VAS 1 (2016) 4–8

Contents lists available at ScienceDirect

Veterinary and Animal Science

journal homepage: www.elsevier.com/locate/vas

Relationship between the inflammatory infiltrate and the degree of differentiation of the canine cutaneous squamous cell carcinoma

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ARTICLE INFO

Keywords:
Skin neoplasm
Inflammation
Lymphocytes
Macrophages
Plasma cells
Immunohistochemistry

ABSTRACT

The inflammatory response may have pro or anti-neoplastic effects in tumors, depending on the histological type and malignancy level of the tumor. This study aimed to evaluate the profile of predominant inflammatory cells by immunohistochemistry in cutaneus squamous cell carcinoma (SCC) of dogs, comparing it with the degree of differentiation of the tumor. Twenty samples of SCC were analyzed. The tumors were histologically classified into two groups, differentiated SCC (SCCd=12) and undifferentiated SCC (SCCu=08). The tumor inflammatory infiltrate was determined by immunohistochemistry, in order to identify macrophages, lymphocytes and plasma cells. The comparison between groups, SCCd and SCCu, was not significant concerning the density of macrophages (P=1.0), T lymphocytes (P=0.335) and plasma cells (P=0.075). However, when comparing the inflammatory infiltrate in each group, the macrophages were the predominant cell type in both groups, a significant difference was found in the SCCd with plasma cells ($P < 0.0001$). In the SCCu the difference occurred with lymphocytes ($P < 0.05$) and plasma cells (P=0.0006). It could be concluded that the presence of inflammation in cases of SCC does not play a role in the differentiation of the neoplasm, since the inflammatory infiltrate was similar in both groups of SCC.

1. Introduction

Squamous cell carcinoma (SCC), also known as epidermoid carcinoma, is one of within the most frequent skin tumors, corresponding to 5% of skin tumors in dogs (Daleck, De Nardi, & Rodaski, 2007). Even though it may appear in any part of the body, SCC is most commonly observed in the head, abdomen, limbs and perineum (Conceição & Santos, 2010).

Microscopically, it can be observed that the cells present an increased hyperchromatic nuclei with aggregated chromatin. The nucleolus may or may not present variation in size, but is prominent. The histological pattern of SCC varies according to the differentiation degree of these cells. In the degree of differentiation tumors a formation of "keratin pearls" can be observed, these are depositions of keratin concentric lamellae at the center of nests of neoplastic cell cords. In less differentiated areas, keratinization of individual cells may occur, without the formation of keratin pearls. Cells may not produce keratin in more aggressive cases (Goldschmidt et al.,1998).

The participation of the immune system in the growth of tumors is still controversial. There are divergent studies regarding the participation of these cells in the suppression or in the development of neoplastic cells. Some studies have reported the contribution of inflammation to the development of cancer (Giercksky, 2001). The combination of immuno-tolerance mechanisms and evasion of the host immune system enable neoplastic cells to create a favorable environment for growth and tumor aggressiveness (Dunn, Old, & Schreiber, 2004; Satyam, Singh, Badjatia, Seth, & Sharma, 2011).

A significant infiltration of macrophages, lymphocytes and plasma cells can be observed in cases of mammary tumors in female dogs. Some studies have reported that the presence of these lymphocytes improve tumor prognosis, while in others it is believed that these cells favor the proliferation of neoplastic cells. The data regarding the role of the inflammatory cells in the development of the neoplastic cells is still uncertain (Damasceno et al., 2008).

Many studies performed in medicine and veterinary medicine highlight the importance of the immune response against neoplastic cells. On the other hand, studies evaluating an inflammatory response in skin tumors are scarce, regardless of the significant incidence of

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http://dx.doi.org/10.1016/j.vas.2016.10.001
Received 18 January 2016; Received in revised form 8 September 2016; Accepted 10 October 2016
Available online 15 October 2016

these neoplasms in dogs. Current studies in dogs have a higher focus in mammary tumors in female dogs. Thus, the characterization of the inflammatory response in canine cutaneous squamous cell carcinoma is important, since it aims to contribute to the understanding of the complex biology of this tumor and with the prognosis and development of oncological immunotherapy studies. The objective of the present study was to evaluate the inflammatory cells profile which is predominant in cutaneous squamous cell carcinoma in dogs, using immunohistochemical analysis, and comparing it to the tumor's differentiation degree.

2. Material and methods

Twenty samples of canine cutaneous squamous cell carcinoma from the Department of Veterinary Pathology, College of Agricultural and Veterinary Sciences, Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP, Jaboticabal, São Paulo State, Brazil) were analyzed. The samples after surgery were sent for histopathology. All surgical samples, were considered to be excisional and representative, because in each sample, when evaluated morphologically (hematoxylin and eosin) and by immunohistochemical techniques, different regions of the neoplasm were represented.

The histological classification of squamous cell carcinoma was performed according to Goldschmidt and Hendrick (2002), and the samples were divided into two groups: Group SCCd (keratinized tumors, well differentiated and with formation of various keratin pearls, n=12) and Group SCCu (tumors with individual cell keratinization or no keratinization at all, poorly differentiated, n=8).

2.1. Histopathological analysis

The SCC samples were kept in paraffin packs and cut within a section thickness of 5 μm, and stained with hematoxylin and eosin, in order to be examined in light microscopy. The tumor organization pattern, the presence of mitosis figures, the intensity of the inflammatory response, the proportion of keratin pearls and the presence of fibrous stroma associated to the tumor were considered for a final classification of the tumors.

2.2. Immuno-histochemical analysis

Sections were embedded in paraffin, cut into 5 μm specimens and immunohistochemical analysis was performed, for immuno-detection of macrophages (mouse monoclonal antibody anti-macrophage, Serotec, MCA874G, cod. 1209, dilution 1:4000), T lymphocytes (rabbit polyclonal antibody, anti-CD3, Daki, cod. M7254, dilution 1:200) and plasma cells (rabbit polyclonal antibody, anti-CD138, Spring, cod. E4564, dilution 1:25). The sections were deparaffinized in a stove, at 60 °C, for one hour. They were then plated in absolute xylene (20 min), followed by soaking in decreasing ethylic alcohol solutions, until the tissue was hydrated in distilled water. In order to proceed with immunostaining of the referred antibodies, an antigenic recuperation by heat was performed (Pascal pressure chamber, Dako) in a sodium citrate buffer solution 10 mM (pH 6.0). Afterwards, the blocking of endogenous peroxidase with hydrogen peroxide 30 volumes (Merk) was performed, added to methyl alcohol, achieving a final solution with a concentration of 8%, for 30 min. The blocking of unspecific proteins was then carried out with a commercial product (Protein Block, Dako, cod. X0909), for 30 min, at room temperature. The incubation of primary antibodies for macrophages and T lymphocytes was performed in a humid chamber, at 4 °C, for 18 h. For plasma cells, the incubation occurred in a humid chamber, at room temperature, for 18 h. The Streptavidin Biotin Peroxidase Complex kit (Kit LSAB, Dako, cod. K0690) was used as a secondary complex for macrophages and lymphocytes, and the Envision Dual Link System-HRP (Dako Cytomation, cod K406189-2) was used for immunostaining of plasma cells. Diaminobenzidine was the chromagen used (DAB, Dako, cod. S-3022-2) and the counterstaining was elaborated with Harris Hematoxylin. The negative control reaction was performed by replacing the primary antibody by anticorpo diluent (Dako Cytomation, reference S302283-2). The positive control reaction was done according to the manufacturer's recommendation.

Five microscope fields (Nikon Eclipse E200) were examined under a 40× objective lens, with an area of 0.197 mm^2, in order to determine the number of immunostained cells per animal for each antibody, of according to the protocol described by Moreira et al. (2010). The same areas of the tumor were evaluated for the different types of inflammatory cells and a mean was determined for each animal by antibody.

2.3. Statistical analysis

Non-parametric Kruskal-Wallis and Dunn Multiple Comparison tests, were used in order to compare the immunostaining of macrophages, lymphocytes and plasma cells in the groups, considering $P < 0.05$. The comparison between SCCd and SCCu groups, for the cell types, was evaluated by the Mann-Whitney non-parametric test. The analyses were developed in the Graphpad Prism program (version 4.00, 2003).

3. Results

The characteristics of the tumor malignancy allowed distribution of the samples into two groups (Table A.1). The presence of mitotic figures was predominant in the SCCu group, which presented the highest averages of mitotic figures. The variation in volume and cell shape (pleomorphism) was similar between the groups and the pattern of distribution of the tumor was compatible with the tumor's differentiation degree (Table A.1).

The tumor inflammatory infiltrate varied from discrete to moderate, mainly in deeper layers of the dermis, comprising macrophages, lymphocytes and plasma cells. Mast cells associated with this infiltrate were observed occasionally. In the superficial section, when the epidermis was ulcerated, degenerated neutrophils prevailed. In more differentiated tumor samples was noted little inflammatory infiltrate and in undifferentiated samples a higher inflammatory infiltrate associated with the tumor was observed. Desmoplasia around nests of neoplastic cells was frequently found, varying from moderate to severe intensity.

The immunodetection of macrophages (Fig. A.1.A/A.1.B) and lymphocytes (Fig. A.1.C/A.1.D) was observed in the cytoplasm of cells and for plasma cells (Fig. A.1.E/A.1.F) this occurred in the cytoplasmic membrane in SCC samples.

The comparison between groups (SCCd and SCCu) was not significant (Fig. A.2) concerning the density of macrophages ($P=1.0$), T lymphocytes ($P=0.335$) and plasma cells ($P=0.075$). However, when comparing the predominant cellular profile within each group (Fig. A.2), a significant predominance of macrophages in relation to the other types of cells was observed in SCCd ($P < 0.001$) and in SCCu ($P=0.0006$). However, the SCCu group had a greater involvement of lymphocytes associated with macrophage (Fig. A.2.B).