Short paper

Evidences of vasculogenic mimicry in a palpebral melanocytoma in a Doberman dog

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Abstract

A female spayed, 7-year-old Doberman dog was presented to the ophthalmologist with a palpebral nodule on the haired eyelid of the left eye. The nodule was surgically removed and submitted for histopathology. Histologically, the nodule was consistent with eyelid melanocytoma. The neoplasia was also characterized by an unusual histological feature: interspersed throughout the neoplastic melanocytes, numerous lacunar and slit-like spaces filled by erythrocytes were observed. On immunohistochemistry, these spaces were lined by cells PNL2-positive and Factor VIII and CD31 negative, consistent with neoplastic melanocytes without endothelial cell participation. This feature was interpreted as vasculogenic mimicry (VM), a mechanism of tumour angiogenesis well-recognized in human melanomas that, to the authors' best knowledge, has not yet been reported in melanomas in veterinary medicine.

Keywords: dog; melanocytoma; vasculogenic mimicry.

Vasculogenic mimicry (VM) is a well-known feature of human melanoma (Maniotis et al., 1999). VM has been defined as the *de novo* generation of vascular channels by tumour cells and is not considered a strictly vasculogenic event because it does not result in *de novo* formation of endothelial cell-lined vessels (Spiliopoulos et al., 2015). Rather, these microcirculatory networks or capillary-like structures are made of extracellular matrix and are lined by neoplastic cells instead of endothelium (Spiliopoulos et al., 2015). It is still not well understood if these vascular structures are functional, however these channels can be detected angiographically and red blood cells are histologically recognizable within their lumens (Maniotis et el., 1999). Lining endothelial cells have not been identified by light microscopy, by transmission electron microscopy, or by the use of immunohistochemical endothelial cell markers (Factor VIII-related antigen, CD31, CD34, and KDR[Flk-1]) (Maniotis et el., 1999; Spiliopoulos et al., 2015).

VM was firstly described in human melanoma by Maniotis and coauthors in 1999 (Maniotis et el., 1999) and, thereafter, it has also been reported in different types of human tumour, such as malignant mesothelioma (Pulford et al., 2016), cancer of the liver (Zhao et al., 2015), pancreas (Guo et al., 2014), stomach (Zang et al., 2015), prostate (Wang et al., 2016), ovaries and breast (Hendrix et al., 2003). The presence of VM patterns in patients with malignant tumours, e.g. human uveal and cutaneous melanomas, has been correlated with a worse prognosis and shorter survival than their non-VM-forming counterparts (Spiliopoulos et al., 2015).

To date, in dogs, VM has been reported only in canine inflammatory breast cancer (Clemente et al., 2010; Rasotto et al., 2012) but, to the best of the authors' knowledge, it has not been previously described in canine melanocytic tumours. In the present report, VM pattern is described in a melanocytic neoplasm of the eyelid in a dog.

A female spayed Doberman 7-year old dog was presented with a 5 mm, brownish, palpebral nodule on the skin of the upper eyelid of the left eye. The nodule had been present for one year and was slowly enlarging. Complete ophthalmic examination was otherwise unremarkable, except for mild bilateral epiphora. Pre-operative diagnostic tests, i.e. complete blood count and serum chemistry,

were within normal limits. The nodule was surgically removed with “V" full-thickness excision and submitted for histopathology. The sample was fixed in 10% buffered formalin and routinely processed for histology. Microtomic section were obtained and stained with hematoxylin and eosin (H&E) for histopathological examination.

Histologically, the dermis of the eyelid was expanded by a multilobular nodular neoplasm, which was moderately well demarcated and not encapsulated. Neoplastic cells were arranged in lobules and nests with multifocal areas of junctional activity. Neoplastic cells were epithelioid or, less commonly, spindle-shaped, with indistinct cell borders and high nucleus/cytoplasmic ratio. Cytoplasm was moderate, granular and eosinophilic, occasionally vacuolated and multifocally filled by melanin pigment. Nuclei were oval, with marginated chromatin and single prominent central nucleolus. Anisocytosis and anisokaryosis were moderate and mitotic count in 10 HPF was 1. Multifocally, throughout the neoplasm, numerous irregular slit-like or lacunar spaces were observed (Figure 1). These spaces were filled by a moderate number of red blood cells, were supported by fine fibrous stroma and were lined by polygonal, epithelioid or spindle cells with moderate cytoplasm and a round to oval nucleus with a prominent nucleolus. The neoplasm was diagnosed as a mixed-type sparsely pigmented dermal melanocytoma, .

With Periodic acid–Schiff (PAS) special staining of serial sections, performed to characterize the lacunar spaces, there was moderately positive staining of the thin fibrous septa delimiting the blood-filled spaces.

Immunohistochemistry (ABC standard method) was also performed to further characterize the cells lining the blood-filled lacunar spaces. The endothelial cell markers Factor VIII (FVIII) and CD31 and the melanocytic marker PNL2 were specifically investigated.

For immunohistochemistry, serial microtomic sections were obtained and mounted on polylysine coated slides (Menzel-Gläser, Braunschweig, Germany). PNL and FVIII labelling were performed by manual staining with standard ABC method. After heat-induced antigen retrieval in EDTA buffer (PNL2) or enzymatic-retrieval with pepsin (FVIII), slides were immunostained using a

mouse monoclonal anti-PNL2 antibody (Monosan, Uden, Netherlands), incubated at 1:25 overnight at 4°C, and a rabbit polyclonal anti-Factor VIII antibody (Dako, Carpinteria, USA), incubated at 1:200 overnight at 4°C, respectively. CD31 labelling was automatically performed with Ventana BenchMarck ULTRA (Ventana Medical System, Roche, Oro Valley, AZ, USA): the sections were unmasked with Benchmark ULTRA CC1 (pH 8.4) at 95°C for 52 minutes and incubated with the primary antibody mouse monoclonal anti-CD31 Endothelial cell (JC70A; Dako, Carpinteria, USA), at 1:20 for 32 minutes at room temperature. DAB (3,3'-diaminobenzidine) (Roche, Oro Valley, AZ, USA) or AEC (3-amino-9-ethylcarbazole) (Vector Laboratories, Burlingame, USA) substrate-chromogen kit were used as chromogen, and sections were counterstained with Mayer's hematoxylin. Negative controls were prepared by replacing the respective primary antibody with normal rabbit or mouse serum (non-immune serum, Dakocytomation). Endothelium of blood vessels served as internal positive control for FVIII and CD31.

The cells lining red blood cell-filled spaces, as well as the neoplastic cells composing the melanocytoma, labelled strongly with antibody against PNL2 (Figure 2), whereas they were negative to FVIII and CD31 (Figures 3-4). Normal endothelium of pre-existing blood vessels within the same section was diffusely and intensely stained with both FVIII and CD31.

Based on histological and immunohistochemical results, lacunar spaces were interpreted as areas of vasculogenic mimicry within an eyelid melanocytoma.

Eight months after surgery, the dog was in good health conditions with no indications of recurrence of the eyelid mass.

The term vasculogenic mimicry (VM) has been used to describe the ability of aggressive neoplastic cells to acquire an endothelial-like morphology and to form extracellular matrix (ECM)-rich vasculogenic-like networks (Hendrix et al., 2003), which are hypothesized to facilitate tumour perfusion independently from tumour angiogenesis (Maniotis et al., 1999). The present case describes histological evidences of vasculogenic mimicry in a canine cutaneous melanocytoma. The

mass arose on the eyelid and contained widespread microscopic slit-like and lacunar spaces filled with erythrocytes. Histochemical and immunohistochemical staining confirmed the melanocytic origin of the cells lining these lacunar spaces. In fact, these spaces, sustained by PAS-positive fibrous septa, were lined by cells that labelled strongly with antibody against PNL2, marker of melanocytic origin, whereas did not label with antibodies anti FVIII and CD31, markers of endothelial origin. These results confirmed the melanocytic origin of the cells lining the newly formed vascular network and confirmed the presence of a "vasculogenic mimicry" phenomenon. In VM in human melanoma, it has now been recognized that the deeper layer of melanoma cell-lined microvascular structures is composed of extracellular matrix proteins such as laminin, collagens IV and VI, and heparan sulfate proteoglycans, which provide the PAS-positive supportive fibrous stroma (Spiliopoulos et al., 2015). Currently, there are two hypotheses concerning the origin of VM network-forming melanoma cells: they could either be tumour cells that have undergone de-differentiation, resulting in a primitive cell-type which encompasses tumour cell, stem cell, and endothelial cell characteristics, or they may arise from cancer stem cells (Spiliopoulos et al., 2015). VM in melanoma involves several signaling molecules that are also involved in embryonic vasculogenesis, including for example vascular endothelial (VE)-cadherin, erythropoietin-producing hepatocellular carcinoma-A2 (EPHA2), phosphatidylinositol 3-kinase, focal adhesion kinase, matrix metalloproteinases and laminin 5 γ2-chain (Hendrix et al., 2003). Identification of the pathways that regulate this undifferentiated and highly plastic phenotype may be strategic in the development of new therapies for human cancer. In fact, even though the biological implications of VM *in vivo* are still unclear, tumour VM is associated with a poor prognosis (Hendrix et al., 2016). Thus, VM in tumours of human patients with a poor clinical outcome suggests a functionally relevant role in the survival of tumour cells (Hendrix et al., 2016). VM in human melanomas is correlated with a high mitotic index and prevalence of epithelioid morphology, which is associated with worse prognosis (Spiliopoulos et al., 2017).

Nevertheless, VM has also been described in benign melanocytic nevi (Demitsu et al., 1998; Spiliopoulos et al., 2017). In a study by Spiliopoulos and co-authors on human melanocytic tumours of the eye and the periocular area, 10% of benign nevi, mostly arising in the conjunctiva, included areas of VM, without any other atypical features, suggesting the absence of an association with malignant potential. However, in human medicine, conjunctival nevi can occasionally also behave as pre-cancerous lesions and progress into melanomas. The prognostic implication of VM in benign nevi has not yet been elucidated (Spiliopoulos et al., 2017).

There are few reports in the veterinary literature of VM in dogs. VM has been reported in canine mammary carcinoma: specifically, VM has a higher frequency in inflammatory mammary cancer compared to non-inflammatory mammary cancer (Clemente et al., 2010), although VM is not considered predictive of invasion of the lymphatic system (Rasotto et al., 2012).

To the best of the authors' knowledge, VM has not been previously reported in canine melanocytic tumours. To date, there are insufficient cases in the veterinary literature on VM in canine melanocytic tumours to draw prognostic considerations on the significance of VM in dogs, yet it would be interesting to assess if this phenomenon may bear a role in the behavior and invasiveness of this tumour, thus resembling the human cases. The incidence, role and prognostic significance of VM in veterinary species is a field worthy of further investigation.

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193 **Figure legends**

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195 Fig. 1. Dermis is expanded by a multilobular melanocytoma admixed with numerous irregular slit-
196 like or lacunar spaces filled by red blood cells. H&E, bar = 25 μm.

197 Fig. 2. Spaces are lined by PNL2-positive neoplastic cells. IHC anti-PNL2, AEC chromogen, bar =
198 12.5 μm.

199 Fig. 3. Cells lining blood-filled spaces are FVIII negative. IHC anti-FVIII, AEC chromogen, bar =
200 12,5 μm.

201 Fig. 4. Cells lining blood-filled spaces are CD31 negative. IHC anti-CD31, DAB chromogen, bar =
202 12,5 μm.