

Regression of Canine Cutaneous Histiocytoma: Reduced Proliferation or Increased Apoptosis?

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Abstract. *Background/Aim: Canine cutaneous histiocytoma (CCH) is a tumour that undergoes spontaneous regression. The aim of this study was to establish a possible relationship between regression of CCH and tumoural cell proliferation and apoptosis. Materials and Methods: Immunostaining with Ki-67 antigen and the terminal deoxytransferase (TdT) deoxyuridine-5'-triphosphated (dUTP) nick-end labelling (TUNEL) method were performed on 93 specimens of CCH, grouped into four histological groups. Results: The proliferative index evaluated with Ki-67 antigen expression was on average 23.56±7.91%. The apoptotic index determined by the TUNEL method was on average 39.37±5.87%. Neither the proliferative nor apoptotic index differed between histological groups. Moreover, the proliferative and apoptotic indices did not correlate significantly. However, apoptotic activity was higher than proliferative activity in almost all tumours. Conclusion: A reduction of proliferation or an increase of apoptosis does not appear to justify regression of CCH. However, our results suggest that an imbalance between cell proliferation and apoptotic cell death plays a significant role in spontaneous regression of CCH.*

Carcinogenesis comprises a number of changes in the cellular phenotype, based on acquired genetic modifications, including the ability of cancer cells to fail to respond to usual controls such as by proliferation and evasion of cell death (1). The deregulation of cell proliferation and apoptosis is one of the fundamental features in tumorigenesis and is considered one of the hallmarks of cancer (2). Canine cutaneous histiocytoma (CCH) is a benign epidermal neoplasm of Langerhans' cell

origin, which usually displays spontaneous regression (3, 4). However, the cause of spontaneous regression has not been clearly explained. Spontaneous regression of benign and malignant tumours is recognized in different tumours in humans (5, 6) and animals (7, 8) and is commonly attributed to a decrease of tumoural cell proliferation (9, 10), an induction of apoptosis, or to an activation of the immune system (11, 12). Regarding CCH, the regression has been associated with a shift of major histocompatibility complex class-II (MHC-II) molecules to the cell periphery (13) and recruitment of antitumor effector cells (14). In spite of these studies, the understanding of the underlying molecular mechanism(s) of spontaneous regression of CCH is still limited. The aim of this work was to study the proliferation and apoptosis in CCH cells and investigate a possible role of their imbalance during tumour regression.

Materials and Methods

Tissue samples. Ninety-three CCHs and five samples of normal canine skin were obtained from the archive of the Histopathology Laboratory of the University of Trás-os-Montes and Alto Douro (UTAD). For the microscopical study, sections of 4 µm were stained with haematoxylin and eosin (HE). Each sample was re-examined by two independent pathologists (IP and AA) in order to confirm the diagnosis according to the WHO criteria (15) and samples were then allocated to one of four groups representing diverse stages of tumour regression. Based on the degree of lymphocytic infiltration (24), 15 CCHs were classified as groups I, 15 as group II, 42 as group III and 21 as group IV, where in group I lymphocytic infiltrate was minimal and peripheral; in group II, lymphocytic infiltrate was moderate, nodular and peripheral; in group III, a marked nodular infiltrate was seen both at the periphery and in the center of the tumour and in group IV, the lymphocytic infiltrate was diffuse and outnumbered the histiocytic tumour cells.

Proliferation study. Proliferation was assessed by the evaluation of Ki-67 immunolabelling in tumoural cells. Ki-67 immunoreaction was carried out using the streptavidin-biotin-peroxidase complex method, with a commercial detection system (Ultra Vision Detection System; Lab Vision Corporation, Fremont, CA, USA), following the manufacturer's instructions. Antigen retrieval was carried out in citrate

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buffer (pH 6.0), in a microwave at 750 W, three times for 5 min each. The sections were incubated overnight at 4°C with primary antibody specific for Ki-67 (clone MIB1; Dako®, Carpinteria, CA, USA) diluted 1 in 100 in phosphate buffered saline (PBS, pH 7.4, 0.01 M). Labelling was visualized by incubation with 3,3'-diaminobenzidine tetrahydrochloride (DAB) at 0.05% with 0.01% H₂O₂ as the final substrate for 5 min. The sections were counterstained with haematoxylin, dehydrated, cleared and mounted. Positive controls consisted of sections from canine skin, and for negative controls, the primary antibody was replaced with PBS.

Apoptosis study. Apoptotic index was determined by terminal deoxytransferase (TdT) deoxyuridine-5'-triphosphated (dUTP) nick-end labelling (TUNEL) assay. Fragmented DNA was visualized with the *In Situ* Cell Death Detection Kit, POD (Roche Diagnostics®, Penzberg, Germany) according to the supplier's instructions. Negative controls included the substitution of the TUNEL reaction mixture for label solution without terminal transferase. Positive controls included canine small intestine mucosa, canine thymus and canine skin.

Proliferative and apoptotic indices. A positive reaction was indicated by the presence of distinct brown nuclear labelling in neoplastic cells. For the TUNEL assay, positive cells with and without morphological features of apoptosis were counted. The proliferative index (PI) and the apoptotic index (AI) were calculated as the number of positive cells to the total number of neoplastic cells, counting 1000 cells, in 8 to 10 randomly-selected homogeneous high-power fields, and expressed as the percentage of positive cells.

Statistical analysis. The statistical software SPSS version 12.0 (SPSS Inc., Chicago, Illinois, USA) was used for statistical analysis. ANOVA test and Spearman's rank correlation coefficient were used. A level of $p < 0.05$ was considered statistically significant for all tests.

Results

Proliferative index. Ki-67-positive nuclei were observed both in tumoural and non-tumoural cells, such as the keratinocytes of the basal layer of epidermis. The PI of CCH was 23.56%±7.91%, with values ranging from 6% to 41%. The values obtained in the different histological groups were similar and the PI did not reveal significant differences between the four groups ($p=0.69$), with a mean of 23.4%, 22.3%, 24.6% and 22.5% in groups I, II, III and IV, respectively.

Apoptotic index. TUNEL positivity was observed in some isolated nuclei of the cells of apocrine and sebaceous glands, and in the uppermost layers of the epidermis. In the tumour cells, we observed a strong reaction in non-pycnotic nuclei and in structures with features of apoptotic bodies. A weakly-cytoplasmatic diffuse staining of CCH cells was occasionally seen. The mean AI of CCH, estimated by the TUNEL method, was 39.71%±5.87%. The number of positive cells ranged from 26% to 54%. The mean AI was similar in all four histological groups with 42.5%, 39.0%, 39.4% and 37.2% in groups I, II, III and IV, respectively. Although the tumors of group I were those that presented the highest AI and group IV the lowest,

the statistical analysis revealed no significant difference between the groups ($p=0.14$).

Correlation between PI and AI. There was no correlation between the PI and AI ($r=-0.39$, $p=0.838$). However, in most cases (97.8%, $n=91$), AI was higher than the PI (Figure 1). The PI was higher than the AI only in two cases of histological group I.

Discussion

Each cell contains internal monitoring programs that determine when it has reached the end of its life. If this mechanism fails and even one cell evades the death signal and continues to divide, cancer may result (16). Molecular events regulating cell survival and apoptosis are important contributors to the overall kinetics of benign and malignant cell growth and play a role in tumour development, progression and regression (1, 17).

CCH is a benign histiocytic disease that can be considered as a unique model to study some forms of human Langerhans cell histiocytosis (18). Regression of CCH is the natural evolution of this lesion, by an effective immune response (14); however, the role of proliferation and apoptosis in this process remain unknown. Only two groups describe simultaneously study of proliferation and apoptosis in a few CCHs (19, 20), and only one other investigated a possible role of cellular death in regression of 30 CCH (14). However, as far as we know, this is the first work that investigated proliferation and apoptosis simultaneously in a considerable number of CCHs ($n=93$) and also examined a possible imbalance between proliferation and apoptosis in the regression of CCH.

Ki-67, a cell proliferation-associated nuclear antigen expressed in G₁, G₂, S and M phases of the cell cycle (21), is widely used to study tumour proliferation. In our study, the PI estimated by Ki-67 was 23.56%, a higher value than those reported previously (19, 20). However, these studies were carried out on only few cases ($n=2$ and 10 respectively) and regardless of histological group. When we analyzed the tumour proliferation during CCH regression, no significant differences were noted between the four histological groups considered. This suggests that CCH regression does not occur simply by a decrease of cell proliferation, unlike spontaneous regression of some human tumours (22-24). A comparison with other studies on CCH could not be made because, to the Authors best knowledge, this is the first study analysing the association between proliferation and regression of CCH.

One of the most frequently used methods to quantify apoptosis of cancer cells is the TUNEL method. TUNEL is an *in situ* method used for the detection of apoptotic DNA fragmentation, which usually involves terminal nucleotide transferase-mediated polymerization of labeled dUDP, in a template-independent manner, at the site of breaks at the 3'

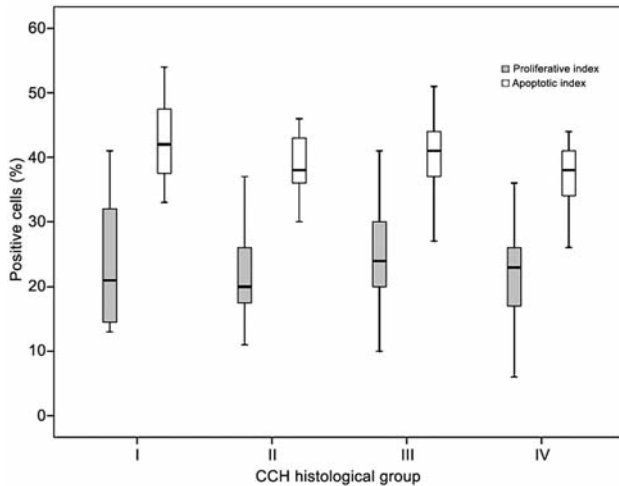


Figure 1. Proliferative and apoptotic index in the four histological groups of canine cutaneous histiocytoma. Group I: Lymphocytic infiltrate scarce and peripheral; group II: lymphocytic infiltrate moderate, nodular and peripheral; group III: abundant nodular infiltrate at the periphery and in the center of the tumour; group IV: lymphocytic infiltrate diffuse and outnumbered the histiocytic tumour cells.

end of DNA (25, 26). Besides typically nuclear staining, diffuse cytoplasmatic staining was also observed in some preparations, as referred in other studies of CCH (19, 20). This could represent leakage of DNA fragments out of the nucleus of apoptotic cells (27).

In our study, the AI was 39.71%. This value is higher than those reported previously (14, 19, 20). This could be due to the larger number of tumours used in our work or could be the result of the different techniques used (reagents and incubation times or sample preservation). However, interestingly, our results are closer to the AI observed for a larger group of other canine benign skin tumours (46.1%) (20).

In spite of the discrepancies in values, our results are partially in concordance with Kaim *et al.* (14). The proportion of TUNEL-positive cells was also similar in all four histological groups. These were not the expected results, because an increase of cell death would be expected in tumours under regression (22-24). Indeed, CCH regression seems to be mediated by CD8⁺αβ lymphocytes (3), and cell-mediated immune attack is known to contribute to the occurrence of apoptosis. Cytotoxic lymphocytes can kill their target cells through activation of the FAS receptor or by the action of granule proteins granzyme and perforin. Natural killer cells preferentially utilise the granule exocytosis pathway, while CD8⁺ cytotoxic T-lymphocytes can use any of the available mechanisms. Both processes activate the cysteine aspartyl proteases (caspases) that are the effector proteases of apoptosis, which ultimately leads to the disassembly of the cell through hydrolysis of cytoplasmic and nuclear proteins (16, 28, 29).

In the human counterpart, the role of apoptosis in tumour regression is not consistent. There are two published studies reporting apoptosis in regression of Langerhans cell histiocytosis. One study reported an AI of 25.7% and the other described the presence of few apoptotic cells. However, both consider apoptosis to be a key process in tumour involution (30, 31).

Despite the high AI found in our study, apoptosis *per se* would not justify CCH regression. Similarly, the proliferation studies do not explain tumour involution. Thus, other mechanisms must be involved. Firstly, tumoural cell death could occur by necrosis. CCH is a tumour with little fibrovascular stroma, which could compromise tumour vascularisation and consequently cell survival. Hypoxia could, thus, be a mechanism also implied in tumour regression (32), by inducing ischaemic necrosis. On the other hand, an imbalance between proliferation and apoptosis could be present. In spite of the absence of correlation between proliferation and apoptosis, this hypothesis could be supported by the fact that AI was higher than the PI in the majority of the studied cases (91 out of 93 cases). If cell death is in excess of cell proliferation, the tumoural mass must decrease in size. Although this is true for the majority of the cases, in two lesions (both of group I), the number of cells positive for Ki-67 antigen was higher than the number of cells stained by the TUNEL method. This could suggest that the rate of proliferation and apoptotic activities alter during tumour development, as proposed for other tumour types such human infantile haemangioma (33). In an early stage, CCH growth is rapid and the proliferative activity is likely to be greater than apoptotic activity. Later, the apoptotic activity becomes greater than the proliferative activity and consequently the lesion decreases in size, leading eventually to involution and complete regression. In our study, 91 tumours had higher AI than PI, hence they must all have been in an involution phase. The two cases with PI greater than AI may thus represent an early phase in histiocytoma development. However, it is necessary to study more of these cases, and early biopsies are required to clarify this process.

In conclusion, the present study strongly suggests that an imbalance between cell proliferation and apoptotic cell death, rather than a decrease in cell proliferation or an increase of apoptosis alone, is a driving force leading to CHH regression.

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