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Canine hemangiopericytomas: cell proliferation and apoptosis in the perivascular, storiform and epithelioid histological subtypes and their significance for prognosis.

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Abstract

Canine hemangiopericytomas (CHP) are soft tissue neoplasms, originary from pericytes. They are frequently diagnosed in dogs and can be seen more frequently in limbs as circumscribed and firm nodules. The histopathology of CHP reveals the presence of spindle cells around blood vessels, forming whorls. In this study, cases of CHP from the Animal Pathology Service of the Veterinary Hospital of the School of Veterinary Medicine and Animal Science, registered from 1990 to 2003, were studied. All cases were reviewed and classified in the three histological subtypes recently described – perivascular (PVHP), storiform (SHP) and epithelioid (EHP). Immunohistochemical markers (vimentin, S-100 protein, CD34, Factor VIII and glial fibrillary acidic protein - GFAP) were applied to all tumors. Samples were submitted to the immunostaining of PCNA, and the labeling indexes were obtained. The mitotic index was also quantified in each subtype. Apoptosis bodies were morphologically identified in H&E stained sections, subsequently confirmed by fluorescence microscopy and then quantified in the three histological subtypes. Dog owners were contacted by telephone, and asked to inform about the outcome of their dogs. The survey revealed 61 cases of CHP, in which 21 (34.43.%) belonged to the perivascular subtype, 18 cases (29.51.%) belonged to the storiform subtype, and 22 cases (36.06.%) belonged to the epithelioid subtype. PCNA labeling index, mitotic and apoptotic indexes were significantly higher in the epithelioid subtype. Records of the follow-up of each case revealed 59% of recurrence rate in the epithelioid subtype, 55% in the storiform subtype and 17% in the perivascular subtype. According to these results, epithelioid hemangiopericytomas seem to be the most aggressive CHP subtype. Therefore, we conclude that the histopathological analysis with classification in subtypes, as well as the quantification of cell proliferation and apoptosis rates, can help in the establishment of prognosis for CHP.

Key Words: Hemangiopericytomas; dogs; PCNA; prognosis; mitosis; apoptosis.

Introduction

Canine hemangiopericytoma (CHP) is a soft tissue neoplasm from dogs (1, 2, 3). It is reported that the CHP originates from pericytes, cells located around blood vessels (2, 3, 4, 5). The pericytes are mesenchymal cells that have contractile capacity, and control the capillary blood flow (2, 3, 5). It is reported that these cells express muscle actin of alfa type, which can be identified by immunohistochemistry (6). The use of markers such as

factor VIII and CD34 confirms the vascular origin of hemangiopericytomas and helps to exclude other mesenchymal tumors (6, 4). However, another study proposed the CHP diagnosis based on tumors morphology that don't bear whorling growth and are characterized by prominent myxoid to staghorn aspects in association with variable expression to actin CD34 and CMG-3G5 (cell membrane ganglioside), but negative to desmin and all mature muscular markers (1). CHP are usually presented as a solitary, multilobular, firm or soft mass, sized between 1 and 25 cm, attached to the subjacent tissue (5). The skin is normally alopecic, hyperpigmented and ulcerated. The preferential localization is in limbs, most specifically in regions of the elbows and knees. Females are predisposed to the development of this tumor, and the large breeds are the most affected. The treatment of choice is surgical excision, and this is not totally effective due to the high rates of recidive, characteristic of the sarcomas (2, 1).

Histopathological examination of CHP reveals that the neoplasm is composed of spindle cells that are characteristically organized in whorls around blood vessels. Recently, three morphological subtypes of CHP were described: epithelioid (EHP), storiform (SHP) and perivascular (PVHP) by (4). Although the authors informed the general recurrence of CHP, they did not make the correlation with the morphological subtype of CHP. Yet, to the best of our knowledge, there are no reports on the cell proliferation and apoptosis rates in the histological subtypes of CHP.

The aims of this study were to evaluate the effects of histopathologic subtypes, cell proliferation and apoptosis as factors of tumor behavior and prognosis, thereby contributing to the existing knowledge of CHP.

Material and Methods

Samples

A survey from 1990 to 2003 of CHP cases was performed in the Animal Pathology Service of the Department of Pathology, Faculty of Veterinary Medicine and Zootechny of the University of Sao Paulo. All cases were reexamined by two pathologists {SVS & MLZD} and classified according to published criteria (4, 3). Representative portions of each tumour were fixed in 10% buffered formalin and routinely embedded in paraffin wax. The hematoxylin and eosin was used for staining and it was examined in an optical microscope.

Immunohistochemical staining of selected markers of hemangiopericytoma and of proliferating cell nuclear antigen (PCNA).

Additional 5 μm sections of CHP from all cases were collected in silanized slides. They were routinely deparaffinized in xilol and hydrated in alcohols. For the antigenic retrieval, the slides were boiled in a solution of citrate buffer for 12 minutes in a microwave owen. After cooling, the slides were washed three times with PBS. The endogenous peroxidase was blocked by incubation, for 30 minutes, in a solution containing 80% of methyl alcohol and 20% of hydrogen peroxide. After that, the slides were washed three times with PBS. The sections were, then, incubated with different antibodies, according to Table 1, and were kept overnight in a wet chamber at 4ºC. The incubation with a secondary antibody for 1 hour, and the kit LSAB (Dako®) were applied afterwards. Then, a solution containing diaminobenzidine at 0.05.% in PBS and hydrogen peroxide was applied. Negative controls were obtained by omitting the primary antibody (1, 4). Tissue sections of normal canine skin were used as positive control (4). After being counterstained with hematoxylin and eosin, the slides were mounted with a synthetic resin.

Table 1 - Antibodies and dilutions used in the study of hemangiopericytomas.

Antibody	Source	Dilution
Vimentin (polyclonal)	Dako	1:200
GFAP (polyclonal)	Dako	1:200
CD34 (monoclonal)	Dako	1:100
S-100	Dako	1:200
Fator VIII (polyclonal)	Dako	1:200
Cytokeratins (AE1-AE3)	Dako	1:40
Proliferating cell nuclear antigen (PCNA)	Dako	1:1600

Quantification of PCNA-positive nuclei and determination of the mitotic and apoptotic indexes.

PCNA positive nuclei, mitotic and apoptotic cells and were quantified in a Nikon E-800 light microscope, by using objective of 20X. For each CHP subtype, the cells present in the 6 central squares in 8 random histological fields were counted. The percentage of PCNA positive cells was calculated by the following procedure: number of PCNA positive cells x 100 / number of total cells. At least 1000 cells of each slide were counted.

For the evaluation of the apoptotic index, the apoptotic cells were morphologically identified in H&E sections. Then the apoptotic nature of the bodies was confirmed through the fluorescence of the eosin (Fig. 1), with the help of a Nikon E-800 microscope, according to the described technique (7).

Analysis of the tumor recurrence

To the determination of the outcome of each CHP case, owners were contacted by telephone twice a year, in June 2002, November 2002; June 2003, November 2003, and four times in 2004, in the months of February, June and November. Follow up time totalized 3 years. Twelve cases were personally visited due to the telephonic interview being considered doubtful. The owners were

asked about tumor recurrence, additional disease, and/or death.

Statistical analysis

For the statistical analysis, the ANOVA and Tukey – Kramer tests were used. The results were considered significant when $p<0.05$.

Results

Histopathological evaluation of CHP

The archives of the Service of Animal Pathology revealed 61 cases of CHP during the proposed time (1990 – 2003). From these, 21 (34.43%) belonged to the perivascular subtype (Fig.2), 18 cases (29.51.%) belonged to the storiform subtype (Fig. 3), and 22 cases (36.06.%) belonged to the epithelioid subtype (Fig.4).

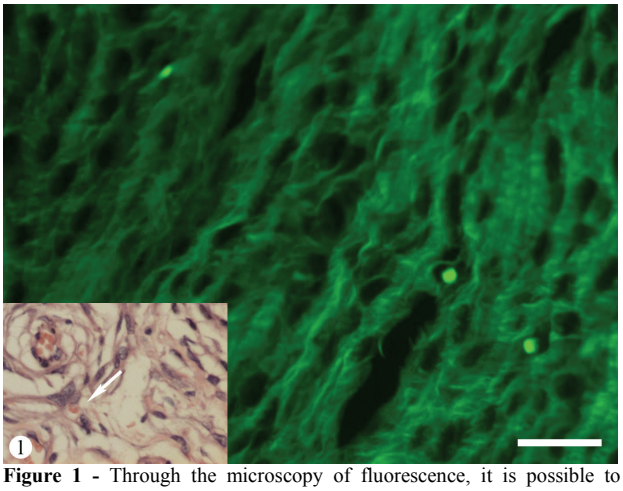

Figure 1 - Through the microscopy of fluorescence, it is possible to visualize the refringency of the apoptotic bodies (arrows) in the H&E stained slides. Bar = 10µm.

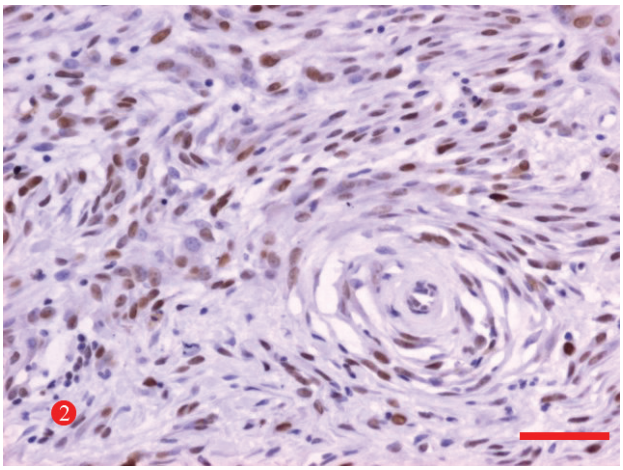

Figure 2 - Perivascular hemangiopericytoma stained with PCNA antibodies. Noticed as histological patterns (tumour cells arranged in a concentric fashion around central lumen). Bar = 10µm.

At microscopic examination, the three morphological variations of the CHP were thoroughly researched, due to the important result that it could have in the clinic prognostic: perivascular (PVHP): fusiform cells arranged in concentric form around a central lumen, which may or not have erythrocyte; - storiform (SHP) : tumor cells set in small multidirectional beams, almost forming the typical "whirlpool" of these tumors, intermingled or not by intercellular collagen; - epithelioid (EHP): the cells present a large vesicular nuclei with eosinophilic cytoplasm, indistinct cellular border and possible lymphoid migration, manifesting or not extensive intercellular collagen areas, that are associated with the perivascular subtype.

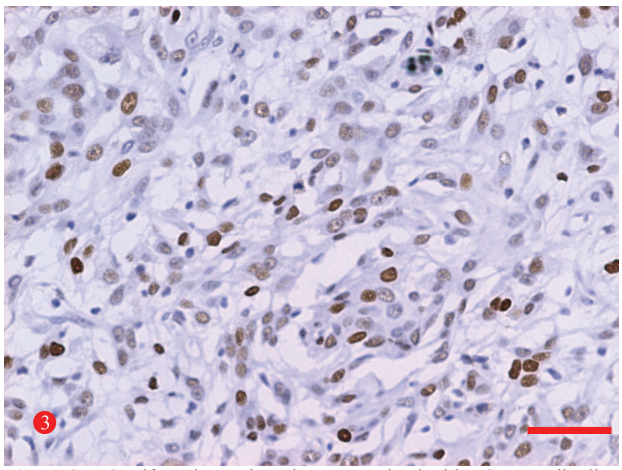

Figure 3 - Storiform hemagiopericytoma stained with PCNA antibodies. Noticed as histological patterns (short fascicles arranged in tumour cells). Bar = 20µm

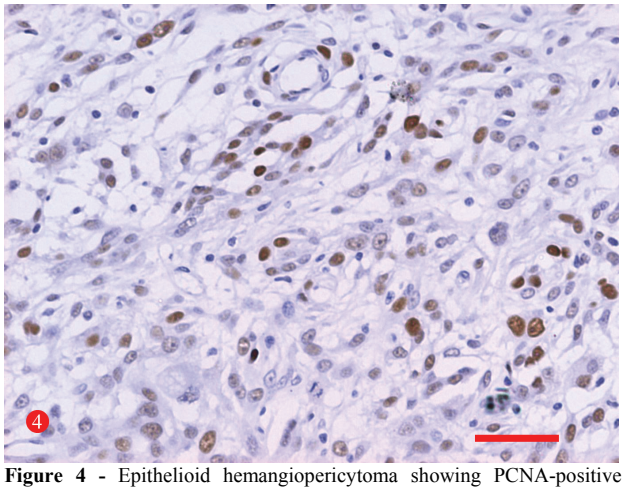

Figure 4 - Epithelioid hemangiopericytoma showing PCNA-positive cells. Noticed as histological patterns (neoplastic cells have indistinct borders and large vesicular nuclei). Bar = 20µm

Our study reported that females are more subject than males, on all CHPs subtypes; the more predisposed age is from 5 ~ 10 years/old; the more susceptible breed

are the mixed breed, with mid to large size; the neoplasms are with moderated size (from 5 ~ 10 cm), being the limbs more affected, specially on joints region are also depicted in Table 5 and 6.

Cases were submitted to immunhistochemistry for selected markers to confirm the diagnosis of hemangiopericytoma. Results are presented in Table 2. All PVHP (perivascular) and SHP (storiform) were positive to vimentin and only 90% of EHP (epithelioid) were positive for this mesenchymal intermediate filament marker. 100% of the PVHP and 70% of SHP and EHP were positive to Factor VIII. 60% of PHVP, 50% of SHP and 30% of EHP were positive to S-100 protein. 6.67% of PVHP, 10% of SHP and none of the EHP were positive for CD 34. None of the tumors were positive for glial fibrillary acidic protein (GFAP) or cytokeratins (AE1/AE3).

PCNA labeling index, mitotic and apoptotic indexes.

PCNA labeling index, as well as mitotic and apoptotic indexes are presented in Table 3. The epithelioid subtype showed the significantly highest proliferation (as evaluated by PCNA immunostaining or mitosis countings) and apoptotic indexes, when compared with the other morphological subtypes.

Recurrence rate

Results of the recurrence rate of each case of CHP, according to the histological subtype, are presented in Table 4. Only 17% of the perivascular type of hemangiopericytomas recurred; while comparatively elevated rates of recurrence (55 and 59%) were demonstrated with the storiform and epithelioid subtypes of CHP, respectively".

Six dogs died or were euthanized: three for nontumour-related causes, whereas in three subjects necropsy findings documented that the cause of death was related to metastasis (one PVHP/ spleen; two EHP cases/liver), but not as confirmed by histological examination.

We also noticed that approximately 55% of the CHP cases were associated with other neoplasms, such as: mast cell tumors, lipomas, adenomas, adenocarcinomas, carcinomas, fibrossarcomas, hemangiosarcomas and mainly seminomas, representing about 7% of the cases.

Discussion

CHP are common soft tissue tumors, and, to the best of our knowledge, no studies have focused on the correlation between the three morphological subtypes, cell proliferation and apoptosis rates and their significance to tumor behavior. The results from this study suggest that collectively these factors can predict tumor behavior and their possible recurrence potential.

The results suggest that the epithelioid form is the most aggressive subtype, from the clinic (greater recurrence rate) view and greater PCNA, mitosis and apoptosis rate.

This aggressiveness can also be investigated with more validity through the results of the counting of the criterions of malignity where the mitotic and apoptotic indexes of each subtype have resulted in an epithelioid type with bigger incidence, as the data shows.

In our retrospective study, the most frequent CHP subtype was the epithelioid, followed by perivascular and storiform, with no great relevance, pointing numeric homogeneity on each diagnosis. These results differ from those another study (4) that showed a higher incidence of the perivascular type followed by the storiform and epithelioid types. These results can be explained by the microscopic examination of the three morphological variations.

Table 2 - Rate of positive cases after immunostaining of canine hemangiopericytomas (CHP).

CHP type	Vimentin	Fat VIII	S100	CD34	GFAP	AE1/AE3
Perivascular	100%	100%	60%	6.67.%	0%	0%
Storiform	100%	70%	50%	10%	0%	0%
Epithelioid	90%	70%	30%	0%	0%	0%

Table 3 - PCNA labeling index, mitotic and apoptotic indexes in the histological subtypes of canine hemangiopericytomas (CHP).

Hemangiopericytomas subtype	PCNA labeling index	Mitotic index	Apoptotic index
Perivascular (n=21)	30.04 ± 10.82	6.15 ± 2.38	1.15 ± 1.44
Storiform (n=18)	39.12 ± 11.15	7.54 ± 1.94	1.89 ± 1.99
Epithelioid (n=22)	41.94 ± 9.70*	12.54 ± 8.63**	2.40 ± 1.65***

n = number of samples *p<0.01 when compared with perivascular type, ANOVA followed by Tukey- Kramer ** p<0.05 when compared with storiform type, ANOVA followed by Tukey- Kramer ***p=0.0599 – not quite significantly different when compared with perivascular type, ANOVA followed by Tukey- Kramer.

Table 4 - Clinical prognosis of the cases of canine hemangiopericytomas, according to the histological subtypes.

Hemangiopericytomas subtype	Recurrence	Non Recurrence
Perivascular (n=21)	17%	83%
Storiform (n=18)	55%	45%
Epithelioid (n=22)	59%	41%

Table 5 - Canine hemangiopericytomas sex and anatomic predilection.

Histologic Classification	***# of dogs***	***Sex***		***Principal Anatomic Location***						
		M	**F**	**Head**	**Neck**	**Trunk**	**Limbs**	**Tail**	**Perianal**	**Digit**
Perivascular	21	9	12	0	1	3	15	1	0	1
Storiform	18	5	13	0	1	1	14	0	2	0
Epithelioid	22	14	8	2	1	1	15	1	1	1

Table 6 – Canine hemangiopericytomas breed predilection.

Breed	***Histologic Classification***		
	Perivascular	**Storiform**	**Epithelioid**
Mix	6	10	7
German Shepherd	5	0	1
Poodle	4	1	2
Boxer	2	3	4
Siberian Husky	2	1	4
Beagle	1	0	0
Pinsher	1	0	0
Pitbull	0	0	1
Rottweiler	0	1	0
Belgian Shepherd	0	0	2
Collie	0	1	0
Whippet	0	0	1
Akita	0	1	0
Total	**21**	**18**	**22**

Our study points a relatively important frequency case rate on small animals, counting sixty one morphologically classified cases in thirteen years, which yields near five cases by year. However, another study indicated that CHPs are less frequent than previously believed and that canine PWT (perivascular wall tumors) represents a mixed group of distinct biologic entities comprising hemangiopericytomas, angioleiomyomas, myopericytomas, and most lileky angiomyofibroblastomas and angiofibromas. (1)

We also found that after evaluating the clinical records, 10% of the CHP cases were subjected to orthopedic implants. Other authors also reported orthopedic implants on CHP cases, however, they didn't quantified that findings (3, 4, 6).

The quantification of cell proliferation can help in the determination of the aggressiveness of the neoplasm. In this study, cell proliferation in CHP subtypes was quantified both by counting the PCNA positive cells and mitotic index. It was verified that the higher PCNA index was obtained in the epithelioid, followed by storiform and perivascular subtypes. Similar results were obtained for the mitotic index. The quantification of apoptotic bodies revealed a higher index within the epithelioid subtype, followed by storiform and perivascular subtypes. These results suggest a similarity between cellular proliferation by PCNA positivity and mitosis; while the apoptotic indexes were similar in the CHP subtypes evaluated".

The evaluation of cell proliferation by counting mitotic index in hemangiopericytomas and fibrosarcomas was described (2). Authors found that dogs with a tumour of mitotic index 9 or more had a median survival time of 49 weeks, compared with 118 weeks for those with a tumour of mitotic index less than 9, regardless of tumour morphology. Tumour recurrence rate for hemangiopericytomas in their cases was of 25%.

The malignancy of neoplasms is the result of the combination of well known factors like proliferation activity, diminished apoptosis, infiltrative and invasive growth. Apoptosis rate can represent an index of the tumoral regression An interesting aspect that must be pointed out is the evaluation of the proliferative activity through the monoclonal anti-PCNA, which shows clear correlation to the conventional (morphological) data. The PCNA information of use as a score for the proliferative cellular activity in the sarcomas are scarce, and an

extension of its study has to be done. Therefore, we conclude that the histopathological analysis with classification in subtypes, as well as the quantification of cell proliferation and apoptosis rates, can help in the establishment of prognosis for CHP.

Our study aimed to increase awareness and help with the morphological diagnostic on the CHP's three subtypes. The use of a panel of immunocytochemical markers can help in the exclusion of other soft tissue sarcomas. Through the evaluation of the criterions of malignity, including the PCNA, the epithelioid subtypes suggested to be the most aggressive, therefore contributing with the instituted therapeutics, which consequence will be a better prognosis.

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