CHAPTER 4

Relation between cell proliferation and tenascin expression in canine gastrointestinal tumours and normal mucosa

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Abstract

Tenascin is an extracellular matrix glycoprotein that has been implicated in cell proliferation and adhesion by in vitro experiments. Its expression is known to be increased in canine and human gastrointestinal tumours. The aim of this study was to investigate the possible relationship between cell proliferation and tenascin expression in these tumours. In tissue sections of normal stomach, small intestine and colon, and gastrointestinal epithelial tumours, the monoclonal antibody Ki-67, which is directed against a proliferation-associated nuclear antigen, was used to identify proliferating cells. Serial sections were also stained for tenascin. Serial sections stained for tenascin and Ki-67 were compared to determine whether there is a correlation between tenascin expression and tumour cell proliferation. In the normal gastric mucosa, Ki-67 positive cells were confined to the neck region and in the normal small intestinal mucosa positive cells were confined to the lower parts of the crypts. In adenomas and carcinomas, the frequency of positive cells was increased at the edges of adenomas and invasive tumour margins of carcinomas and there was inter- and intra- tumoural heterogeneity. Carcinomas with lymphatic invasion showed a high Ki-67-index. There was no relation between cell proliferation and tenascin expression in both normal tissues and tumours studied. The absence of a correlation between tenascin and Ki-67 expression suggests that the main function of tenascin in both normal tissues and tumours of the canine gastrointestinal tract is anti-adhesion rather than proliferation.
Introduction

Epithelial tumours are composed of two discrete interdependent compartments: the neoplastic epithelial cells and the reactive stroma. Identification of tumour properties that may help identify tumours that have malignant behaviour is one of the major aims in tumour research. The tumour properties can basically be divided into two: properties of the tumour cells and properties of the tumour stroma or extracellular matrix components. These properties are not totally independent.

There is increasing evidence that proliferative activity measured by Ki-67 expression may be a useful measure of the malignancy potential for a variety of gut tumours (Yonemura et al 1990, Johnston et al 1989). It has been used to study proliferative activity of human tumour cells of different origin (Yonemura et al 1990, Porschen et al 1991, Kim et al 2001). Quantitative measures of cellular proliferation have been tested in several canine tumours as prognostic indicators (Pêna et al 1998, Phillips et al 2000). Ki-67 antibody is directed against a nuclear antigen that is only expressed in proliferating cells of G1, G2, S and M phases of cell cycle.

Tenascin is a large polymorphic glycoprotein of the extracellular matrix, expressed transiently during embryogenesis, wound healing and neoplasia (reviewed by Mukaratirwa and Nederbragt 2002). In vitro experiments have shown that tenascin promotes proliferation of tumour cells (Chiquet-Ehrismann et al 1986). In human breast carcinomas, tenascin expression was found to be associated with tumour cell proliferation (Jahkola et al 1998). Increased tenascin expression has been demonstrated in canine gastrointestinal adenomas and carcinomas with a regional variation between different areas of the same tumour (Mukaratirwa 2003). It would therefore be of great interest to study the regions of tumours with increased tenascin expression with proliferation markers to determine whether there is a correlation between tenascin expression and tumour cell proliferation as seen in vitro experiments.

While tenascin is a key player in the development of small intestine mucosa during embryogenesis (Beaulieu et al 1993), its role in the regulation of adult intestinal functions such as proliferation, migration of enterocytes and specific tissue gene expression remains to be established. Studies on cell kinetics in relation to tenascin expression pattern in tumours are rare (Goussia et al 2000, Kim et al 2000).

The objective of this study was to assess the proliferative activity of the tumour cells and determine whether there is a correlation between the proliferative activity of tumour cells and tenascin expression around the tumour cells.
Material and methods

Case Materials
Blocks of formalin-fixed, paraffin wax-embedded tissue from cases of normal canine stomach (n=10), small intestine (n=10) and large intestine (n=10), and epithelial tumours of the gastrointestinal tract (11 adenomas, 14 adenocarcinomas, 5 undifferentiated carcinomas) from dogs were retrieved from the Department of Pathology, Utrecht University. Table 1 shows a summary of the number, types and sites of tumours examined. Haematoxylin and Eosin-stained slides were re-evaluated by two pathologists and tumours were classified according to World Health Organisation classification of tumours of the lower alimentary tract (Head 1976).

Table 1. Summary of the number, type and site of canine gastrointestinal tumours studied

<table>
<thead>
<tr>
<th>Tumour</th>
<th>Stomach</th>
<th>Small intestine</th>
<th>Colon</th>
<th>Rectum</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubular</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Tubulovillous</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucinous</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Tubular</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Solid/ signet ring</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Carcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>30</td>
</tr>
</tbody>
</table>

Immunohistochemistry
To study whether tenasin expression correlates with tumour cell proliferation, tenasin expression in tumour stroma was compared with Ki-67 expression in tumour cells in serial sections. Serial sections of each case were stained for tenasin and Ki-67. Tenasin labelling was carried out as previously described (Mukaratirwa 2000) using a monoclonal antibody (Clone TN2; Dako, Glostrup, Denmark) and Ki-67 immunohistochemistry was performed using monoclonal antibody MIB-1 (Biogenex, CA, USA). After de-
waxing through xylol and graded alcohols, the sections were heated in a pressure cooker at 120°C for 10 minutes. The slides were cooled at room temperature for 60 minutes. Endogenous peroxidase activity was blocked by immersing the sections in 3% H₂O₂ in methanol for 30 minutes. Non-specific binding of secondary antibody was blocked by incubating the slides with normal horse serum diluted 1 in 50 for 30 minutes. Sections were then incubated with primary mouse anti-human Ki-67 antibody diluted 1 in 200 overnight at 4°C. Biotinylated horse anti-mouse secondary antibody was applied for 30 minutes at room temperature. Bound antibody was visualized with avidin-biotin-peroxidase complex (Vector, Burlingame, USA). The colour was developed by 3,3’-diaminobenzidine. The slides were counter-stained with haematoxylin. Positive control slides consisted of sections from human lymph nodes with follicular hyperplasia. Negative control slides were prepared by omitting the primary antibody.

**Microscopic analysis**

In sections immunolabelled for Ki-67, the cell nuclei were identified as positive or negative and counted using a 400x objective lens. A Ki-67 index was defined as the number of tumour cells immunolabelled divided by the total number of tumour cells counted x 100. A total number of 1000 nuclei were counted in at least four random fields. The sections were scored as weakly positive when less than 20% of the nuclei were positive, moderate when 20-50% of the nuclei were positive and as strong when greater than 50% of the nuclei were positive. Serial sections stained for Ki-67 and tenascin were compared to determine whether there was a high Ki-67 index in tumour regions expressing tenascin.

**Results**

**Tenascin Expression**

Tenascin expression pattern in the normal gastrointestinal mucosa and epithelial tumours was as described previously (Mukaratirwa, 2003). Briefly tenascin expression was absent in the normal mucosa of the stomach, but expressed in the normal intestine with a gradual increase from the cryptal glands to the surface epithelium. Tenascin expression was increased in both adenomas and carcinomas. Two different patterns of tenascin expression were observed in carcinomas. In well-differentiated tumour regions, a fibrillary sub-glandular expression was present and in poorly differentiated tumour regions a diffuse network expression pattern was present. In areas of incomplete invasion of the muscularis mucosae, the muscularis mucosae was thickened and tenascin expression was increased in these areas.
Ki-67 expression

Normal mucosa
Ki-67 positive cells exhibited a clearly recognizable reaction, which was located in the nucleus. In the normal gastric mucosa, cells in the glandular neck region were positive for Ki-67 and cells at the surface of the mucosa and glands were negative. This is consistent with the fact that in the gastric mucosa, cells proliferate from the neck region to the surface mucosa and down to the gastric glands. Stromal cells of the lamina propria and submucosa were negative. In the normal small intestine and colon, epithelial cells of the crypts were positive with a gradual decrease to the top of villi or surface mucosa (Fig 1). Cells at the surface were negative. This is consistent with the fact that in the intestine, cells proliferate from the crypts to the top of the villi or surface mucosa.

Tumours
Of the 11 adenomas studied Ki-67 expression was increased compared to the normal mucosa (Fig 2). Three cases were weakly positive, four were moderate and four were strongly positive. There was no relation between Ki-67 expression and the histological type of the adenoma. Some well-differentiated areas of the tumour were completely negative. Of the 14 adenocarcinomas, four were weakly positive, four were moderate and six were strongly positive. All the undifferentiated carcinomas were strongly positive for Ki-67 (Fig 3a). Stromal cells were negative for Ki-67. There was no relationship between Ki-67 index and the depth of tumour invasion or differentiation.

Fig 1: Section of normal colon mucosa immunolabelled for Ki-67. Cryptal cells are positive and cells at the surface are negative for Ki-67 (1b). Avidin-biotin peroxidase (ABC), haematoxylin counterstain. x 100

Fig 2: Colon adenoma immunolabelled for Ki-67. Increased number of moderately Ki-67 positive cells. ABC, haematoxylin counterstain. x 200
Fig 3: Serial sections of a colon undifferentiated carcinoma immunolabelled for Ki-67 (3a) and tenascin (3b). Increased number of Ki-67 positive cells in (3a) and absence of tenascin expression in (3b). ABC, haematoxylin counterstain. x 200

Fig 4: Serial sections of a colon adenocarcinoma immunolabelled for Ki-67 (4a) and tenascin (4b). Increased number of Ki-67 positive cells at the invasive tumour margins (4a) is not related with increased tenascin expression at the tumour margins (4b). ABC, haematoxylin counterstain. x 100

of the tumour. Ki-67 expression was increased on the edges of adenomas and at the invasive tumour margins of carcinomas (Fig 4a).

Relation Between Tenascin and Ki-67 Expression

Normal tissue
There was no correlation between tenascin expression in the stroma and Ki-67 expression in epithelial cells in the normal gastrointestinal mucosa. In the gastric mucosa there was no tenascin immunoreactivity, but Ki-67 was consistently expressed in cells of the glandular neck region. In the small intestine and colon tenascin was expressed with a decreasing gradient from crypts to surface epithelium, but Ki-67 was expressed in a
decreasing gradient from crypts to surface mucosa (Fig 1).

Tumours
In 8/11 adenomas there was no relation between tenascin and Ki-67 expression. In 2/11 adenomas the intensity of tenascin expression was increased in tumour regions with high Ki-67 expression. In adenocarcinomas and undifferentiated carcinomas there was no relation between tenascin and Ki-67 expression (Fig 3a, 3b and Fig 4a, 4b), except in three cases (21%). Tenascin expression was not increased at invasive tumour margins where Ki-67 index was high (Fig 4a, 4b).

Discussions
Previous studies on cell kinetic parameters in human gastrointestinal tumours have shown that high proliferative activities measured by Ki-67 are associated with a poor prognosis (Johnston et al 1989, Yonemura et al 1990, Porschen et al 1991). In this study the pattern of Ki-67 expression in the normal gastrointestinal mucosa and tumours is in line with the reported pattern in humans (Johnston et al 1989). In adenomas and carcinomas, proliferating cells as identified by Ki-67 labelling were not uniformly distributed throughout the section and this was also observed in tenascin expression. Intra-tumoural heterogeneity of Ki-67 expression has also been demonstrated in human colon carcinomas (Johnston et al 1989). This phenomenon can be explained by the different tumour cell subpopulations within the carcinomas, and this is reflected by the differences in histological differentiation (Jass et al 1986), DNA content (Hiddemann et al 1986) and sensitivity to cytotoxic drugs (Kimball and Brattain 1980).

In vitro studies have shown that tenascin plays a role in foetal and tumour cell proliferation (Chiquet-Ehrismann et al 1986). In our study, there was no relation between tenascin expression and cell proliferation in the normal gastrointestinal mucosa. While cells around the neck zone of the gastric mucosa expressed Ki-67, tenascin was not expressed at all. In the normal small intestine and colon Ki-67 positive cells were situated at the cryptal zone, while tenascin was expressed mainly at the mucosal surface. Our results suggest that the main function of tenascin in the normal gastrointestinal mucosa is cell shedding rather than proliferation.

In adenomas and carcinomas there was no clear correlation between tenascin expression and cell proliferation, except in three adenocarcinomas. These findings are in contrast with the findings in human intra-ductal carcinomas where peri-ductal tenascin expression was associated with increased Ki-67 expression (Jahkola et al 1998). The
absence of a clear correlation between tenascin expression and proliferation in our study might be due to the presence of different subpopulations of tumour cells within the same tumour as these might have different paracrine influence on the stromal components. Another more likely reason for the absence of correlation might be that isoforms of tenascin in gastrointestinal tract have different functional properties. The functional properties reported for tenascin are numerous and in some instances contradictory, largely depending on cell type and experimental conditions (Bélanger and Beaulieu et al 2000). In vitro studies with several gastrointestinal cell lines are required to assess the effects of different isoforms of tenascin on cell adhesion, proliferation, differentiation and migration.

In conclusion, our study demonstrates that gastrointestinal carcinomas exhibit a wide variability in their proliferation rate with inter- and intra-tumoural differences, and that tenascin expression is not correlated to cell proliferation in tumours and normal mucosa.

References


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