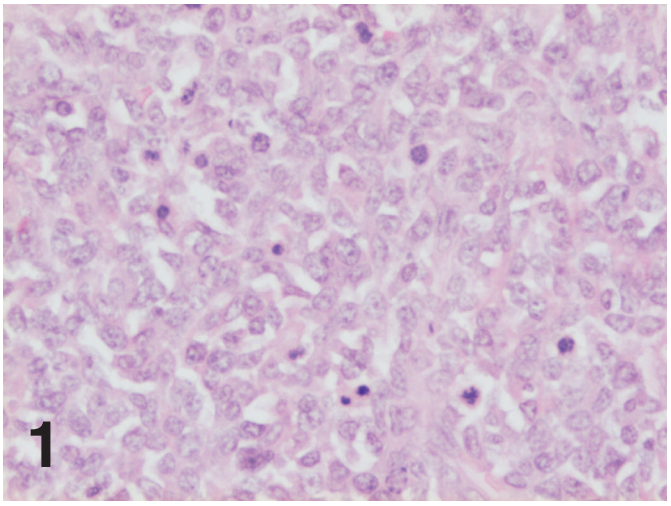
Evaluation of labelling and statistical analysis

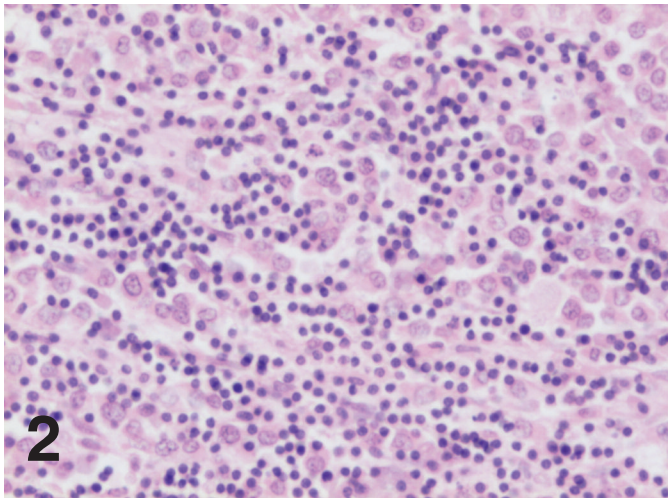
The sections were examined using a dual-head microscope simultaneously by two observers. Evaluation of the labelling intensity or the number of cells labelled was performed by either scoring the entire lesion or at least 10 representative high power fields. The labelling of α-SMA in stromal cells was graded as negative (no stromal cells positive), weak (<25% cells positive), moderate (25-50% cells positive), and strong (>50) and recorded as 0-3, respectively. The level of stromal tenascin-C, hyaluronan, chondroitin sulphate and versican in the tumours was graded as negative, weak, moderate or strong and recorded as 0-3, respectively. The expression of hyaluronan in tumour cells was graded as weak (<25% cells positive), moderate (25-50% cells positive), and strong (>50%) positive and recorded as 1-3, respectively. The relationship between the stage of the tumour and stromal cell type or tenascin-C, chondroitin sulphate, versican and hyaluronan intensity was tested using χ^2 analysis of SAS (1996).

Results

Histology

In the progressive stage, the tumours consisted of round cells, with little connective tissue stroma (Fig. 1). The stromal cells were spindle shaped with an elongated nucleus. During the early regression stage, there was infiltration of the tumours by tumour-infiltrating





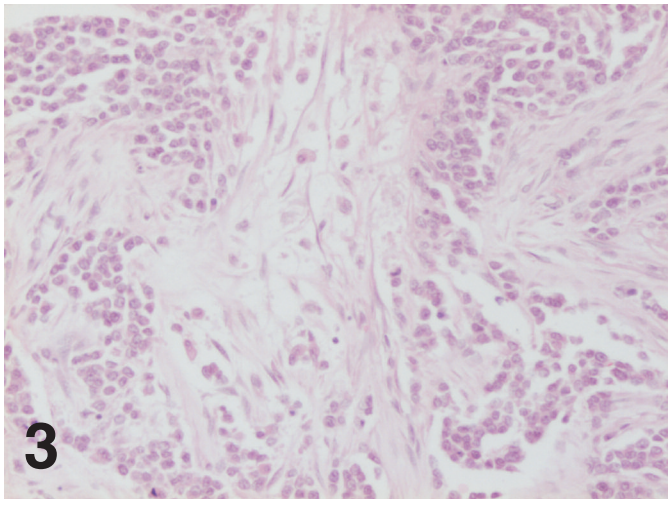


Fig. 1. Canine transmissible venereal tumour (CTVT) during the progression stage. The tumour consists of round cells, with little connective tissue stroma. Heamatoxylin and eosin (HE). x 200

Fig. 2. CTVT during early regression stage. Presence of tumour-infiltrating lymphocytes (TILs). HE. x 200

Fig. 3. CTVT during late regression stage. Collapse of the tumour parenchyma and replacement by fibrous stroma. HE. x 200

lymphocytes (TILs). These TILs were either diffusely distributed (Fig. 2) or associated with areas with fibrovascular stroma. In the late regression stage, there was collapse of the tumour parenchyma and replacement by fibrous stroma (Fig. 3).

Stromal cells; Vimentin, Desmin, α-smooth muscle-actin expression

In all the tumours, all the stromal cells were strongly immunolabelled by vimentin and negative for desmin. α-smooth muscle actin was positive in the media of all blood vessels. Stromal cells were positive for α-SMA in 4/13 (30%) of the tumours in the progressive stage (Fig. 4), and all (100%) of the tumours in the regression stage (Fig. 5). The number of stromal cell expressing α-SMA was significantly higher (P=0.001) in regressing tumours than progressing tumours (Table 2). In all the cases, tumour cells were positive for vimentin and negative for desmin and α-SMA.

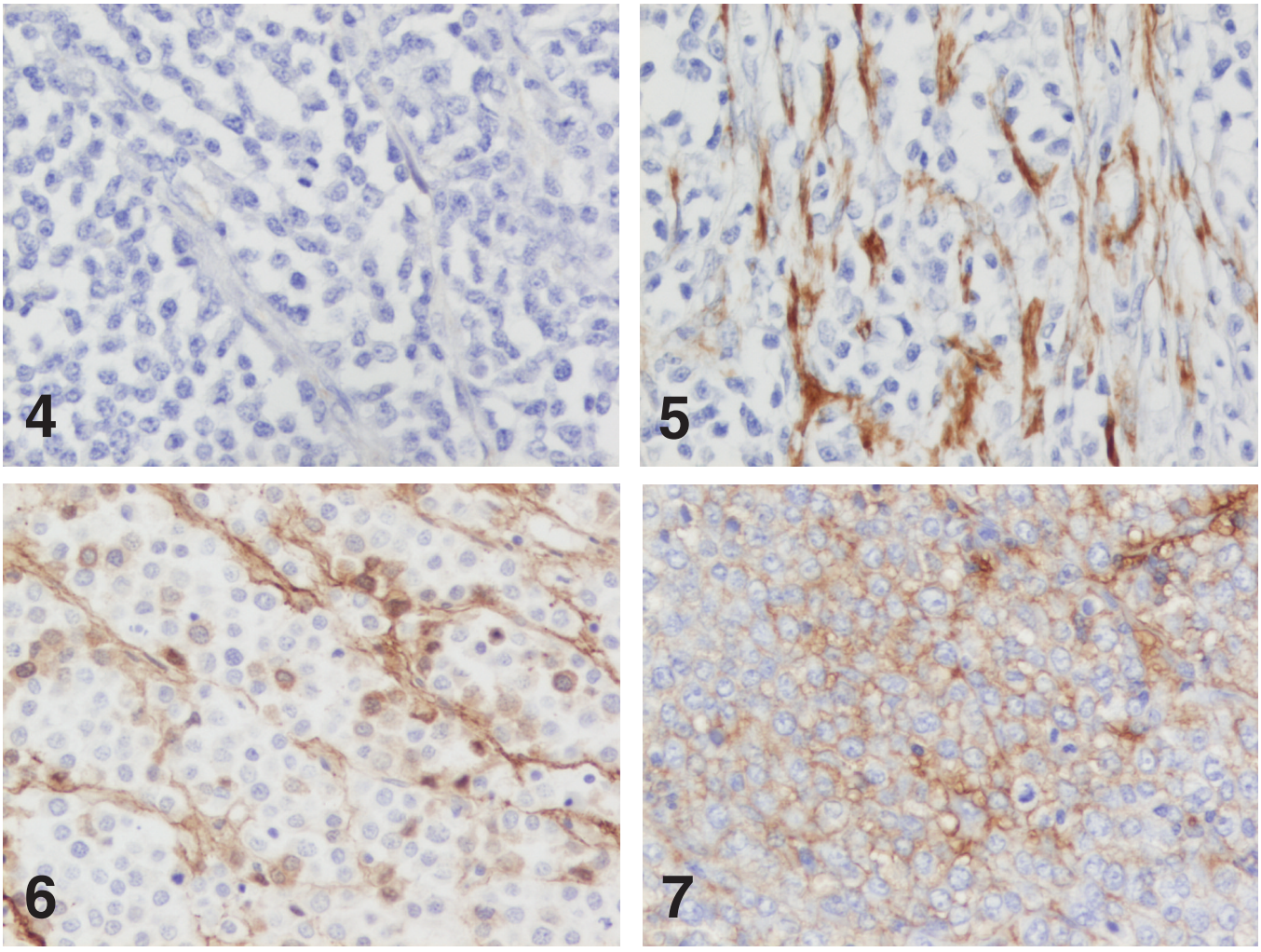


Fig. 4. CTVT during progression stage immunolabelled for α-SMA. There is no immunolabelling of stromal cells by α-SMA. Avidin-biotin peroxidase complex (ABC), haematoxylin counter-stain. x 200

Fig. 5. CTVT during regression stage immunolabelled for α-SMA. There is a strong immunolabelling of stroma by α-SMA. ABC, haematoxylin counter-stain. x 200

Fig. 6. CTVT during regression stage immunolabelled for tenascin-C. There is a strong immunolabelling of stroma by tenascin-C. Note there is also granular cytoplasmic labelling of some tumour cells. ABC, haematoxylin counter-stain. x 200

Fig. 7. CTVT during the progression stage immunolabelled for hyaluronan. There moderate is membranous and cytoplasm labelling of tumour cells. ABC, haematoxylin counter-stain. x 200

Tenascin-C expression

Tenascin-C labelling was present in the stroma in 9/13 (69%) of the tumours in the progressing stage. In the early regression stage, tenascin-C was labelled in the stroma of all the tumours except in areas with TILs. In the late regression phase, tenascin-C was intensely labelled in the stroma of all tumours (Fig. 6). There was no tenascin-C labelling in the tumour cells during the progressing stage, while 8/16 (50%) of the regressing tumours expressed granular cytoplasmic tenascin-C (Fig. 6). A strong stromal tenascin-C intensity was significantly associated with regressing tumours (P=0.001).

Hyaluronan, chondroitin sulphate and versican expression

Hyaluronan was immunolabelled in the stroma of all tumours. Strong stromal hyaluronan intensity was significantly associated with progressing tumours (P=0.001). In addition to stromal labelling, hyaluronan was labelled on cell membranes and cytoplasm in all the tumours (Fig. 7). A high proportion of hyaluronan-positive tumour cells was significantly associated with progressing tumours (P=0.001). In no cases were TILs positive for hyaluronan.

Chondroitin sulphate was expressed in the stroma of all tumours and on the outer membrane of tumour cells regardless of their stage of progression. Versican was expressed in the stroma of only 5/29 (17%) tumours (2 regressing and 3 in the late progressive stage), and was not expressed in tumour cells. The intensity of stromal chondroitin sulphate and versican was not significantly related to the growth stage of the tumour.

Discussion

This study, to our knowledge, is the first study on the role of stromal cells and extracellular matrix (ECM) components on the progression of CTVT. The study shows that the stromal cells and ECM components are different in the different stages of growth of CTVT, and this may influence tumour progression.

We used immunolabelling with vimentin, desmin and α-smooth muscle actin (α-SMA) to identify myofibroblasts, and our results showed that there was a higher number of myofibroblasts during the regression than the progression stage. Myofibroblasts have been implicated in a variety of pathological conditions involving tissue remodelling, including wound healing, and stromal reactions of tumours (Martin, 1997; Serini and Gabbiani, 1999). As the appearance of myofibroblasts occurred during the regression stage and coincided with TILs, it seems quite likely that mediators produced by TILs are important for controlling and development of myofibroblasts, or the increase in myofibroblasts and TILs may be the consequence of the same factor produced by tumour cells. The mediators that direct the stromal reaction were not determined in this study. TGF-ß emerges as a strong candidate because of its key regulatory role in wound repair and stromal cell biology (Roberts and Sporn, 1993). It is also possible that TILs communicate with fibroblasts through intimate physical contacts.

Tenascin-C is a multifunctional glycoprotein, participating in cell adhesion, repulsion, and epithelial shedding from surfaces. In our study, we observed an increase in stromal tenascin-C expression from progressive to regressive stage. During the regression stage of CTVT, there is destruction of the tumour

Table 2. Association between α-smooth muscle, tenascin-C, hyaluronan, chondroitin sulphate and versican expression in the tumour stroma and the stage of tumour.

EXTRACELLULAR MATRIX COMPOONNNENT	TUMOUR STAGE	EXPRESSION IN THE EXTRACELLULAR MATRIX OF CANINE TRANSMISSIBLE VENEREAL TUMOUR CASES				
		Negative	Weak	Moderate	Strong	Significance*
α-SMA	Progressing	9(31.03%)	3(10.34%)	1(3.45%)	0(0.00%)	P=0.001
	Regressing	0(0.00%)	2(6.90%)	6(20.69)	8(27.59%)	
Tenascin-C	Progressing	4(13.79%)	8(27.59%)	1(1.45%)	0(0.00%)	P=0.001
	Regressing	0(0.00%)	2(6.90%)	7(24.14%)	7(24.14%)	
Chondroitin sulphate	Progressing	0(0.00%)	1(3.45%)	3(10.34%)	9(31.03%)	P=0.368
	Regressing	0(0.00%)	0(0.00%)	2(6.90%)	14(48.28%)	
Versican	Progressing	11(37.93)	1(3.45%)	1(3.45%)	0(0.00%)	P=0.471
	Regressing	11(37.93)	4(13.79%)	1(3.45%)	0(0.00%)	
Hyaluronan	Progressing	0(0.00%)	1(3.45%)	4(13.79%)	8(27.59%)	P=0.001
	Regressing	0(0.00%)	11(37.93)	5(17.24%)	0(0.00%)	

χ^2: square test

parenchyma and extensive remodelling of the stroma. Tenascin-C expression is increased in processes like wound healing (Mackie et al., 1988) and embryogenesis (Chiquet-Ehrismann et al., 1986), where there is extensive tissue remodelling. The increased expression of tenascin-C in regressing tumours probably enhances proliferation of stromal cells (myofibroblasts). There was co-localization of tenascin-C and myofibroblasts, suggesting that these cells are responsible for the secretion of tenascin-C. These observations in CTVT accorded with the reported tenascin-C expression in canine gastrointestinal epithelial tumours, where tenascin was co-localized with myofibroblasts (Mukaratirwa et al., 2003). In tumours, myofibroblasts are responsible for the elaboration of most components of the extracellular matrix including tenascin-C (Hanamura et al., 1997). The presence of cytoplasmic tenascin-C immunolabelling in tumour cells suggests that CTVT cells also secrete tenascin-C.

In areas with TILs, tenascin-C expression was absent. Since tenascin-C suppress T cell proliferation and cytokine production (Puente Navazo et al., 2001), its absence in these areas would allow TILs to play a primary role in the spontaneous regression of CTVT. Similar results have been observed in transgenic mice, where in tenascin-C-null mice, stromal components of spontaneous mammary tumours contained more monocytes/macrophages than from tenascin-C wild type mice (Talts et al., 1999). TILs are considered to be of primary importance in the spontaneous regression of CTVT (Mizuno et al., 1989).

The results indicate that hyaluronan is expressed in the stroma of all CTVTs, irrespective of the stage of growth. The presence of hyaluronan in the stroma may give tumour cells a growth advantage since hyaluronan creates hydrated matrices, which enhance cell proliferation and locomotion (Knudson and Knudson, 1990). There was a significant relation between tumour cell-associated (membranous and cytoplasmic) hyaluronan expression with the stage of the tumour. Cell membrane-associated hyaluronan may give CTVT cells a growth advantage, as hyaluronan shields cells against apoptosis (Yasuda et al., 2001), and possibly also mask tumour-associated antigens and major histocompatibility antigens on tumour cell surface. When glioma cells were cultured with lymphocytes, it was observed that the cancer cells were surrounded by a pericelluar coat containing hyaluronan, which increased the resistance of them to immune attack (Gately et al., 1984). A similar process possibly occurs during the progressive stage of CTVT. The cytoplasmic hyaluronan may support tumour cell proliferation, perhaps through its intracellular receptors RHAMM and CD44. RHAMM interact with microtubules and actin filaments in the cell replication interphase and dividing cells, possibly interfering with cell proliferation (Assmann et al., 1999). The accumulation of hyaluronan in the stroma and CTVT cells may offer possibilities of therapeutic interventions using hyaluronan degrading agents or agents which specifically inhibit hyaluronan synthesis, as suggested by Auvinen et al., (2000). Hyaluronidase has been successfully used to enhance the penetration of cytostatic drugs in human bladder carcinomas (Höbarth et al., 1992), squamous cell carcinoma (Kohno et al., 1994) and breast cancer (Beckenlehner et al., 1992). It will be interesting to test the effect of combined use of hyaluronidase with the commonly used chemo-theraupetic drugs (vincristine and cyclophosphamide) in the treatment of CTVT.

Our results showed that chondroitin sulphate proteoglycans were expressed in the stroma and tumour cells of both progressing and regressing tumours. The nature of the proteoglycans involved in the increase in chondroitin sulphate proteoglycans in these tumours is unknown, but our results suggest that versican is not responsible for this chondroitin sulphate proteoglycans accumulation

In conclusion, the stromal reaction occurring in regressing CTVTs appears to be comparable, if not identical, to generic wound repair, and stromal tenascin-C and hyaluronan, and cell associated hyaluronan may be markers of the growth stage of the tumour. We, therefore, suggest that further clinical studies on the synthesis and regulation of tenascin-C and hyaluronan in CTVT are clearly warranted.

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