Thyroglobulin, Calcitonin, Ki 67 and CEA Immunohistochemical
Staining of Dog Thyroid Carcinomas

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SUMMARY

Immunohistochemical studies were conducted in 20 cases of canine thyroid tumours. On histological grounds the tumours included four well-differentiated follicular and four well-differentiated medullary carcinomas. Four tumours were suspected to be of possible follicular origin but were poorly-differentiated or anaplastic (undifferentiated); similarly six tumours that were poorly-differentiated or anaplastic were suspected to represent medullary tumours. Two tumours were so poorly differentiated that they could not be readily classified. Fifteen of the tumours stained positively for thyroglobulin. Eleven of the tumours stained for thyroglobulin but not calcitonin; they included eight that were of follicular origin, two tumours that had been thought to be of medullary origin and one that was a poorly differentiated tumour not thought to be of medullary origin; immunohistochemical staining allowed their reclassification as follicular carcinoma. Four other tumours that were stained with thyroglobulin had been thought to be of medullary origin and were also stained with calcitonin; immunohistochemical staining allowed their reclassification as mixed tumours. Immunohistochemistry is essential for accurate diagnosis of canine thyroid tumours.

INTRODUCTION

In dogs, thyroid gland tumours have been reported to represent 1 to 2% of all tumours, and 10 to 15% of all head and neck tumours (LOAR, 1986). Ninety per cent of thyroid gland tumours are malignant whereas thyroid adenomas are uncommon as a clinical entity in dogs (LOAR, 1986).

Immunohistochemical studies in canine thyroid gland tumours have included investigation of expression of thyroglobulin and calcitonin (LEBLANC et al, 1991; MOORE et al, 1984; PATNAIK and LIEBERMAN, 1991), calcitonin gene-related peptide, neuron-specific enolase, somatostatin and neurotensin (LEBLANC et al, 1991). Differences in thyroglobulin reactivity may reflect degrees of differentiation of tumour cells but in humans there is no absolute correlation between thyroglobulin positivity and grade of differentiation (KAVISHWAR et al, 1998). Tumour differentiation is only one parameter of malignancy and the present study was performed to extend the immunohistochemical investigation of canine thyroid carcinomas...
by including staining for carcinoembryonic antigen (CEA) and the proliferation marker Ki-67 (MIB-1). Such markers have not been previously investigated in canine thyroid tumours.

MATERIALS AND METHODS

Tumour samples

Twenty cases of thyroid carcinoma were selected from the archives of the Comparative Pathology Laboratory of the University of Bristol Veterinary School. All specimens were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin. Five consecutive sections 4-5 µm thick were cut from each block. One section was stained with haematoxylin and eosin (H&E), and the others were used for immunohistochemistry.

Histological examination

Sections stained with haematoxylin and eosin (H&E) were reviewed to confirm the diagnosis and to classify the tumours within categories of the WHO classification of thyroid tumours in domestic animals (VON SANDERSLEBEN and HANICHEN; 1974) and in humans (HEDINGER et al 1988); in most cases the original diagnosis had been of a thyroid carcinoma with no identification of a likely follicular or parafollicular (C cell) origin.

Immunohistochemistry

Tissues were immunostained according to the avidin-biotin-peroxidase complex (ABC) method (HSU et al 1981). The following primary antibodies were used: rabbit anti-human thyroglobulin (polyclonal) diluted 1:700 (Dakopatts, Copenhagen, Denmark), rabbit anti-human calcitonin (polyclonal) diluted 1:800 (Dakopatts), rabbit anti-human carcinoembryonic antigen (CEA - polyclonal) diluted 1:1500 (Dakopatts) and a monoclonal antibody Ki-67 clone MIB-1 (Immunotech, Marseille, France) diluted 1:100. Briefly, the formalin-fixed sections were deparaffinized, dehydrated and then treated with 0.3% hydrogen peroxide in methanol for 30 min to quench the endogenous peroxidase activity. Sections were then briefly rinsed in distilled water and incubated for 10 minutes at 750W in a microwave oven (Miele Supratronic, model M754) in a 10mM citrate buffer in a thermostable container to prevent drying during the incubation process. The slides were cooled in buffer for 20 min at room temperature, washed in distilled water and rinsed in phosphate-buffered saline. The primary antibodies were applied to the sections and incubated overnight at 4°C. This was followed by incubation with a 1:100 dilution of biotin-labelled anti-mouse secondary antibody (Dakopatts) for 30 min and ABC (Dakopatts) for 60 min. Careful rinses with PBS were done between each step of the procedure. The colour was developed with diaminobenzidine and the sections were lightly counterstained with H&E, dehydrated and mounted. Negative controls for the immunostaining were carried out by substituting the primary monoclonal antibody with mouse IgG1 antibody (Dakopatts), or with a rabbit immunoglobulin fraction (Dakopatts) for the polyclonal antibodies. Positive controls were sections from dog mammary tumours known to express MIB-1, human thyroid tumours known to express thyroglobulin and calcitonin, and a human colon cancer known to express CEA.

Evaluation of immunohistochemical data

The MIB-1 immunostaining (MIB-1 labelling index) was scored by counting 1000 cells in 10 to 20 fields per histological section, depending on their cellularity. Every stained nucleus was considered positive regardless of the intensity of staining. When there was a clear-cut variability in the numbers of stained cells in different areas of the sections, the field examined included areas with the highest and lowest percentages of stained cells.

The presence and distribution of positive brown immunostaining for thyroglobulin, calcitonin and CEA was assessed qualitatively as positive or negative.

RESULTS

Tumour samples

The tumours examined came from a variety of breeds and sex; age ranged from 5-13 years. There was no obvious association between age, breed or sex of dog and either the histological classification of the tumours or their immunohistochemical characteristics. Table 1 lists the histological classification of the tumours based on H&E stained sections according to the human WHO classification; the table also includes revised classification based on immunohistochemical staining.

A follicular origin was suspected in eight of the cases, four of which were anaplastic or poorly differentiated; in all cases there were degrees of follicular formation within solid sheets or cords of tumour cells (Fig. 1) which showed differing degrees of pleomorphism. Two cases were poorly differentiated with no particular indication of either a follicular or medullary origin. A probable medullary origin was suggested in six anaplastic or poorly differentiated tumours and a confident diagnosis of medullary carcinoma was possible in four cases; those cases consisted of clusters of cells separated by thin strands of connective tissue (Fig. 2).
**Immunohistochemistry**

The results of staining for thyroglobulin, calcitonin, CEA and Ki-67 are summarised in table 1 and are described below:

**Thyroglobulin**

Fifteen of the tumours stained positively for thyroglobulin. Positive staining was present in many tumour cells of all tumours in which a follicular origin was suspected on histological grounds and in some localised areas of a poorly differentiated tumour that was not thought to be of medullary origin. Positive thyroglobulin staining was also seen in six cases that were thought to be of medullary origin, four of these tumours stained positively also with calcitonin. Thyroglobulin-positive cells, of variable intensity, occurred in distinct or ill-defined groups separated by unstained tumour tissue (Figure 3); occasional scattered thyroglobulin-positive cells corresponded with cells recognised in H&E sections as macrophages containing brown pigment rather than isolated remnants of normal follicles.

**Calcitonin**

Six cases stained positively with calcitonin, all of which had been considered to be medullary tumours based on histological features. Four of the calcitonin-positive tumours also contained groups of thyroglobulin-positive staining cells. Calcitonin-positive cells, with variable intensity of staining, were scattered through sheets of tumour tissue (Figure 4). Four other tumours that had been suspected to be of medullary origin were not stained with calcitonin. Two of the calcitonin-negative tumours suspected to be of medullary origin stained with thyroglobulin; two other tumours were not stained with either calcitonin or thyroglobulin.

**Ki-67 (MIB-1)**

All tumours showed positively stained cells (Figure 5), regardless of histological appearance or other immunohistochemical properties. The propor-

<table>
<thead>
<tr>
<th>Breed Age Sex</th>
<th>Tumour type</th>
<th>Thyroglobulin</th>
<th>Calcitonin</th>
<th>CEA</th>
<th>*Ki-67</th>
</tr>
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<tbody>
<tr>
<td>Crossbreed</td>
<td>7 MN</td>
<td>Follicular</td>
<td>Follicular</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Rough Collie</td>
<td>10 FN</td>
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<td>Follicular</td>
<td>+</td>
<td>-</td>
</tr>
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<td>-</td>
</tr>
<tr>
<td>Shetland Sheepdog</td>
<td>7 F</td>
<td>Follicular</td>
<td>Follicular</td>
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<td>-</td>
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<tr>
<td>Collie cross</td>
<td>13 M</td>
<td>Poorly differentiated</td>
<td>Follicular</td>
<td>+</td>
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<tr>
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<td>+</td>
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<td>N/A*</td>
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<td>-</td>
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<tr>
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<td>-</td>
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<tr>
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<td>Medullary</td>
<td>Medullary</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
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<td>+</td>
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<tr>
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<tr>
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<td>Mixed</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Retriever</td>
<td>7 F</td>
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<td>Poorly differentiated</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>+</td>
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<td>Poorly differentiated</td>
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<tr>
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<tr>
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<td>Poorly differentiated</td>
<td>Poorly differentiated</td>
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</table>

* N/A not available
tion of stained cells varied from less than 10% to 84%; there was no obvious relationship between Ki67 labelling index and histological tumour type.

**CEA**

There was weak staining of a few tumour cells in only one tumour; it had been considered to be a poorly differentiated tumour on histological grounds but was calcitonin-negative and thyroglobulin-positive. There was no staining of any other tumours or of normal thyroid gland cells.

**DISCUSSION**

The WHO (1974) classification of malignant thyroid tumours of domestic animals distinguishes five morphological types of carcinoma of follicular origin (follicular; solid and solid-follicular; papillary; squamous; anaplastic) and C-cell (parafollicular) carcinomas. In humans (HEDINGER et al, 1988) medullary carcinoma is a malignant tumour of the thyroid gland composed of cells showing evidence of C cell differentiation and usually containing calcitonin; in contrast, any malignant thyroid tumours showing features of follicular cell differentiation and, specifically, forming thyroid follicles are regarded as follicular carcinoma. In the cases investigated here approximately half the cases appeared to be predominantly of follicular or medullary origin of varying degrees of differentiation.
An association between calcitonin and CEA has been demonstrated in human thyroid medullary carcinomas (TALERMAN et al, 1979; TAKEICHI et al, 1989). In the present study only one of the tumours stained for CEA, and thyroglobulin; none of the calcitonin-positive tumours was stained. CEA-staining has been reported in a variety of other canine carcinomas including pancreatic (RABANAL et al, 1992) and hepatic tumours (MARTIN DE LAS MULAS et al, 1995).

Ki-67 expression has been investigated in several different types of neoplasia in dogs including mammary tumours (GERALDES et al 2000; LÖHR et al, 1997), testicular tumours (SARLI et al, 1994) and lung carcinomas (GRYFFEY et al, 1999); there has been good correlation between Ki-67, and other proliferation indices, and mitotic rate. In the present study the percentage of stained cells ranged from 7.9 to 84%; there was no obvious association between the percentage of staining and histological tumour type among the other tumours. In human thyroid tumours expression of Ki-67 was considered to be low, ranging from 0.2-3.9% of carcinoma cells (WALLIN et al, 1992). In another report, the level of expression (mean 57.6+/-3.3) in anaplastic carcinomas was up to three times greater than in other types of tumour and could distinguish follicular adenomas from follicular carcinomas (ERICKSON et al, 1998; RIGAUD and BOGOMOLETZ (1991) however found no correlation between Ki-67 staining and histological typing or pTNM classification in 11 thyroid carcinomas.

In the present study only three, poorly-differentiated, tumours failed to stain with either thyroglobulin or calcitonin, alone or in combination. MOORE et al (1984) reported positive staining for thyroglobulin in thyroid carcinomas and described different patterns of immunoreactivity and some variation between different histological types; as in the present study, all follicular carcinomas were thyroglobulin-positive. LEBLANC et al (1991) also reported all follicular carcinomas to be positive for thyroglobulin but negative for somatostatin and neurotensin; half of the tumours were stained with antisemum to neuron specific enolase. A thyroid oncocytoma was also thyroglobulin-positive (TANG et al, 1994) and HOLSCHE et al (1986) have demonstrated that thyroglobulin was also positive in an ectopic thyroid tumour in a dog. PATNAIK and LIEBERMAN (1991) have shown that all of 16 medullary thyroid carcinomas were negative for thyroglobulin but positive for calcitonin. In the present study six of 20 cases stained for calcitonin; all of the cases were considered to be medullary carcinomas and in four of these cases there was dual staining for both thyroglobulin and calcitonin. The nature and origin of dual staining is of great importance. MOORE et al (1984) reported only occasional calcitonin-positive cells that were interpreted as entrapped non-neoplastic C-cells, while thyroglobulin-positive cells in C-cell carcinomas were considered to represent entrapped remnants of normal thyroid tissue. An ectopic thyroid gland tumour was negative for calcitonin (HOLSCHER et al, 1986). The danger of misinterpretation of false-positive staining due to passive absorption or phagocytosis by tumour cells has been highlighted by SOBRINHO-SIMOE S and FONSECA (1994) and scattered thyroglobulin-positive cells in some cases in the present study appeared to represent thyroglobulin in macrophages; VENKATRAMAN et al (2001) have recently highlighted possible confusion with metastasis of thyroglobulin immunoreactivity in lymph node histiocytes. The first description of mixed medullary-follicular carcinoma in humans was by HALES et al in 1982; subsequently thyroglobulin staining of three of ten (WILSON et al 1986) and eight of 14 (HOLM et al; 1987) medullary carcinomas, all of which were also stained with calcitonin, was reported. Dual staining for thyroglobulin and calcitonin in the ultrastructural study by HOLM et al (1986) was usually in separate cell populations; more recently, PAPOTTI et al (1997) have reported investigations by in situ hybridisation as well as immunohistochemistry; in most cases tumour cells expressed only either calcitonin or thyroglobulin genes (PAPOTTI et al, 1997) although dual gene expression occurred in rare tumour elements. According to the human WHO classification, mixed medullary-follicular carcinomas are tumours “showing both the morphological features of medullary carcinoma together with immunoreactivity for calcitonin, and the morphological features of a follicular carcinoma together with immunoreactivity for thyroglobulin”. SOBRINHO-SIMOE S (1993) has suggested that most of the tumours exhibiting thyroglobulin and calcitonin immunoreactivity are most likely medullary carcinomas with thyroglobulin immunoreactivity when such staining is confined to the glandular foci.

The tumour typing applied in the present study was based on the predominant histological pattern and, even though a mixed pattern was not obvious, dual staining with thyroglobulin and calcitonin in four cases of medullary carcinoma could also be explained by the presence of elements of a mixed tumour rather than dual expression of both proteins. This is the first description of possible mixed medullary-follicular carcinomas in animals; mixed tumours are not included in the WHO classification of thyroid tumours in domestic animals (VON SANDERSLEBEN and HANICHEN; 1974) in which malignant tumours are separated only into five forms of malignant epithelial tumour (follicular, solid and solid-follicular, papillary squamous and anaplastic/undifferentiated) or C-cell carcinomas.
ACKNOWLEDGEMENTS

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