Metallothionein Expression in Canine Cutaneous Apocrine Gland Tumors

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Abstract. Background: the aim of the present study was to evaluate, by immunohistochemical staining, metallothionein expression in normal, benign and malignant canine apocrine gland tissues and to correlate the protein expression with the histological grade of malignancy. Materials and Methods: MT immunostaining was evaluated in 25 formalin-fixed and paraffin-embedded canine apocrine glands (2 normal and 23 neoplastic). Moreover, we evaluated quantitative expression of MT in normal and neoplastic cells by western blotting. Results: a marked increase in MT expression was observed in neoplastic, compared to normal samples as well as in malignant compared to benign tumors. Western blotting analysis revealed one major protein band of approximately 14 kDa in normal, as well as in the neoplastic, tissues. Conclusion: We conclude that MT expression appears to be a potential biomarker for the diagnosis of canine apocrine gland tumors and may also assist in the better understanding of the evolution of this neoplasia.

Metallothioneins (MTs) are a family of intracellular, low-molecular-weight proteins (6-7 kDa), characterized by a very high proportion of cysteine residues, resulting in their close affinity for essential (zinc and copper) and toxic metals (cadmium and mercury) (1). The mammalian MT family consists of four similar but distinct isoforms, designated MT1 to MT4 (2, 3). MT1 and MT2 are the major isoforms and are expressed in most tissues, including liver, kidney and brain (2, 3), whereas MT3 (also called growth inhibitory factor) and MT4 are minor isoforms which are normally found in specialized cells [central nervous system (4) and keratinizing epithelia of skin, tongue and upper alimentary tract (5), respectively]. MT synthesis can be induced by many endogenous and exogenous stimuli, including physical and chemical stress, hormones and heavy metals. Generally considered as housekeeping proteins, MTs are involved in many physiological and pathological processes, most of which are related to their metal-binding property (6). Thus, MTs are known to have a protective role on cells against apoptosis induced by alkylating agents, oxygen radicals and ionizing radiation (7); have metalloregulatory function in cellular repair processes, growth and differentiation (1, 8); and are involved in fundamental cellular processes, such as absorption, transport, intracellular storage and detoxification of metals (9, 10). In recent years, further interest in the study of these proteins has been raised after demonstrating their association with spontaneous mutagenesis, response to anticancer drugs and tumor progression, and after proving their potential application in the prognosis of human neoplasia (11-14). In this respect, the correlation between high levels of MT expression and poor prognosis for numerous human neoplasias has been shown by many authors (11, 15-20). However, only few studies about MT have been until now reported in veterinary oncology (21-24).

Tumors of cutaneous apocrine gland are rare in the dog, accounting for approximately up to 2% of all canine skin tumors (25, 26). These tumors have been reported everywhere in the body, although most develop on the head, neck and limbs, and are usually found in dogs more than 8 years old, and in golden retrievers (26-28). Approximately 70% of canine apocrine tumors are benign, but malignant ones tend to recur locally and metastasize to regional lymph nodes and lung (26, 29). The World Health Organization (WHO)
classification of apocrine gland tumors of domestic animals (26) subdivides these tumors into adenoma/carcinoma, complex or mixed adenoma/carcinoma, and ducal adenoma/carcinoma. The aims of the present study were: i) to investigate MT expression by the immunohistochemical method in a subset of spontaneous cutaneous canine apocrine gland tumors, classified by WHO criteria; ii) to correlate MT expression with the histological grade of tumors and iii) to evaluate MT expression quantitatively, in normal and neoplastic cells by western blotting.

Materials and Methods

Sample. Twenty-five samples consisting of 2 normal canine cutaneous apocrine glands and 23 cutaneous apocrine gland tumors (11 benign and 12 malignant) were examined. Each sample, removed at surgery, was divided into two. One piece was immediately resected and a part tissue cooled in isopentane in liquid nitrogen for western blotting analysis. The other piece was fixed in 10% formalin and paraffin wax-embedded for routine hematoxylin and eosin (HE) staining and immunohistochemistry. The HE-stained slides were classified by WHO criteria (25) (Table I) and malignant tumors were graded by two observers as well- (4), moderately- (3), or poorly-differentiated (5) using the Misdorp parameters (30).

Western blot analysis. Frozen tissues were homogenized in a homogenization buffer [50 mM Tris HCl, pH 7.4; 150 mM NaCl; 1% Triton X-100; 0.5% Na-deoxycholate; 100 mM NaF; 2 mM Na3VO4; 10 mg/ml leupeptin; 0.1 U/ml aprotinin; 1 mM PMSF] using an Ultra-Turrax homogeniser and centrifuged at 16,000 × g for 20 min at 4˚C. Aliquots of the supernatant were subjected to electrophoresis on 18% sodium dodecyl sulphate (SDS)-polyacrylamide gel (Bio-Rad, Hercules, CA, USA). After electrophoresis, the gel was transferred to a nitrocellulose membrane by using a semidry apparatus (Bio-Rad) according to the manufacturer’s instructions. The membrane was washed three times with TBST, was then incubated for 1 h with anti-mouse IgG peroxidase conjugate (cod. NA931V; GE Healthcare, UK) diluted 1:2000 in TBST-1% BSA and was washed three times with TBST. Proteins were visualized by an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK). Marker proteins (coloured protein molecular weight markers; Prosieve Quadcolor, Lonza, Rockland, ME, USA) were used to estimate the molecular weight of each band.

Immunohistochemistry. Sections were de-waxed in xylene, dehydrated in graded alcohol and were washed in 0.01 M phosphate-buffered saline (PBS), pH 7.2-7.4. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in absolute methanol for 30 min. The streptavidin-biotin-peroxidase method (LSAB Kit; Dako, Glostrup, Denmark) was used for staining. The primary antibody was a monoclonal mouse anti-human MT (UC1-MT; Abcam), which reacts to epitope of MT1 and MT2, at a concentration of approximately 14 kDa from homogenates. The membrane was washed three times with TBST, was then incubated for 1 h with anti-mouse IgG peroxidase conjugate (cod. NA931V; GE Healthcare, UK) diluted 1:2000 in TBST-1% BSA and was washed three times with TBST. Proteins were visualized by an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK). Marker proteins (coloured protein molecular weight markers; Prosieve Quadcolor, Lonza, Rockland, ME, USA) were used to estimate the molecular weight of each band.

Scoring of immunoreactivity. MT immunoreactivity was scored as previously described by Bier et al. (31): –, negative cells; +, individual MT-positive cells; ++, foci from moderate to strong positivity; ++++, > 50% of cells moderately to strongly positive. The immunoreactivity was scored by two observers (PM and MM).

Results

Western blot analysis. The results of the western blot analysis are shown in Figure 1. Tissue extracts of the normal and neoplastic tissues reacted with the antibody to MT. The antiserum recognised one major protein band of approximately 14 kDa from homogenates.
Immunohistochemistry. Results of immunohistochemical findings are shown in Table II. MT immunopositivity was detected in normal and in almost all (23/25) neoplastic samples. The MT expression was generally observed in the cytoplasm, occasionally in the nucleus and was highly heterogeneous.

Normal apocrine gland tissue. Epithelial cells lining ducts exhibited diffuse MT cytoplasmic positivity from moderate to strong (Figure 2A).

Benign apocrine gland tumours. Among benign tumors, 3/11 (27%) samples exhibited individual MT-positive cells (+), while 8/11 (72%) showed foci with moderate to strong immunolabelling (++). In the latter samples the positivity was observed in both cytoplasm and nucleus with equal density (Figure 2B).

Table II. Classification of apocrine gland tumors based on positivity to MT antibody (31).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Number</th>
<th>Positivity of immunostaining to MT antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>Benign tumor</td>
<td>11</td>
<td>3/11 (C)</td>
</tr>
<tr>
<td>Malignant tumor</td>
<td>12</td>
<td>4/12 (H)</td>
</tr>
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</table>

C: Cytoplasmic immunolabeling; H: heterogeneous immunolabeling.

Malignant apocrine gland tumours. Among malignant tumors, 4/12 (33%) samples, which were classified as well-differentiated, showed foci with moderate to strong immunolabelling. A mosaic pattern was observed for MT
immunolabeling in some tumors, with neoplastic cells showing high heterogeneity of staining, from negative to strongly positive, and positivity in both cytoplasm and nucleus (Figure 2C). The intensity of expression increased progressively as the carcinomas proceeded from well-differentiated (4/12; 33%) to moderately-(3/12; 25%) and poorly-differentiated (5/12; 42%) phenotypes, in which more than 50% of cells were moderately to strongly immunolabeled (Figure 2D).

Discussion

The results of the present study have demonstrated an increased expression of MT in canine apocrine gland tumors and suggest an association between the degree of MT expression and the histological grade of the tumors. These findings are therefore similar to those observed in a wide variety of human tumors [liver (16), breast (17, 31-33), salivary glands (18), lung (19), ovary (20), thyroid (34), melanoma (35), stomach (32)] and in canine and feline cutaneous melanomas, as well as in feline mammary tumors, but not canine mammary tumors (21, 22, 24), in which MT overexpression was associated with tumors of low-grade malignancy. To explain these interspecies differences, it was postulated that there might be an association with the state of metal occupancy of the expressed MT, which has been advocated as a cause of the tumor progression of human and canine tumors (21, 22). MT occurs either as apo (metal-free) or holo (metal-bound) forms in mouse tumor cell lines (33). While holo-MT has an anticarcinogenesis effect, by protecting cells against DNA-damaging agents, apo-MT was closely correlated with growth and progression of tumor, whereby the interaction of its free sulphydryl groups with metal ions, such as Zn-associated tumor suppressor protein p53, leading to its inactivation and uncontrolled proliferation of tumor cells (21, 36-38). It is possible that during neoplastic transformation, there is an increase in apo-MT concentration, while holo-MT concentration remains unchanged. The results of the present study have shown a marked increase in MT expression proceeding from benign to malignant tumors, as well as from well-differentiated to moderately and poorly differentiated phenotypes. This observation seems to suggest that in canine apocrine gland tumors, the expressed MT in malignant tumor cells would appear to be the metal-free MT (apo-MT).

In our study, in addition to a marked increase in MT expression, there was a marked difference in the cellular localization. Although MT was localized mainly in the cytoplasm, it was also possible to observe staining in nuclei of normal and neoplastic apocrine gland tissues. A mosaic pattern was observed for MT immunolabeling in some tumors, with neoplastic cells showing high heterogeneity of staining, from negative to strongly positive and with both cytoplasmic and nuclear positivity. The significance of the presence of MT in the nucleus of tumor cells is not yet understood but may be related to its significant role in the proliferative phase of the cell cycle. In fact, localized in the nucleus of tumor cells, this protein can effectively donate zinc to several enzymes involved in the synthesis of nucleic acid and protein and chelate zinc from transcriptional factors (16, 39). The increase of nuclear expression of MT in malignant tumors could be explained by the increase of tumor growth by elevated proliferative activity in malignant tumors compared to their benign counterparts (16, 39). Moreover, the observed mosaic pattern could be attributed to the phenotypic differences of neoplastic cells, acquired throughout tumor progression (40).

To confirm the specificity of the MT antibody, western blot analysis was performed, for the first time to our knowledge, in veterinary medicine and the antibody recognized one major protein band of approximately 14 kDa in normal, as well as in neoplastic tissues.

These results confirm that MT plays an important role in carcinogenesis but are not sufficient to explain its role in tumor progression. In humans, several authors reported that tumor cells with high expression of MT were more aggressive and more resistant to the toxic effects of anticancer agent, such as cisplatin, as well as to radiation exposure (41, 42). Moreover, MT expression has been associated with a low number of apoptotic cells (43) and a poor prognosis of the cancer (44). Many authors supposed that an increase of MT produced by tumor cells could have the capacity to protect the tumor against oxidative stress and apoptosis, leading to an increase in the malignancy of the tumor (35). Additional experimental data on MT and its isoforms in canine neoplastic tissues are needed to elucidate the biological role of MT in neoplastic progression, along with other tumor markers and especially to help in choosing the type of chemotherapy to use as, for example, apo-MT, with its free sulphydryl groups, can react with electrophilic species associated with various drugs, including cisplatin, which are in clinical use for the treatment of many canine neoplastic diseases, leading to chemoresistance by tumor cells (41, 42).

In conclusion, MT expression appears to be a potential biomarker for the diagnosis of canine apocrine gland tumors and may also assist in better understanding of the evolution of these neoplasms.

References


