EXTENT OF METALLOTHIONEIN EXPRESSION IN CORRELATION WITH EXPRESSION OF KI-67 ANTIGEN IN SOFT TISSUE FIBROSARCOMAS IN DOGS

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Abstract

This study is aimed at an immunocytochemical demonstration of metallothionein and Ki-67 expression, and to define the correlation between the markers in spontaneous fibrosarcomas in dogs. Material for the study was sampled during operative procedures from 27 dogs of various breeds, aging 5 to 16 years. The neoplastic tumours were histopathologically verified and immunohistochemical reactions were conducted to detect MT and Ki-67. Microphotographs of the preparations were subjected to computer-assisted image analysis. In light of the numerous established studies for a high prognostic value of Ki-67, for this reason we have identified in our studies a weak correlation between the expressions of MT and Ki-67 (r = 0.15), which allows us to conclude that the presence of metallothionins in the cells of soft tissue fibrosarcomas in dogs does not manifest an unequivocal relationship with augmented proliferative potential of tumour cells, as it was demonstrated in similar tumours in humans.

Key words: dog, metallothionein, Ki-67 protein, fibrosarcoma.

Metallothioneins (MT) represent a group of low molecular weight proteins of around 7 kDa in size. They consist of polypeptide chains of 61-68 amino acids, including around 30% cystein residues. The residues represent a significant component of MT, due to their thiol (-SH groups), which permit binding of metal ions (9). The proteins contain 4 –12 atoms of heavy metals, linked through the sulphhydryl groups, and demonstrate highly conservative amino acid composition. MT isolated from various animal organs exhibit only slight variability in amino acid composition. The metallothionein structure involves two domains (α and β) linked by a lysine dimer. The α domain binds four Cd ions while the β domain binds three metal ions: two Zn ions and one Cd ion (14). Four principal types of metallothioneins were distinguished: MT-I, MT-II, MT-III, and MT-IV, which in humans are coded by a group of 12-15 genes, located in the chromosome 16.

The earliest recognised function of MT involves their effect on cellular homeostasis of metals. MT is thought to form the principal element of cell protection against strongly toxic Cd, Pb, Hg, and Cu ions. The proteins are capable of binding them into the safe for cells inactive complexes (32). Apart from a detoxication function, MT have been shown to bind Zn and in this manner to control the activity of Zn-dependent enzymes, involved in DNA replication, transcription, translation, and several other metabolic processes in the cell. MT has also been proven to exert a significant controlling influence for the growth and proliferation of both normal and neoplastic cells (3). In cases of neoplastically transformed cells, augmented expression of MT may be linked to enhanced resistance of the cells to cytostatic drugs, due to the capacity of the proteins to bind certain chemotherapeutic agents and to transform them into inactive forms. The phenomenon used to be called, multi drug resistance (MDR) (7, 21, 22). The presence of MT was demonstrated in cells of various human tumours of either epithelial or mesenchymal origin (4, 12, 15, 30). In tumours of epithelial origin, a strong relationship was demonstrated between MT expression and grade of histological malignancy (G), regarded to represent one of the most significant prognostic indicators in oncological therapy (19, 25, 28). Similarly, a positive correlation was noted between expression of MT and that of antigen Ki-67, accordingly confirming the involvement of MT in proliferative processes of cells for epithelial tumours (11, 20). Until now, the expression of MT has been examined in few studies only, in various types of human...
sarcomas (12, 13, 15, 31). Since the proteins are detected mainly in cells in S phase of the cell cycle, MT are thought to provide a good index of proliferative activity of cells and, thus, to represent an index of neoplastic progression (6, 8, 20, 29).

In the study of tumour aggressiveness, including tumours of soft tissues, an evaluation of cell proliferative activity by estimation of expression of Ki-67 antigen involves one of the principal elements (18). The protein belongs to the group of non-histone compounds present in the cell nucleus (16). Its expression is detected already in G1 phase of cell cycle; and it increases in intensity in S and G2 phases to reach peak intensity in M phase and rapidly vanishes in G0. Thus, the protein is detectable only in proliferating cells (5,17). The ratio of cells with expression of Ki-67 to cells without such expression is called the proliferative index; and it reflects the extent of cell mitotic activity (2).

The present study aimed at an attempt to demonstrate MT expression in cells of soft tissue fibrosarcomas in dogs and to correlate it with expression of Ki-67 antigen.

Material and Methods

Material for the studies was sampled during surgical procedures from 27 dogs of various races, aging 5 to 16 years. The neoplastic tumours were verified by histopathology to represent fibrosarcomas. The tumour samples were fixed in 10% buffered formalin, dehydrated and embedded in paraffin blocks. In order to estimate MT expression (isoforms MT-I and MT-II) and expression of Ki-67 antigen in paraffin sections, mouse monoclonal MT antibodies (clone E9), and mouse monoclonal Ki-67 antibodies (clone MIB-1) were used. The reaction product was visualised using LSAB2+ kit of reagents and diaminobenzidine (DAB). Sections, in which an expression of the nuclear Ki-67 antigen was studied, were earlier subjected to a procedure of boiling in Antigen Retrieval Solution, in a microwave oven, in order to expose the antigenic determinants. The tests were always accompanied by negative controls using Primary Negative Control. All the antibodies and reagents originated from DakoCytomation.

The preparations were used to obtain microphotographs. The latter were subjected to computerised image analysis in a stand consisting of a computer coupled to Axioptot microscope (Carl Zeiss). The set allowed us to record the images, and to analyse them using numerical technique. In the analysis, the MultiScaneBase V 14.02 software was used, working in the Windows environment. In order to evaluate MT expression, a modified semiquantitative IRS scale (26) was employed (Table 1).

The method takes into account both percentage of positive cells and intensity of the reaction product, and the final score represents product of the point allotted in the evaluation of the two variables, ranging from 0 to 12 points (no reaction: 0 pts (-), weak reaction: 1-2 pts (+), moderate reaction: 3-4 pts (++), strong reaction: 6-12 pts (+++)). Also the expression of Ki-67 antigen was appraised semi-quantitatively evaluating the proportion of positive cells: 0-5% - no reaction (-), 6-25% - weak reaction (+), 26-50% - moderate reaction (++), above 50% - intense reaction (+++). The obtained results were subjected to statistical analysis employing Statistical PL software (StatSoft, Poland) and using Spearman’s correlation analysis.

Results and Discussion

Soft tissue sarcomas account for around 1% of all malignant tumours in humans. However, their etiopathogenesis remains to be fully recognised, and the relatively ineffective treatment causes that the group of tumours poses a significant problem for oncology (33). In veterinary medicine, tumours of mesenchymal origin included to the group of soft tissue sarcomas, account for slightly more than 14% of all diagnosed malignant lesions in dogs and; thus, almost every sixth malignant tumour belongs to the group (23). In dogs, fibrosarcomas form a dominating subgroup among soft tissue sarcomas. All the remaining types of tumours, like mucosarcomas, adiposarcomas, sarcomatous, and smooth cell myomas collectively, do not exceed 3% of all mesenchymal tumours of skin and subcutaneous tissue (1, 27).

Table 1

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<td>0 pts – no cells with positive reaction</td>
<td>0 pts – no colour reaction</td>
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<td>1 pts – to 10% cells with positive reaction</td>
<td>1 pts – low intensity of colour reaction</td>
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<tr>
<td>2 pts – 11-50% cells with positive reaction</td>
<td>2 pts – moderate intensity of colour reaction</td>
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<td>3 pts – 51-80% cells with positive reaction</td>
<td>3 pts – intense colour reaction</td>
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<td>4 pts – &gt; 80% cells with positive reaction</td>
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The relatively late detection of tumours in the group creates problems both for veterinarians and physicians: the patient reports to the doctor frequently with highly advanced tumour. In such a situation accurate evaluation of the grade of histologic malignancy (G) forms a very important element of histological diagnosis, justifying attempts to define prognosis of the disease course. However, appraisal of the G grade on the basis of subjective evaluation of cell pleomorphism, mitotic activity and/or extent of necrosis in the tumour, yield frequently insufficient grounds for reliable prognosis. In such a situation attempts are conducted to use novel markers of cell proliferation (e.g. MT), which might provide more precise determination of prognosis. In our studies we have attempted to demonstrate presence of MT in cells of fibrosarcomas and to correlate their presence with the accepted in oncology marker of cell proliferation such as Ki-67. One of the most frequently applied techniques for the detection of MT in cells, both normal and neoplastic ones, involves immunocytochemistry. This technique allows to evaluate, under a light microscope, the site of the protein expression, its cellular distribution (nucleus, cytoplasm), and intensity of the colour reaction (14). Expression of MT was noted in cell nucleus and/or in cytoplasm and it varied depending upon the type of studied normal or neoplastic tissue (4).

In most of studies on humans a clear-cut positive correlation was disclosed between expression of MT and grade of tumour’s malignancy (G) and expression of Ki-67 antigen (14). Moreover, increase in MT expression was linked to less advantageous prognosis, expressed by shorter survival after the surgical procedure (12).

In our studies, performed on soft tissue fibrosarcomas in dogs, also the positive correlation was demonstrated between MT expression and expression of Ki-67 (Fig. 1), but its level was very low (r = 0.15), in contrast to clearly higher values of correlations obtained in analogous studies of soft tissue sarcomas in humans (12).

Only in 22% the studied by us sarcomas shown expression of both MT (Figs 2-4) and Ki-67 (Figs 5-7) valued at least (+).

In the entire material, 74% of tumours demonstrated expression of MT, and 26% of them showed no such expression. In similar studies on skin melanomas in dogs, Dincer et al. (10) obtained completely different results: only 25% of melanomas exhibited expression of MT and the remaining 75% were MT (-). In our investigations, quantitative analysis in 41% of tumours demonstrated MT expression at the + level, 29% at the ++ level, and 4% at the +++ level. In the cases of Ki-67, 22% of tumours shown expression of the antigen at the + level, 4% at the ++ level, and 4% at the +++ level (Fig. 8).

Only in one of the 27 examined tumours the two examined markers were shown to be co-expressed at the +++ level. It should be added that in almost 30% of cases the mean expression of MT (3-4 pts in IRS scale) correlated with absence (-) of Ki-67 antigen, recognised as a reliable prognostic index in neoplastic diseases.

In view of the recognised prognostic value of Ki-67 antigen index, the detected in our studies weak correlation between expressions of MT and Ki-67 allows us to conclude that the presence of metallothionein in the cells of soft tissue sarcomas in dogs, and shows an unequivocal relationship with the augmented proliferative potential of neoplastic cells, in contrast to results obtained in similar tumours in humans. It should be added that low level correlations between the above-mentioned markers documented also in studies on mammary adenocarcinomas in bitches, and also in such cases the results proved to be completely different from analogous results obtained in mammary tumours in women (24). Most probably, this might be related to distinct mechanisms of induction and control of genes responsible for metallothionein synthesis in humans and in animals, and to variable biological function of various isoforms of the protein in individual mammalian species.

![Fig. 1. Spearman’s correlation between intensities of expression of MT and Ki-67. Correlation coefficient r = 0.15](image-url)
Fig. 2. Weak expression (+) of metallothionein in fibrosarcoma of a dog. 400x

Fig. 3. Moderate expression (+++) of metallothionein in fibrosarcoma of a dog. 400x

Fig. 4. High expression (+++) of metallothionein in fibrosarcoma of a dog. 400x

Fig. 5. Low expression (+) of Ki-67 protein in fibrosarcoma of a dog. 400x

Fig. 6. Moderately high expression (++) of Ki-67 protein in fibrosarcoma of a dog. 400x

Fig. 7. Intense expression (+++) of Ki-67 protein in fibrosarcoma of a dog. 400x
Fig. 8. Intensity of expression of Ki-67 antigen and metallothionein (MT) in fibrosarcoma of a dog.

References

activity (MIB-1 index) is an independent prognostic parameter in patients with high-grade soft tissue sarcomas of subtypes other than malignant fibrous histiocytomas: a retrospective immunohistological study including 216 soft tissue sarcomas. Histopathology 1998, 32, 536-546.


