IMMUNOHISTOCHEMISTRY WITH KERATIN, VIMENTIN, DESMIN, AND \( \alpha \)-SMOOTH MUSCLE ACTIN MONOCLONAL ANTIBODIES IN CANINE MAMMARY GLAND: BENIGN MAMMARY TUMOURS AND DUCT ECTASIAS

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Veterinary Quarterly 1993; 14:89-95

SUMMARY
Duct ectasias (n=2) and different types of benign canine mammary tumours (n=19) were studied immunohistochemically with monoclonal antibodies (MoAbs) directed against various human keratin types (K), \( \alpha \)-smooth muscle actin, vimentin, and desmin. In the duct ectasias and in most tumours the epithelial structures revealed an inner and outer cell layer. The inner cell layer was characterized by labelling with K 7, 8, 18, 19 and mostly also with K 4 and/or K 10 MoAbs. The outer cell layer was almost invariably labelled by K 14, K 14 and 17, and \( \alpha \)-smooth muscle actin MoAbs. The labelling patterns of both duct ectasias and tumours corresponded largely to the patterns observed in normal mammary gland tissue, although a more distinct heterogeneity was seen. Tumours histopathologically assumed to be of a myo-epithelial origin did not show immunohistochemical features of myoepithelial cells. The myoepithelial nature of the vast majority of spindle-shaped cells present in the adenomas of the complex type and in the fibroadenomas of the benign mixed type could not be confirmed immunohistochemically. These cells, however, unequivocally expressed vimentin, suggesting proliferation of stromal cells in these tumours, which in the fibroadenomas of the benign mixed type may show metaplasia to bone or cartilage.

In the duct ectasias and in some tumours, a fraction of elongated stromal cells, probably representing myoepithelial cells, was labelled with the \( \alpha \)-smooth muscle actin MoAb.

INTRODUCTION
Compared to other mammals, including humans, dogs have the highest incidence of mammary tumours (15). They are by far the most common tumours in female dogs (8,41), representing 25 to 50 per cent of all neoplasms (8,15,35,36). Mammary tumours occur in male dogs only occasionally (35,41). In female dogs these tumours are commonly found from 6 years onwards and occur only incidentally in dogs younger than 2 years (32,41).

Data on the frequency of benign mammary tumours in dogs vary from 9% (32) to about 65% (41). This considerable variation is due to the existence of different methods of tumour classification (8,18,31,34,41) and the lack of uniform criteria to differentiate between benign and malignant mammary neoplasms (8,42). Particularly tumours characterized by the presence of large numbers of spindle-shaped, myoepithelial, cells may show histomorphological features compatible with malignancy, despite a benign clinical behaviour (24). As a result, the histological designation of these tumours may be inconclusive (6). Careful examination of apparently normal mammary glands of older dogs may reveal numerous small benign lesions (10,24) which are rarely examined histomorphologically (35).

Histogenetically, mammary tumours may either arise from luminal epithelial cells lining the ducts or alveoli, from myoepithelial cells, or from underlying connective tissue (35). Canine mammary tumours are known for their histomorphological heterogeneity (8,18,35,42). Most benign canine neoplasms are complex or mixed tumours (6,24,26,41,42), characterized by the presence of spindle-shaped, myoepithelial cells, as well as cartilage, bone, or fat in addition to proliferated luminal epithelial cells (24,41). The prominent presence of spindle-shaped, myoepithelial cells is considered to be characteristic of canine mammary neoplasms (34), although these cells have also been observed in feline and rat mammary tumours (24,50). In contrast to the situation with dogs, myoepithelial proliferations constitute only a minor component in human breast tumours (24) and pleomorphic adenomas, histomorphologically similar to canine mixed tumours (26), are rare in humans (3,4,14,39). In addition, tumour-like lesions, e.g., duct ectasias, can be found in mammary gland tissue (24).

In the canine mammary gland, as in the human breast, luminal and basal/myoepithelial cells can be distinguished immunohistochemically with monoclonal antibodies (MoAbs) directed against specific human keratin types and \( \alpha \)-smooth muscle actin (64).

This paper reports the immunophenotyping of duct ectasias and various benign tumours of the canine mammary gland by using MoAbs directed against different human keratin types, \( \alpha \)-smooth muscle actin, vimentin, and desmin. The results are discussed with particular emphasis on the spindle cell component of complex mammary tumours.

MATERIALS AND METHODS
Tissue specimens of canine mammary gland duct ectasias (n=2) and benign tumours (n=19) were frozen in liquid nitrogen precooled isopentane immediately after surgical excision or euthanasia of the animals. The specimens were stored at -70°C until use. Frozen sections for histological examination were fixed in Ca-formalin Macrodex (NPBI, Amstelveen, the Netherlands) in 0.9% NaCl and labelled with haematoxylin and cosin. The mammary gland lesions

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were classified on cryostat sections according to the World Health Organization classification of tumours of domestic animals (24) (Table 1).

The specificity, dilutions used, source, and relevant references concerning the monoclonal antibodies (MoAbs) used for immunohistochemistry are presented in the accompanying paper (64). Five-micron-thick frozen sections were fixed in cold acetone (-20°C) for 10 minutes and air dried for 5 minutes. Immunohistochemical labelling was performed using the indirect immunoperoxidase technique as described previously (65).

The labelling results are expressed semi-quantitatively as follows:

- ++ = < 10% positive cells
- +++ = > 10% but < 50% positive cells
- ++++ = > 50% but < 90% positive cells
- ++++ = > 90% positive cells

RESULTS

The immunohistochemical labelling patterns of the duct ectasias and the benign mammary tumours labelled with the various monoclonal antibodies (MoAbs) are presented in Table 1 and partially depicted in figures 1-7. In the duct ectasias, RCK 102 (K 5+8) showed homogeneous labelling of both inner and outer cells of the ductal epithelium. Specific labelling of the inner layer was observed with CAM 5.2 (K 8), RGE 53 (K 18), RCK 105 (K 7), LP2K (K 19), which showed almost homogeneous reaction patterns. Antibodies 6B10 (K 4) and RKSE 60 (K 10) exhibited a more heterogeneous labelling pattern, with ductal cells occasionally being labelled. Specific labelling of the outer layer was seen with the MoAbs RCK 107 (K 14), 8.7 (K 14+17) and with Sm-1 (α-smooth muscle actin). The epithelium did not react with the vimentin or desmin MoAbs.

A similar labelling pattern was observed in the benign mammary tumours. Homogeneous or almost homogeneous labelling of virtually all epithelial structures with equal labelling intensity in inner and outer cell layers was seen with RCK 102 (K 5+8); this MoAb did not label spindle cells. More or less homogeneous specific labelling of only the inner layer of the epithelial structures was observed with the MoAbs CAM 5.2 (K 8) and RCK 105 (K 7); more heterogeneous labelling of these cells was seen with the other keratin MoAbs, varying from no labelling at all in some tumours (e.g. with 6B10) or reactivity in a small number of cells (e.g., with RCK 107 or 8.7), to labelling of the majority of the inner cells in some tumours (e.g., with RGE 53 or RKSE 60). The outer cell layer of the epithelial structures in all tumours was invariably labelled with RCK 107 (K 14), 8.7 (K 14+17) and Sm-1 (α-smooth muscle actin) (Figure 1), although in some tumours (e.g., in the simple tubular adenoma) the latter MoAb labelled fewer cells than the two keratin MoAbs. In one of the fibroadenomas of the benign mixed type, RCK 107 labelled fewer cells than the MoAb 8.7 (compare

<table>
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<tr>
<th>Classification</th>
<th>n</th>
<th>cell type</th>
<th>RCK 102 (K 5+8)</th>
<th>CAM 5.2 (K 8)</th>
<th>RGE 53 (K 18)</th>
<th>RCK 105 (K 7)</th>
<th>LP2K (K 19)</th>
<th>6B10 (K 4)</th>
<th>RKSE 60 (K 10)</th>
<th>RCK 107 (K 14)</th>
<th>8.7 (K 14+17)</th>
<th>Sm-1</th>
<th>RV 203</th>
<th>RD 301</th>
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<td>++++/+++++</td>
<td>++++</td>
<td>++++/+++</td>
<td>++++/+++</td>
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<td>+</td>
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<td>OL</td>
<td>++++</td>
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<td>OL</td>
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<td>+++</td>
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n = number of cases; (K) keratin types according to Moll et al. (38); IL = inner layer; OL = outer layer; SC = spindle cells; BCC = bone and/or cartilage cells

+ = < 10% positive cells
++ = > 10% but < 50% positive cells
+++ = > 50% but < 90% positive cells
++++ = > 90% positive cells

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Figures 2A and 2B. In some of the benign tumours, areas were present in which there was a marked absence of labelling of cells at the junction with the surrounding stroma by these antibodies (Figure 3).

With respect to the different tumour types, in most benign tumours positive although heterogeneous labelling was observed with the 6B10 (K 4) (Figure 4) and/or the RKSE 60 (K 10) MoAb, whereas only in one of the fibroadenomas of the benign mixed type the tumour cells were not labelled with these MoAbs. In one of the fibroadenomas of the benign mixed type, the epithelial structures in a small part of the specimen were characterized by homogeneous labelling with MoAbs RCK 107 (K 14), RCK 8.7 (K 14+17) and RCK 102 (K 5+8) and by the absence of labelling with the other keratin MoAbs (Figure 5). Most of these epithelial structures were also labelled with Sm-1. Immunohistochemically, the two duct papillomas showed striking differences in labelling patterns. In one case only a thin layer of outer cells was observed to be specifically labelled with RCK 107 (K 14), RCK 8.7 (K 14+17) and Sm-1, whereas in the other case a much broader layer of outer cells was labelled with these antibodies (Figure 6).

Stromal cells in all cases were invariably labelled with the vimentin MoAb RV 203. In some tumours a restricted number of inner and/or outer layer cells was also labelled with this MoAb.

The tumour types which contained spindle-shaped cells as a distinct component (adenoma of the simple myoepithelial and complex types, and the fibroadenomas) showed homogeneous labelling with RV 203 of these cells (Figure 7A). In some cases, these spindle-shaped cells were also labelled with the keratin MoAb CAM 5.2 (K 8). Additionally, bone and cartilage cells were occasionally labelled with this K 8 MoAb. Only in some tumours a very small number of spindle-shaped cells was labelled with the keratin MoAbs RCK 107 or 8.7. These positive cells were situated directly adjacent to the epithelial tumour cells (Figure 7B).

In some fibroadenomas of the benign mixed type, also a small number of RCK 107- or 8.7-positive cells was observed within the bone or cartilage component (Figure 2). These cells were not labelled with Sm-1. This α-smooth muscle actin MoAb labelled, however, a varying, but small fraction of solitary elongated cells in the connective tissue of duct ectasias and in 11 benign tumours. The desmin MoAb RD 301 labelled vessel walls and small muscles. In addition, a few elongated cells scattered in the connective tissue were also positive.

Figure 2A. Immunoperoxidase labelling pattern of canine fibroadenoma of the benign mixed type with MoAb 8.7, directed against human keratin types 14 and 17. Labelling of outer cells in the epithelial component is noticed. In the spindle cell/chondroid component only small groups or scattered cells are labelled. Frozen section, 125x.

Figure 2B. Immunoperoxidase labelling pattern of canine fibroadenoma of the benign mixed type with RCK 107, directed against human keratin type 14. A similar labelling pattern as in figure 2A is noticed, although fewer cells seem to be labelled. Frozen section, 125x.

Figure 3. Immunoperoxidase labelling pattern of canine fibroadenoma of the benign mixed type with RCK 107, directed against human keratin type 14. Absence of labelling in a high percentage of outer tumour cells. Frozen section, 100x.

Figure 1. Immunoperoxidase labelling pattern of canine adenoma of the complex type with Sm-1 directed against α-smooth muscle actin. An almost continuous layer of outer cells is labelled. Frozen section, 200x.
**DISCUSSION**

The labelling patterns in the duct ectasias were similar to the patterns in the normal mammary gland. Identical labelling patterns for mammary cysts (duct ectasias) and normal breast tissue have been seen in humans (63).

All keratin MoAbs except CAM 5.2 (K 8) reacted exclusively with epithelial cells in the benign canine mammary tumours. CAM 5.2 also reacted with stromal cells in some tumours, an observation also reported for human breast lesions (39, 46). Since these stromal cells were not labelled with any...
of the other keratin MoAbs, including RCK 102, which also reacts with K 8, and since CAM 5.2 also labelled bone and cartilage cells in fibroadenomas of the benign mixed type, this finding may indicate cross-reactivity of CAM 5.2 with a non-epithelial epitope. Also in human tissues, CAM 5.2 has been suggested to react with epitopes of antigens different from keratins (44). MoAb RCK 102 is the only immunoreagent tested that labelled both inner and outer cells in the tumours examined.

The labelling patterns within the benign canine mammary tumours were to a large extent in accordance with the patterns seen in the normal canine mammary gland. MoAbs that exclusively labelled luminal cells and basal/myoepithelial cells in the normal gland (64), almost invariably labelled only inner and outer cells, respectively, in the benign tumours. K 18 and K 19 immunoreactivity in luminal cells of benign canine mammary tumours has been reported previously (13). The number of cells immunoreactive for certain keratins apparently differ when compared to the normal situation. For instance, the MoAbs 6B10 (K 4) and RKSE 60 (K 10) labelled most tumour cells in the tubular adenoma of the simple type and in a fibroadenoma of the benign mixed type, whereas these antibodies only labelled a minority of ductular luminal cells in normal canine mammary gland tissue (64). Although most luminal cells in the mammary gland were labelled with RGE 53 (K 18) (64), one of the adenomas of the complex type showed only a minority of cells that reacted with this MoAb. Since K 4 and K 10 immunoreactivity was found only in ductal cells in normal mammary gland tissue, the presence of cells reacting with both these antibodies in 10 of the benign tumours may indicate a possible ductal origin of these tumours. However, changes in the patterns of keratin expression as a result of neoplastic transformation cannot be excluded (48). In feline benign mammary tumours, luminal cells have been reported to be labelled with K 5 + 8, K 7, K 18 and K 19 MoAbs (37). In human benign mammary lesions, including benign tumours, labelling of inner cells has been reported with K 7, K 8 and K 18, together with a heterogeneous pattern with K 19 antibodies (1.5.2.24.43.60). In general, the benign mammary tumours show a basal compartment of epithelial cells that reacted with the MoAbs RCK 107 (K 14), 8.7 (K 14+17) and Sm-1 (α-smooth muscle actin). These antibodies label exclusively the basal/myoepithelial cells in normal mammary gland tissue (64). Although hypertrophy and slight hyperplasia of basal/myoepithelial cells was observed, in general only a single continuous layer of positive cells was seen at the epithelial stromal junction. In a few tumours these cell layers showed discontinuities and in one of the fibroadenomas of the benign mixed type these cells were absent. In humans, this phenomenon has been observed in malignant breast tumours (9,19,20,49,66). In feline benign mammary tumours basal cells are labelled with MoAbs directed against K 5+8 and K 14+17 (29,37).

In most benign (complex) breast lesions in humans, myoepithelial cells are found as a single layer encircling duct or alveolar structures (12,20,43.49). Such cells express K 5, K 14 and K 17 (60). In some canine tumours the α-smooth muscle actin MoAb labelled fewer cells than the two basal cell keratin MoAbs RCK 107 and 8.7. This observation may be due to loss of differentiation as a result of neoplastic transformation. However, neoplastic transformation can also boost cytoplasmic actin in epithelial cells (23), and malignant cells have been demonstrated to contain an increased amount of contractile proteins, including actin (19). Therefore, these findings may indicate the presence of two different, basally located cell types. Although in the normal canine mammary gland no distinct differences were found in the labelling of basal cells with the three MoAbs (64), the existence of different types of basal cells seems probable considering findings in other species. In human breast tissue, basal cells have been observed which were not labelled for α-smooth muscle actin. These may possibly represent 'reserve' cells with proliferative potential (23). Also in rat mammary tissue, the presence of such precursor cells, able to differentiate into luminal or myoepithelial cells, was suggested (59). The presence of basal cells, different from myoepithelial cells, has also been reported in mammary gland tissue of the mouse (51,55). A minority of luminal cells in some tumours was positive for the basal keratin markers. These cells were mostly negative for α-smooth muscle actin. Similarly, in human benign breast tumours, luminal cells have been shown to be K 14 (63) and K 17 positive (22). Although not observed in the dog (64), in humans the presence of a restricted number of K 17 as well as K 14 reactive luminal cells in large ducts of normal breast tissue has been reported (21,67,68). The presence of luminal cells with a keratin immunoreactivity similar to that of basal/myoepithelial cells in benign mammary gland lesions may indicate the proliferative capacity of precursor cells which are able to differentiate either into luminal or into myoepithelial cells, resulting in heterogeneous immunophenotypic patterns (63). However, an abnormal distribution pattern of basal cells or the acquisition of keratin polypeptides normally characteristic of these basal cells by neoplastic luminal cells cannot be excluded (21).

In the canine tumour types in which spindle-shaped cells are a prominent part of the tumour tissue, i.e., simple myoepithelial and complex adenomas and the fibroadenomas, these spindle-shaped cells were mostly not labelled with the keratin and actin MoAbs. In the simple myoepithelial adenomas spindle-shaped cells were labelled only with the K 8 MoAb CAM 5.2 and the vimentin MoAb RV 203. As already discussed, CAM 5.2 most probably also reacts with a non-epithelial epitope. In humans, myoepitheliomas originating from different sites, including mammary gland, were focally or diffusely labelled with antibodies directed against keratins, actin and vimentin (7,16,28,54), although also vimentin (61) and actin unreactive (11) myoepitheliomas have been reported. Keratin immunoreactivity was considered to be the most useful adjunct to the diagnosis (7). Therefore, the presumed myoepithelial nature of the canine neoplasms, histologically classified as simple myoepithelial solid adenomas, cannot be confirmed immunohistochemically and consequently they should be reclassified. Based on the vimentin reactivity, a classification as benign mesenchymal tumours would be justified, although the immunohistochemical and the histomorphological features of these tumours may also be compatible with giant fibroadenomas, described in humans, which are characterized by a very conspicuous cellular stromal component masking the epithelial part of these tumours (3). In complex adenomas and fibroadenomas, in which spindle shaped myoepithelial cell proliferation is a distinct histomorphological feature (24), the vast majority of the spindle shaped cells was also not labelled with any of the keratin or the actin MoAbs. Spindle-shaped cells reacting with keratin 14 and 17, and with the actin antibodies were only found directly adjacent to epithelial compartments of the tumour, and consequently these cells have to be regarded
as hypertrophic and hyperplastic basal/myoepithelial cells. The vast majority of the spindle-shaped cells was only labeled with the vimentin MoAb, as in human fibroadenomas (21). Therefore, the immunohistochemical findings suggest may be a mesenchymal origin of these cells. Cartilage and/or bone formation is a characteristic feature of canine fibroadenomas of the benign mixed type. In canine benign mixed mammary tumours, transition of myoepithelial cells to cartilage has been documented by electron microscopical and histochemical studies examining chondroitin sulphuric acid and alkaline phosphatase (30,47,58). Transformation of modified myoepithelial cells has also been reported in humans (2,17,28,33) and the origin of pleomorphic adenoma of the breast from a single cell type capable of bidirectional differentiation has been suggested (14). However, our findings strongly suggest that cartilage and/or bone formation in canine mammary tumours results from stromal metaplasia, which is in line with histochemical studies concerning anionic heteroglycans (45). This stromal metaplasia is a well-known phenomenon in the stroma of epithelial tumours at different sites (33). In the canine fibroadenomas of the benign mixed type, a small number of cells positive for the basal cell keratins was found within the chondroid or osteoid matrix. As these cells were unreactive for α-actin, they have to be considered as entrapped epithelial remnants. Similar to observations in the benign canine mammary lesions, benign breast lesions in humans, e.g., intraductal papillomas, fibroadenomas and pleomorphic adenomas, contain elongated cells that are reactive with muscle-specific actin antibodies within the connective tissue of the tumours (4,39,43,44,53,63,66). Since actin antibodies also label myofibroblasts (25,52,53,56,62), and these stromal cells in the canine mammary lesions did not label with the keratin MoAbs known to label basal/myoepithelial cells, they may represent myofibroblasts rather than myoepithelial cells. The vimentin positivity of some outer layer cells as well as inner layer cells observed in several of the canine tumours has also been reported previously in both canine (27) and human benign mammary tumours (13,22,63). It has been postulated that vimentin may be commonly expressed in cells with loss of cohesiveness (57), which may explain the vimentin labeling of tumour cells, e.g., neoplastic myoepithelial cells (40).

In conclusion, the labelling patterns of the various canine benign mammary tumours largely correspond to those observed in normal mammary gland tissue. However, even identical tumour types exhibit heterogeneous immunohistochemical features. Human benign tumours also show some heterogeneity of the tumour cell population, thought to be due to the polyclonal growth of such neoplasms (60). Although various tumours showed hypertrophy and hyperplasia of myoepithelial cells, immunohistochemically these cells appear to constitute a far less prominent component in benign canine mammary tumours than assumed on basis of histomorphological features. The spindle cell component most likely represents a proliferation of mesenchymal stromal cells which may show metaplasia to bone or cartilage.

ACKNOWLEDGEMENTS

The authors wish to thank Mrs. W.E.M. van Dijk-Wijnen for excellent manuscript preparation.

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