IMMUNOHISTOCHEMISTRY WITH KERATIN, VIMENTIN, DESMIN, AND ω-SMOOTH MUSCLE ACTIN MONOCLONAL ANTIBODIES IN CANINE MAMMARY GLAND: MALIGNANT MAMMARY TUMOURS

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SUMMARY
Ten malignant canine mammary gland tumours and five metastases from three of these tumours were studied immunohistochemically with monoclonal antibodies (MoAbs) directed against different human keratin types (K), ω-smooth muscle actin, vimentin, and desmin.

In all tumours the neoplastic epithelium was either homogeneously labelled with the keratin MoAbs RCK 102 (K 5 and 8) and CAM 5.2 (K 8). The adenocarcinomas (n=5), the solid carcinomas (n=2), and the carcinosarcoma (n=1) showed heterogenous labelling with the MoAbs specific for luminal cell antigens in the normal canine mammary gland, i.e., K 18, K7 and K 19 MoAbs. These cells were also immunoreactive with K 4 and K 10 MoAbs. The spindle cell carcinomas (n=2), however, did not react with these MoAbs.

All tumours except one adenocarcinoma were characterized by the absence of immunoreactive labelling with the ω-smooth muscle actin MoAb. In the solid carcinomas this was associated with the absence of labelling with one or both basal cell specific keratin MoAbs, i.e. K 8 (K 14 and 17) and RCK 107 (K 14), respectively. In contrast, the other malignant tumours showed marked labelling of neoplastic epithelium with these MoAbs. Another remarkable finding was the labelling of a limited to moderate number of neoplastic epithelial cells with the vimentin MoAb. The presence of such labelling patterns in canine mammary gland tumours may be indicative of malignancy. Metastatic tumour tissues had a labelling pattern largely similar to that of the primary tumour, although also less of reactivity for some keratin MoAbs was seen.

INTRODUCTION
Mammary tumours are the most frequently occurring neoplasms in the female dog (18,41). Much controversy exists, however, on the incidence of malignant mammary tumours as they have been reported to comprise 28% (28) up to 91%

(29) of the tumours examined. This wide range is most probably due to the lack of uniform histological criteria to assess malignancy (38,40). Morphological criteria alone may be insufficient for a proper diagnosis, as histologically determined benign tumours may incidentally give rise to metastases (38), while, vice versa, canine complex adenomas and mixed mammary tumours often show histomorphological evidence of malignancy despite benign biological behaviour (25).

Malignant mammary tumours are not uniformly classified (20,25,38,40), as there are various classifications for mammary tumours in the dog. Some are based on descriptive morphology corresponding to the World Health Organization (WHO) classification of human breast tumours, i.e., the WHO international histological classification of tumours in domestic animals (25), while others are based on the anatomical site of tumour origin associated with the most prominent morphological features (30,38) or are correlated with differences in biological behaviour (20,35).

In women, malignant breast tumours are the most common form of malignant disease in the Western countries and result in tumour-related death in more than half of the patients (3,31,65). Because of its relevance to the human situation, the various aspects of animal mammary gland tumours are often compared with their human counterparts (20,34,49).

Canine mammary tumours have been proposed as an animal model for human malignant breast neoplasms (8,16,20,40,42), as they have many features in common, e.g., onset in coinciding age groups, morphological appearance, metastatic capacity, and the general course of the disease (38). Various classification methods also exist for human malignant breast tumours (3).

Immunohistochemistry, in particular in combination with the use of monoclonal antibodies (MoAbs), has proven to be a valuable and objective method in tumour evaluation, supplementary to histological examination (19,33,56). Consequently, human breast lesions have been extensively studied immunohistochemically, in particular for the distribution of cytoskeletal proteins such as keratins, vimentin, desmin, and actin (e.g., 4-6,17,22, 43,44,65,71,75). Particularly the study of malignancies has received much attention, since breast carcinomas with different keratin compositions may exhibit different clinical behaviours (65), and the vimentin expression of these tumours has been associated with progressive behaviour (15,46). Rat (41,49), mouse (1,55), canine (10,11,27), and feline (34) mammary gland tumours, either induced or spontaneous, have also been studied immunohistochemically.

The present paper reports our findings for various canine malignant mammary gland tumours immunohistochemically tested with MoAb directed against different human keratin types, ω-smooth muscle actin, vimentin, and desmin. The findings are discussed and compared to the findings in canine normal mammary gland and canine benign mammary tumours, and data obtained in other species, particularly humans.
MATERIALS AND METHODS
Ten malignant canine mammary gland tumours, which all had regional and/or distant metastases, as well as five additional metastases from three of these tumours, were examined immunohistochemically. Tissue specimens 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-formaldehyde (NPBI, Amstelveen, the Netherlands) in 0.9% NaCl and labelled with haematoxylin and eosin. The tumours were classified on cryostat sections according to the World Health Organization classification of tumours of domestic animals (25) (Table 1). The carcinosarcoma was finally classified after additional evaluation of paraffin sections. The metastatic tissues studied comprised lymph node metastases of an adenocarcinoma of the simple type and both lymph node and lung metastases of a spindle cell carcinoma and of a carci nosarcoma. The monoclonal antibodies (MoAbs) used for immunohistochemistry are mentioned in Table 1. Their specificity, dilutions used, source, and relevant references are presented in the accompanying paper (68). Five-micron thick 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 described previously (70). 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 labelling patterns of the various malignant canine mammary tumours are presented in Table 1. In most tumours the epithelial cells were quite homogeneously labelled with the keratin MoAbs RCK 102 (K 5+8) (Figure 1) and CAM 5.2 (K 8). In the spindle cell carcinomas, only about half of the epithelial cells were positive for RCK 102 (K 5+8) (Figure 2). The other keratin MoAbs revealed a heterogeneous labelling pattern within and between the various tumour types. For example, RCK 105 (K 7) showed an almost homogeneous labelling pattern in both adenocarcinomas and solid carcinomas (Figure 3), whereas the spindle cell carcinomas did not label with this MoAb. In fact, the spindle cell carcinomas were also negative with several other keratin MoAbs (Table 1).

A remarkable labelling pattern was observed with RCK 107 (K 14) and 8.7 (K 14+17) as well as the α-smooth muscle actin MoAb Sm-1. The adenocarcinomas showed a rather homogenous labelling in the epithelial cells with these keratin MoAbs (Figures 4 and 5) and no labelling with Sm-1. In one adenocarcinoma only a peripheral cell layer reacted with RCK 107 (Figure 6) and 8.7. This outer layer of cells was also labelled with Sm-1 (Figure 7). Inner cells in this tumour were only incidentally labelled with RCK 107 or 8.7. The spindle cell carcinomas and the carcinosarcoma showed limited, or peripheral labelling (in the complex areas) with both keratin MoAbs, whereas they did not label with Sm-1. The 8.7 (K 14+17)-labelled cells were more numerous than the RCK 107 (K 14)-labelled cells in some tumours of different histological type. A different labelling pattern was found in the solid carcinomas, since they were negative for RCK 107 and Sm-1. Only one case showed 8.7-labelled

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n = number of tumours; (K) = keratin types according to Moll et al. (36).
EC = epithelial cells; SC = spindle cells; * only an outer layer of cells was labeled in one tumour
+ = < 10% positive cells
++ = > 10 and < 50% positive cells
+++ = > 50 and < 90% positive cells
++++ = > 90% positive cells
cells. All malignant tumours, particularly the spindle cell carcinomas, showed labelling of neoplastic epithelial cells with the vimentin MoAb (RV 203). Metastatic tumour tissue in general showed a labelling pattern identical to that of the primary tumour, although the number of labelled cells seemed reduced. MoAbs that labelled only a limited number of cells in the primary tumours sometimes did not label metastatic tumour tissue. Metastatic tissue of the simple type adenocarcinoma did not label with the RCK 107 or 8.7 MoAb, whereas in the primary tumour a moderate number of cells was labelled with these MoAbs. Labelling patterns of spindle cell carcinoma metastases in lymph node and lung were similar to those of the primary tumour. Metastases to the regional lymph node of the carcinosarcoma appeared morphologically to be of pure carcinomatous nature, whereas the lung metastatic tissue showed almost entirely a sarcomatous histomorphology. In the metastases to the lymph node, the labelling pattern corresponded to that of the epithelial cells in the epithelial myxoid component of the primary tumour, although an increased number of metastatic cells was immunoreactive with the vimentin MoAb RV 203 (Figure 8). In the lung metastases only a small number of cells was labelled with keratin MoAbs, whereas the vast majority was labelled with RV 203 and CAM 5.2 (K 8). A small number of cells showed a higher labelling intensity with the latter MoAb as compared to the other cells labelled with this antibody. The localization of these intensely labelled cells corresponded to the localiza-

Figure 3. Immunoperoxidase labelling pattern of canine mammary adenocarcinoma of the simple type with RCK 105, directed against human keratin type 2. Note the homogeneous labelling of the tumour cells. Frozen section, 100x.

Figure 4. Immunoperoxidase labelling pattern of canine mammary adenocarcinoma of the complex type with RCK 102, directed against human keratin types 5 and 8. Note the heterogeneous labelling of neoplastic epithelial structures. Frozen section, 100x.

Figure 5. Immunoperoxidase labelling pattern of canine mammary adenocarcinoma of the simple type with MoAb 8.7, directed against human keratin types 14 and 17. Homogeneous labelling of neoplastic epithelial cells. Frozen section, 100x.
The broad spectrum keratin MoAb RCK 102 as well as the K 8-specific MoAb CAM 5.2 labelled neoplastic epithelium in all tumours. CAM 5.2, however, also labelled spindle cells in the tumours with a complex histomorphology. A similar labelling pattern of spindle cells has been observed in benign, complex canine mammary tumours and is most probably due to labelling of a non-keratin epitope (69). In the normal mammary gland, in duct ectasias, and in benign mammary tumours luminal and inner cells, in addition to their immunoreactivity with RCK 102 and CAM 5.2, are labelled with RGE 53 (K 18), RCK 105 (K 7), LP2K (K 19), and some of these cells are also labelled with 6B10 (K 4) and RKSE 60 (K 10) (68,69). In the malignant canine mammary tumours, a more heterogeneous labelling was observed with these keratin MoAbs. Notably the spindle cell carcinomas did not react with any of the MoAbs that labelled luminal cells in the normal canine mammary gland. Immunoreactivity with MoAbs directed against K 8 (7), K 18 and K 19 (11) has been reported previously in canine malignant tumours. In human malignant breast tumours, biochemical and immunohistochemical studies have shown the presence of K 7 (2,3,2,36,37,65), K 8 (2,2,3,2,36,37,65), K 18 (2,3,2,36,37,65,75) and K 19 (2,3,2,36,37,65). Human malignant breast tumours, including metastases, were reported to be almost homogeneously K 19 immunoreactive as opposed to the very heterogeneous immunoreactivity of benign tumours (4). In contrast, the labelling pattern in the canine malignant tumours with the K 19 MoAb used (LP2K) did not differ from the labelling pattern observed in canine benign tumours (69). In feline carcinomas, fairly homogeneous immunoreactivity patterns have been observed for K 7, K 8, K 19 and K 5+8 MoAbs and heterogeneous labelling patterns ranging from absence to almost homogeneous positivity for K 4, and K 14+17 MoAbs (34).

In the normal canine mammary gland, the K 14 MoAb (RCK 107) and the K 14+17 MoAb (8,7), as well as the α-smooth muscle actin MoAb (Sm-1), react with basal/myo-epithelial cells (68). In benign mammary tumours of the dog, generally a similar labelling of outer epithelial cells is observed (69). Such exclusive labelling of outer layer cells was observed in only one adenocarcinoma (RCK 107, 8,7 and Sm-1 positive), and in the complex part of the carcinomarcoma (only RCK 107 and 8,7 positive). In humans, outer layer cells of carcinomas in situ have been shown to express K 14 (54,65,74,75), K 17 (23) or actin (5,6,44,47,54,59,64,71). These outer cells are considered to represent a non-malignant compartment (23,44,65,66). The absence of outer cells expressing K 14 or actin has been related to invasive behaviour (5,21,54,74). However, outer cells labelled with MoAbs exclusively reacting with myoepithelial cells have also been observed in infiltrative carcinomas in humans (54,59,64). Since benign mammary tumours in the dog show regular or irregular labelling of peripheral cells with the α-smooth muscle actin MoAb, the total absence of α-smooth muscle actin immunoreactivity in almost all canine malignant mammary tumours seems to be related to a malignant nature of canine mammary tumours. The absence of actin-labelled (myoepithelial) cells is often reported in human mammary carcinomas (5,6,12,21,47,54,66,71). The absence of Sm-1 immunoreactivity in the canine tumours seems to be...
associated with a different labelling pattern with the keratin MoAbs that specifically label basal/myoepithelial cells, i.e., RCK 107 (K 14) and 8.7 (K 14 + 17). Fairly homogeneous labelling of neoplastic cells with RCK 107 and 8.7 was seen in the adenocarcinomas that did not label with Sm-1. Also in some human breast carcinomas, tumour cells with basal/myoepithelial keratin immunophenotype (61, 75) expressing K 14 (22, 54, 65, 67, 74, 75) and K 17 (22, 75) have been reported. These findings could be caused by the acquisition of basal cell type keratins by neoplastic luminal cells (22) or result from the selection of a specific cell type (37). It is tempting to speculate about the value of this phenomenon as a possible indicator of malignancy. The focal presence of tumour cells labelled with RCK 107 and 8.7 in a histologically benign canine mammary tumour (69) and in human benign mammary lesions (22) may indicate a local malignant transformation, but would also weaken the suggested association of these findings with malignancy. However, in this benign canine tumour, contrary to the carcinomas, α-smooth muscle actin immunoreactivity was observed.

In the canine solid carcinomas, the absence of α-smooth muscle immunoreactivity correlated with the absence of labelling with RCK 107 (K 14), whereas only in one tumour neoplastic cells were labelled with the MoAb 8.7 (K 14 + 17). This absence of labelling with immunoreagents that normally label basal/myoepithelial cells and the labelling for luminal cell markers may indicate a luminal cell type of differentiation of these malignancies. In humans, two main types of invasive carcinomas have been recognized based on immunohistochemical analysis, namely those that are characterized by the presence of predominantly basal cell-specific or luminal cell-specific keratins (75).

Both canine spindle cell carcinomas were labelled with basal cell antibodies without showing labelling with α-smooth muscle actin. Human mammary gland spindle cell carcinomas, histologically assumed to be of myoepithelial origin, show actin immunoreactivity in addition to keratin labelling (52, 62, 72). Also induced mouse malignant mammary gland myoepitheliomas show both actin and general keratin labelling (48). As the canine spindle cell carcinomas do not react for α-smooth muscle actin, a myoepithelial origin of these tumours seems unlikely. However, as basement membrane interaction has been suggested to be essential for myoepithelial differentiation (60), the selective loss of α-smooth muscle actin expression during malignant transformation of myoepithelial cells cannot be excluded. In human spindle cell carcinomas, the majority of cells was labelled by vimentin antibodies, while the additional expression of keratins is regarded as being indicative for the epithelial origin of these tumours (17, 73). Therefore, the pattern of immunoreactivity of the canine spindle cell carcinomas and the metastatic tissue of one of these tumours indicates a genuine epithelial origin of these tumours.

All canine malignant mammary tumours showed vimentin positive cells, varying from a small number, up to the vast majority of cells, corresponding to previously reported findings (27). This phenomenon has also been observed in about 20% of human malignant breast tumours (13-15, 22, 43, 45-47). Vimentin expression may be associated with rapid proliferation (15, 46) and with differentiation associated with loss of cell-to-cell contact (46). In general, vimentin has been suggested to be a common protein of incohesive cells (57). In humans, vimentin expression has been associated with tumour types and malignancy grades with a poor prognosis (13-15, 46).

The metastatic tumour tissue showed, in general, a labelling pattern identical to that of the primary tumour. This is in agreement with immunohistochemical and biochemical findings in humans when metastases and their primary tumours are compared (4, 36, 37, 62). The canine metastatic tissue of the adenocarcinoma of the simple type did not react with the basal cell keratin MoAbs, however, although the primary neoplastic tissue was largely positive. Differences in patterns of keratin expression between metastatic and primary tumour tissue have been incidentally observed in humans as well (39). These differences may be due to a presumed clonal origin of metastases (58).

In the connective tissue of all malignant tumours, elongated cells were found labelled with the α-smooth muscle actin MoAb. A small number of such cells was also labelled with the desmin MoAb. These cells have to be considered as myofibroblasts, i.e., stromal cells showing immunohistochemically and electron microscopically features of smooth muscle cells (9, 26, 51, 63, 67). Myofibroblasts are involved in a desmoplasmic stromal response to neoplasms (50). Originally, this reaction was regarded as a response to epithelial tumour invasion (53, 63), and in breast tumours in particular as being a characteristic feature of tubular carcinoma (26). However, these cells are observed in many neoplastic and non-neoplastic lesions as a reaction of the supporting stromal component (73). They can also be found in human benign breast tumours (24, 50, 51, 67) and in benign mammary tumours in the dog (69). In conclusion, we can state that the various canine malignant mammary tumours showed differences in their immunohistochemical profile with the keratin MoAbs used. Whether these differences are tumour-type characteristic need to be confirmed in a larger group of tumours. The absence of labelling for α-smooth muscle actin, combined with the presence of inner cells labelled for basal/myoepithelial keratins and/or vimentin-reactive neoplastic epithelium, may be indicative of malignancy. As each of these immunohistochemical features may also occur in benign canine mammary tumours (69), a critical and careful evaluation of these findings is warranted. Immunohistochemistry alone, therefore, as in humans (12, 65), cannot decisively differentiate between benign and malignant mammary gland tumours in the dog.

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REFERENCES

IMMUNOHISTOCHEMISTRY WITH KERATIN, VIMENTIN, DESMIN, AND α-SMOOTH MUSCLE ACTIN MONOCLONAL ANTIBODIES IN CANINE MAMMARY GLAND: NORMAL MAMMARY TISSUE

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SUMMARY
Normal canine mammary gland tissue was studied immunohistochemically with monoclonal antibodies (MoAbs) directed against various human keratin types, vimentin, desmin, and α-smooth muscle actin. Both ductal and alveolar luminal cells were immunoreactive with MoAbs recognizing respectively human keratins no. 7, 8, 18 and 19. In addition, some ductal luminal cells were labelled with a keratin 4 and a keratin 10 MoAb. Basal/myoepithelial cells were immunoreactive only with MoAbs directed against keratin 14, keratins 14 and 17, and α-smooth muscle actin. The vimentin MoAb merely labelled solitary loose intraluminal cells representing macro-phages or sloughed epithelial cells. These findings correspond largely to observations made in human breast tissue.

INTRODUCTION
The mammary gland of all mammals is a complex organ, continuously changing morphology throughout lifetime, mostly due to body growth and cyclic hormonal stimulation (42). This compound tubuloalveolar gland is composed of fibroconnective stromal tissue surrounding parenchyma and consists of a secretory and an excretory ductal component. The secretory component is formed by alveoli and the initial portion of the intralobular ducts (secretory tubules) (3). Both alveoli and ducts are lined by two cell layers, i.e., the luminal cells and basal/myoepithelial cells (42,49). The latter are in direct contact with the basement membrane (42,49). Luminal and basal/myoepithelial cells can be identified his-