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Claudin-5 protein is a new differential marker for histopathological differential diagnosis of canine hemangiosarcoma

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Summary. Aims: Claudin-5 protein is an endothel-specific claudin, present in tight junctions. To evaluate its usefulness as a differential diagnostic marker of canine hemangiosarcomas, the expression of claudin-5 molecule was studied in different canine tumours of vascular origin. Methods and results: Ninety two canine neoplastic tissue samples obtained from necropsies and biopsy specimens were routinely processed and stained immunhistochemically for claudin-5. The neoplastic endothelial cells of canine hemangiosarcomas, hemangiomas, and lymphangiomas showed a strong membrane immunoreactivity for claudin-5, but the other investigated canine malignant and benign tumours, including fibrosarcomas, myxo-, leiomyo-, cardiac rhabdomyo-, neurofibro-, synovial-, osteo-, and chondrosarcomas, spindle cell melanomas, hemangio-pericytomas, benign fibroblast proliferations, and leiomyomas were negative for this endothelial marker. In these non-vascular canine tumours intense immunostaining was detected in the endothelial cells of the incorporated intratumoural vessels and neovasculature. The canine splenic hematomas induced by hemangiosarcomas were distinguished from splenic hematomas induced by non-neoplastic lesions by the means of claudin-5 protein. In hemangiosarcomas the percentage of positive neoplastic endothelial cells was higher, and stronger when using the claudin-5 molecule compared to CD31 and vWf. Conclusion: The results show that claudin-5 molecule can be used as a new differential marker, and could also be of a diagnostic value in the differential diagnosis of canine hemangiosarcomas from sarcomas of other origin with hemorrhages or increased vascularization. Claudin-5 could help to reveal neoplastic proliferation of endothelial cells causing splenic hematomas and differentiate these tumours from non-vascular neoplastic splenic lesion. The immunohistochemical detection of the claudin-5 protein had a higher sensitivity than CD31, and vWf antigen in case of canine hemangiosarcomas.

Key words: Canine hemangiosarcoma, Immuno-histochemistry, Claudin-5, Differential diagnosis, Canine splenic hematoma

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Introduction

Canine hemangiosarcoma (HSA) arises from neoplastic transformed vascular endothelial cells. HSA is characterized by early and aggressive metastatic phenotype (Smith, 2003). HSA occurs most frequently in older dogs with a mean age between 8 and 13 years, most commonly in the spleen, right atrium, or subcutis (Withrow and MacEwen, 2001). Other primary sites of HSA origin that have been reported in the dog include the liver, lung, aorta, kidney, oral cavity, muscle, bone, urinary bladder, intestine, tongue, prostate, vulva-vagina, conjunctiva or peritoneum. It occurs most commonly in large breed dogs (Aronsohn, 1985; Brown et al., 1985; Withrow and MacEwen, 2001). Cutaneous HSAs have been associated with ultraviolet light exposure in dogs and often arise on the ventral abdomen and prepuce, where the hair coat is sparse (Hargis et al., 1992; Ward et al., 1994). HSA tends to metastasize through hematogenous or transabdominal implantation and the most frequent metastatic sites are the liver, omentum, mesentery and lungs (Withrow and MacEwen, 2001).

Treatment and prognosis for HSA vary by location. Surgery and chemotherapy have limited success in prolonging survival times and increasing quality of life in dogs with HSA. Cutaneous HSA is often curable with surgery alone, provided the lesion is small and defined to the dermis (Clifford et al., 2000). Morbidity and mortality are often due to acute internal hemorrhage secondary to tumour rupture. Despite surgery and intensive chemotherapy, the median survival time for dogs diagnosed with HSA is little more than 6 months (Hammer et al., 1991; Sorenmo et al., 1993, 2000).

For diagnostic purposes, von Willebrand's factor (vWf; factor VIII-related antigen, F VIII Rag) and CD31 (PECAM-1, platelet endothelial cell adhesion molecule-1) have been firmly established as neoplastic endothelial markers in paraffin wax-embedded, formalin-fixed tissue sections in human beings (Little et al., 1986; Parmus et al., 1990; Kuzu et al., 1993).

Several veterinary studies have investigated the immunhistochemical phenotype of canine vascular tumours including vWf- (von Beust et al., 1988), CD31- (Ferrer et al., 1995), vimentin- (Moore et al., 1989) and other non-diagnostic immunohistochemical markers (Augustin-Voss et al., 1990; Yonemaru et al., 2006).

The tight junction (TJ) is a specialized membrane domain at the most apical region of polarized epithelial and endothelial cells, which not only creates a primary barrier to prevent paracellular transport of solutes (barrier function), but also restricts lateral diffusion of membrane lipids and proteins to maintain cellular polarity (Gumbiner, 1987; Schneeberger and Lynch, 1992; Anderson and van Itallie, 1995). Claudins are a family of about 17-27 kDa integral membrane TJ proteins which determine the size of molecules that can pass through the paracellular space in epithelial and endothelial tissues. Together with other integral membrane partners, such as occludin and the junctional adhesion molecule 1 (JAM-1), the tissue-specific expression of claudin proteins performs specialized epithelial or endothelial functions, including maintenance of cell polarity and paracellular barrier functions (Furuse et al., 1998; Gonzalez-Mariscal et al., 2003; Harhaj and Antonetti, 2004).

The first vascular-specific claudin was claudin-5, also known as transmembrane protein deleted in velo-cardio-facial syndrome (TMVCF) (Sirotkin et al., 1997; Morita et al., 1999). Claudin-5 is a 22kDa phosphoprotein with four transmembrane domains (Yang et al., 2007). Claudin-5 is an exception because it has not been detected in epithelial compartments, and it is apparently restricted to endothelial cells of most tissues. In support of endothelial-specificity, research has also shown claudin-5 protein in dermal vascular endothelium in mouse and human skin cells (Morita et al., 2003).

Soini (2005), in his study evaluated the immunoreactivity of claudin-1, -2, -3, -4, -5 and -7 in 116 epithelial and 92 non-epithelial human tumours. Of non-epithelial tumours, claudin-5 was found only in angiosarcomas and benign vascular tumours. His results showed that these claudins can be used as markers for epithelial differentiation and to distinguish epithelial neoplasms from lymphomas and selectively also from soft tissue and naevocytic lesions. Additionally, claudin-5 seemed appropriate to be used as a marker for endothelial lesions compared to other soft tissue lesions (Soini, 2005).

The aim of the present study was to evaluate the immunhistochemical detection of claudin-5 protein in formalin fixed paraffin-embedded canine HSAs, and other canine tumoural/non-tumoural tissues for the possible differential diagnostic value of the claudin-5 protein expression during the investigation of the canine HSAs. The authors investigated the immunohistochemical expression of claudin-5 in different malignant and benign canine tumours including hemangiosarcoma, hemangioma, lymphangioma, hemangiopericytoma, fibrosarcoma, myxosarcoma, leiomyosarcoma, cardiac rhabdomyosarcoma, malignant peripherial nerve sheath tumour, spindle cells melanoma, synovial sarcoma, osteosarcoma, chondrosarcoma, benign fibroblast proliferation, leiomyoma, and in normal canine spleen, splenic hematoma, granulation tissue, and intact skin. This is the first report of the detection of claudin-5 in canine vascular tumours especially in HSAs. Overall, we would use claudin-5 to enhance differential diagnosis of HSA and other non-vascular tumours, especially the solid type ones.

Materials and methods

Samples of canine tumours

Tissue samples were collected between 2004 and 2008 from Szent István University, Faculty of Veterinary Medicine, Department of Pathology and Forensic Veterinary Medicine (Budapest, HU). A total of 26 HSAs (16 samples were primary spleen tumours [12 from 16 were with splenic hematoma], 4 samples were primary right atrium lesions, and 6 samples were primary subcutaneous neoplasias), 6 hemangiomas (4 samples were subcutaneous tumours, 2 samples were splenic lesions), 3 cutaneous lymphangiomas (2 hindlimbs, 1 trunk), 7 hemangiopericytomas, 11 fibrosarcomas (6 samples were primary skin tumours, 3 samples were spleen lesions, and 2 were primary oral tumours), 3 myxosarcomas (skin lesions), 3 leiomyosarcomas (1 vagina, 1 stomach, 1 jejunum tumour samples), 3 cardiac rhabdomyosarcomas, 3 malignant peripheral nerve sheath tumours (n. hypoglossus, plexus brachialis, unidentified cranial nerve), 4 spindle cell melanomas (2 legs, 1 head, 1 trunk), 4 synovial sarcomas, 5 osteosarcomas (non-productive type from bone), 4 chondrosarcomas, 5 benign fibroblast proliferations (3 collagen nevus from head, and neck, and 2 acrochordon, from trunk, and foreleg), 5 vaginal leiomyomas were examined. In addition 14 spleen (4 normal samples, 5 acute intrapulpar hematomas, and 5 fibroblastic organisations

of splenic hematoma), 4 granulation tissues (skin lesions) in an active phase of angiogenesis, 6 normal skin samples (dermal vessels) were investigated. Eighty four samples were removed surgically, and 32 samples (4 normal spleen samples, 4 atrial HSAs, 6 splenic HSAs, 5 osteosarcomas, 4 vaginal leiomyomas, 2 synovial sarcomas, 3 leiomyosarcomas, 3 cardiac rhabdomyosarcomas, and 1 malignant peripheral nerve sheath tumours) were collected during necropsy. The samples were fixed in 8 % neutral buffered formalin for 24 hours at room temperature, dehydrated in a series of ethanol and xylene, and embedded in paraffin. The 3-4µm thick sections were routinely stained with hematoxylin and eosin (HE), Masson's trichrome and van Gieson staining.

We investigated the expression of claudin-5 in different canine epithelial tissues including 6 normal skin, 20 normal mammary gland (Jakab et al., 2008a), 5 prostatic gland, 5 lungs, 5 kidney, 5 liver samples and 5 cardiac muscle, 5 thymus, 5 lymph nodes samples were collected during necropsy.

Breed, sex, and age of the dogs

The canine HSA samples were collected from 10 females, 16 males dogs with an average age of 8.8 years (range, 5-16 years). The most common breeds were mixed breeds (n=8), German Shepherds (n=6), Golden Retrievers (n=3), Rottweilers (n=3), Schnauzer (n=2), Hungarian Vizsla (n=1), English Setter (n=1), Dalmatian (n=1), Labrador Retriever (n=1). The hemangioma study group comprised 4 females, 2 males with an average age of 8.16 years (range, 6-12 years). The most common breeds were mixed breeds (n=3), German Shepherds (n=2), and Rottweilers (n=1). The cutaneous lymphangioma study group comprised 1 female, 2 males with an average age of 7.3 years (range, 6-8 years). Breeds: Caucasian, mixed breed, Beagle. The hemangiopericytoma study group comprised 4 females, 3 males with an average age of 8.7 years (range, 4-13 years). Four different breeds were represented: Dobermanns (n=3), Hungarian Vizsla (n=2), Schnauzer (n=1), and German Shepherds (n=1). The fibrosarcoma study group comprised 3 females, 8 males with an average age of 9.1 years (range, 8-11 years). The most common breeds were mixed breeds (n=5), German Shepherds (n=3), Rottweilers (n=2), and Chow-chow (n=1). The myxosarcoma study group comprised 2 females, 1 male mixed breed dogs with an average of 8 years (range 6-10 years). The leiomyosarcoma study group comprised 2 females, 1 male with an average age of 8.3 years (range, 8-9 years). Breeds: poodle, rottweiler, and mixed breed dog. The cardiac rhabdomyosarcoma study group comprised 2 females, 1 male with an average age of 5.6 years (range, 2-8 years). Breeds: Golden Retriever, Irish Setter, and German Shepherd Dog. The malignant peripheral nerve sheath tumour study group comprised 2 females, 1 male with an average age of 10 years (range, 9-11 years). Breeds were: Hungarian Vizsla, Dobermann, and mixed breed dog. The spindle cell melanoma study group comprised 1 female, 3 males mixed breeds with an average 8.7 years (range, 8-10 years). The synovial sarcoma study group comprised 1 female, 2 males with an average age of 10.3 years (range, 9-12 years). Breeds were: Mixed breed dogs (n=2), and Mastiff. The osteosarcoma study group comprised 4 males with an average age of 6.7 years (range, 6-8 years). The samples arrived from Tike, Rottweilers (n=2), German Dog, and Schnauzer. The chondrosarcoma study group comprised 2 females, 2 males with an average age of 10 years (range, 6-13 years). The samples were taken from Hungarian Vizsla, Husky, Dobermann, and mixed breed dog. The benign fibroblast proliferations group comprised 2 females, 3 males with an average age of 7.2 years (range, 6-8 years). The samples arrived from a mixed breed dog, German Shepherd, Poodle, Beagle, and Spaniel. The vaginal leiomyoma study group comprised 3 Dachshunds, 2 Poodles with an average age of 8.2 years (range, 6-10 years). The non-neoplastic tissue samples were collected from different canine breeds, sexes and ages.

Immunohistochemical analysis

The immunohistochemical antibodies used were claudin-5 (diluted 1:100, 4C3C2, mouse anti-human monoclonal, Zymed Inc., San Francisco, CA, USA - #18-7364), CD31 (diluted 1:80, JC/70A, mouse anti-human monoclonal, DAKO), von Willebrand's factor (diluted 1:50, F8/86, mouse anti-human monoclonal, DAKO) vimentin (diluted 1:200, 3B4, mouse anti-bovine monoclonal, DAKO), α-smooth muscle actin (α-SMA) (diluted 1:8,000, 1A4, mouse anti-human monoclonal, Sigma), desmin (diluted 1:50, N1526, monoclonal mouse anti-human, DAKO), S-100 protein (diluted 1:50, rabbit anti-ovine polyclonal, DAKO), and neuron specific enolase (NSE) (diluted 1:100, BBS/NC/VI-H14, mouse anti-human monoclonal, DAKO).

For immunohistochemistry 3-4 µm thick sections were cut. The slides for the claudin-5 immunohistochemical reaction were deparaffinized in xylene and graded ethanol. The deparaffinized sections were treated with primary antibody claudin-5 for 60 minutes at room temperature after treatment with appropriate antigen retrieval (Target Retrieval Solution, DAKO, Glostrup, Denmark, pH 6; microwave - 800W - oven for 30 minutes). Immunohistochemical staining was performed using the streptavidin-peroxidase technique. Antigen-bound primary antibody was detected using standard avidin-biotin immunoperoxidase complex (DAKO LSAB2 Kit). The chromogen substrate was 3, 3 - diaminobenzidine tetrahydrchloride (DAB substrate-chromogen, DAKO, Denmark). Mayer's hemalaun was used for counter-staining. The external positive control was the endothelial cell component of the dermal capillaries and small vessels from human skin. The

staining pattern was strong membrane. The reactions were carried out in a Ventana ES automatic immunostainer (Ventana Medical System Inc., Tucson, AZ, USA) using the raegents provided by the manufacturer.

The 3-4 µm slides for the all of the other antibodies immunohistochemical reactions were deparaffinized in xylene and graded ethanol. Before application of anti-vimentin, α-SMA, CD31, vWf, desmin, S-100, and NSE, the sections were treated in microwave - 800W - oven in 10 mM citrate buffer, pH 6.0, for 15 min. Each slide was treated with 0,3% hydrogen peroxide in methanol for 30 minutes at room temperature. The deparaffinized sections were treated with primary antibodies for 60 minutes at room temperature. Immunohistochemical staining was performed using the streptavidin-peroxidase procedure. Antigen-bound primary antibody was detected using standard avidin-biotin immunoperoxidase complex (DAKO LSAB2 Kit). Antibody binding was revealed using H_2O_2 as a substrate and diaminobenzidine as chromogen. Mayer's hemalaun was used for counter-staining.

For each immunohistochemical reaction, a negative control with omission of the primary antibody was included. The external positive controls were the endothelial cells of vessels in the normal human skin for claudin-5, human fibroma for vimentin, human leiomyoma for α-SMA, and desmin, human hemangiosarcoma for CD31, and vWf, human schwannoma for S-100 and NSE .

In our study we compared claudin-5 protein and CD31 staining within canine HSAs. We compared the percentage of positive neoplastic endothelial cells of both endothelial markers. Scoring for semiquantitative analysis was independently evaluated by two pathologists (CSJ, AK). The number of claudin-5, and CD 31 positive cells was calculated as follows: 10 randomly selected areas per slide were analyzed using x20 objective with 100 cells counted in each field. Reactions were scored positive where linear membrane staining was seen. The scoring standardized for each group was as follows: 5 = 80 to 100%, 4 = 60 to 80%, 3 = 40 to 60%, 2 = 20 to 40%, 1 = 5 to 20% and 0 = 0 to 5% showed positive reactions.

Paired t-test was used, because both methods were used on the same samples, and the difference of the two variables was normally distributed in both cases (P=0.707 and P=0.155 using Shapiro-Wilk normality test).

Results

Histopathology of HSA

In the HSA study group the tumour tissues were composed of immature endothelial cells forming solid areas, or vascular spaces as small clefts and cavernous channels separated and supported by collagen which were detected with Masson's trichrome and van Gieson stainings. The morphology of the neoplastic endothelial cells was variable and ranged from a spindle shape to polygonal and ovoid. Individual cells were characterized by scant to moderate eosinophilic cytoplasm, and pleomorphic, euchromatic nuclei with medium-sized nucleoli. There usually were one to two mitotic figures per high power field. In our study we considered the HSAs to be cavernous (11 samples) and capillary (4 samples) types when the cells had a fairly uniform morphology and were arranged in cords and vascular channels (Fig. 1A,C). HSAs were considered to be of the solid types (11 samples) when the cells were pleomorphic and formed clefts or solid sheets rather than channels (Fig. 2A).

The specificity of claudin-5 in neoplastic and non-neoplastic endothelial cells

In all HSAs, intense staining of claudin-5 was detected in the membrane of neoplastic endothelial cells, both in cavernous and capillary types (Fig. 1B,D), and solid types as well (Fig. 2B). Due to strong membranous, and diffuse immunoreactivity of claudin-5 we were able to detect the structural orientation of the enlarged neoplastic endothelial cells. The differentiated neoplastic endothelial cells of hemangiomas (5 cavernous, 1 capillary types) stained strongly similarly to cutaneous lymphangioma (cavernous types) samples. The immunreactivity of claudin-5 in HSA cells was moderately stronger than in the external positive controls (human dermal vessels), and in the hemangioma, and lymphangioma samples, indicating an increased expression of claudin-5 in HSA samples. The neoplastic cells of the fibrosarcoma, myxosarcoma, leiomyo-sarcoma, cardiac rhabdomyosarcoma, malignant peripheral nerve sheath tumour, spindle cell melanoma, synovial sarcoma, osteosarcoma, chondrosarcoma, benign fibroblast proliferation, and vaginal leiomyoma groups were negative for claudin-5, but intense membrane staining was detected in the endothelial cells of the neoangiogenetic, incorporated intratumoural, and peritumoural preexisting vessels in case of each claudin-5 negative tumoural groups. These vessels were used as internal positive controls (Fig. 2C,D). In the hemangiopericytomas the neoplastic cells are arranged as interlacing bundles, sheets, and concentric whorls in varying proportions. The whorls generally have a single capillary blood vessel at their centers (Withrow and MacEwen, 2001). As expected the tumour cells of hemangiopericytomas were negative for claudin-5, only the differentiated endothelial cells of the central collapsed capillaries showed intense membrane immunoreactivity, as well as the endothel of the incorporated existing vessels in the tumour parenchyma (Fig. 3A,B) (Table 1).

We investigated 4 canine normal spleen samples. In the canine normal spleen the endothelial cells of the sinusoids, trabecular and central arteries, and capillaries showed intense immunoreactivity for claudin-5. The

strongest expression of claudin-5 was found in endothelial cells of sinusoids, followed by trabecular arteries, central arteries, and sheathed capillaries (Fig. 3C,D). The lymphoid-macrophage cellular population of the white pulp, the stromal fibroblast cells of the spleen, smooth-muscle cells of trabeculae, the arteries and erythrocytes were negative for claudin-5 in all cases. The original endothelial cell population of the sinusoids and trabecular arteries of the intact spleen can be used as internal positive controls during the immunohistochemistry (Fig. 4A,B).

In the dermis of the normal skin of the dog the endothelial cells of the intact vessels showed intense membrane staining for claudin-5. The strongest expression of claudin-5 was found in endothelial cells of small arteries, followed by arterioles, venules, and capillaries.

Intense membrane staining of claudin-5 in the granulation tissues was detected in the endothelial cells of newly formed capillaries, but not in the case of fibroblasts, or inflammatory cells, including neutrophil granulocytes, lymphocytes, and macrophages.

In the canine intact skin samples the epidermal cells, follicules and sebaceous glands were negative for claudin-5, but the apocrin glands showed a membrane-bound positivity. In an earlier study we have described

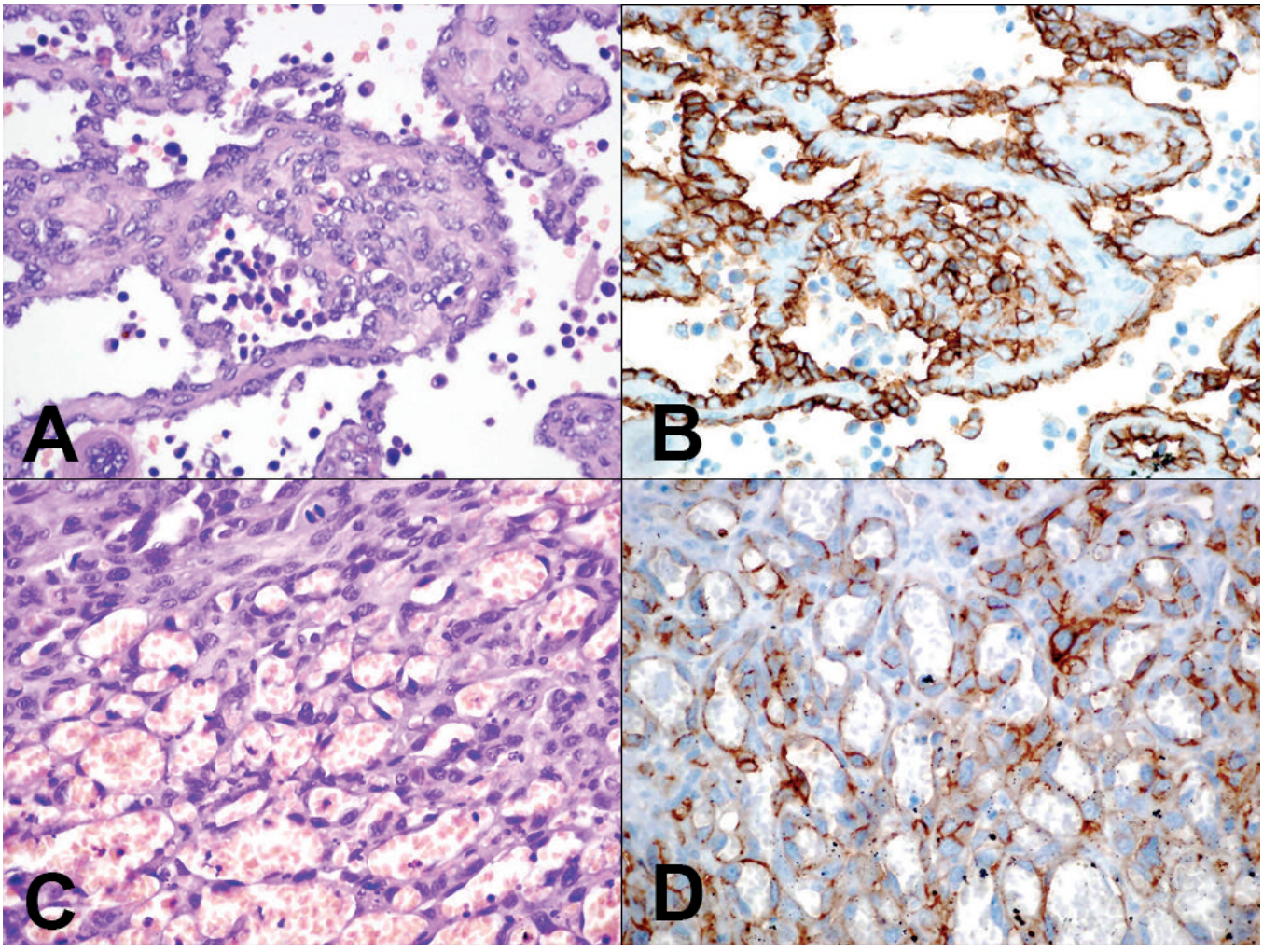

Fig 1. Canine hemangiosarcoma. **A.** Cavernous type HSA from spleen of an 8 year old German Shepherd bitch. The neoplastic endothelial cells present in the well differentiated tumour have pleomorphic nuclei and often protrude into the cavernous vascular lumens. The lumens of the cavernous channels contain numerous hemopoetic cells. At the left corner of the picture a megacaryoblast can be seen. HE. **B.** In this similar HSA sample, strong membrane-bound positivity of the neoplastic endothelial cells for claudin-5 can be seen. The hemopoetic cells present in the cavernous vascular channels are negative for claudin-5. **C.** Capillary type HSA from spleen of an 11 year old mixed breed male dog. This lesion shows capillary-like growth pattern of the newly formed vessels. HE. **D.** This similar HSA sample demonstrates a strong membrane-bound positivity for claudin-5 of the neoplastic endothelial cells. The erythrocytes present in the cavities of the neoplastic vessels are negative for claudin-5. x 60.

that the claudin-5 protein showed weak lateral membrane reaction in the nomal canine mammary glands (Jakab et al., 2008a). The epithelial cells of the canine prostatic gland showed intense membrane immunoreactivity for this protein. In the canine lung samples the epithelial cells of the alveoli, bronchi and bronchioli were negative, but the mesothelial cells of the pleura were positive for claudin-5 molecule. In the canine kidney samples the epithelial cells of the nephron showed weak cytoplasmic reaction, but the glomerulums showed a strong positivity for this TJ-protein. In the liver samples the liver cells and biliary epithelial cells were negative for claudin-5. The canine cardiac muscle cells, the lymphoid and macrophage cells of the lymphatic tissues were negative for this TJ-protein. In all canine organs the endothelial cells of the original vessels and lymphatics showed a strong positivity for claudin-5.

Immunohistochemical analysis of claudin-5 antibodies in splenic hematomas

We investigated 12 splenic hematomas induced by splenic HSAs (3 cavernous, 1 capillary and 8 solid types), 3 splenic hematomas induced by splenic fibrosarcomas, 5 acute intrapulpar hematomas induced by lymphoid hyperplastic nodules, 5 fibroblastic organisations of splenic hematoma without tumoural lesions. In all splenic hematomas induced by HSAs, intense staining of claudin-5 was detected in the membrane of enlarged neoplastic endothelial cells, both

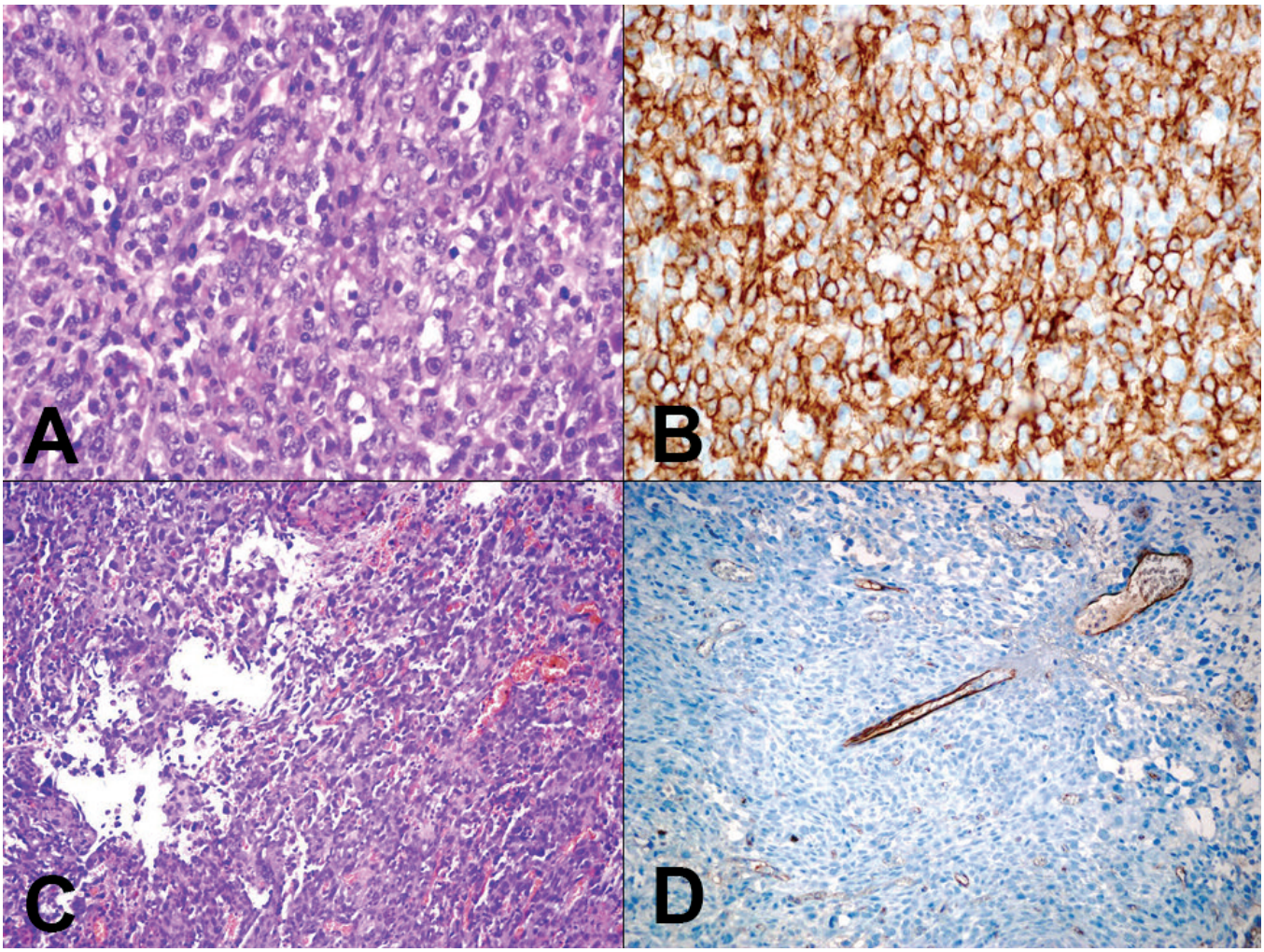

Fig 2. Canine solid HSA and fibrosarcoma. **A.** Solid type HSA from the spleen of an 11 year old mixed breed male dog. In this poorly differentiated tumour the loss of obvious vascular structures is the predominant histological feature. HE. **B.** In this similar solid type HSA, strong membrane-bound positivity of the tumoural endothelial cells for claudin-5 can be seen. **C.** Canine HSA-like fibrosarcoma, from the skin of an 8 year old mixed breed male dog, with good vascularization and multiple hemorrhages. HE. **D.** Canine fibrosarcoma lacking claudin-5 expression. The endothelial cells of the intratumoural microvessels show strong positivity for claudin-5 (internal positive controls). A, B, x 60; C, D, x 20.

in cavernous and capillary types, and solid types as well. The neoplastic, enlarged endothelial cells of HSAs, which overexpressed the claudin-5 protein, formed cohesive cell populations among the claudin-5 negative accumulated red blood cells (Fig. 4C). In the cases of the splenic hematomas induced by fibrosarcomas the neoplastic cells were negative for claudin-5 but the endothelial cells of the nearby, compressed red pulp (internal positive controls) and the intratumoural microvessels showed a membrane-bound positivity for this protein. In the case of the acute hematoma samples induced by lymphoid hyperplastic nodules we did not detect irregular proliferation of the neoplastic endothelial cells. Only the claudin-5 negative hyperplastic lymphoid nodules and the claudin-5 positive opened, disorganized marginal zone capillaries, original vascular sinusoids and blood accumulations were visible (Fig. 4D). The hemosiderin in the cytoplasms of the siderocytes showed false positivity for claudin-5. In cases of the organisation samples we detected a strong immunoreactivity of one layer endothelial cells of the original, regular sinusoids of the red pulp, which were compressed by the granulation tissues, as well as the newly formed angiogenetic vessels. In the splenic hematoma with fibroblastic organisation specimens the stromal fibroblasts were negative for claudin-5. Van Gieson stained the abundant collagen fibers in the splenic hematoma with fibroblastic organisation specimens red and the Masson's trichrome stained the collagen fibers blue.

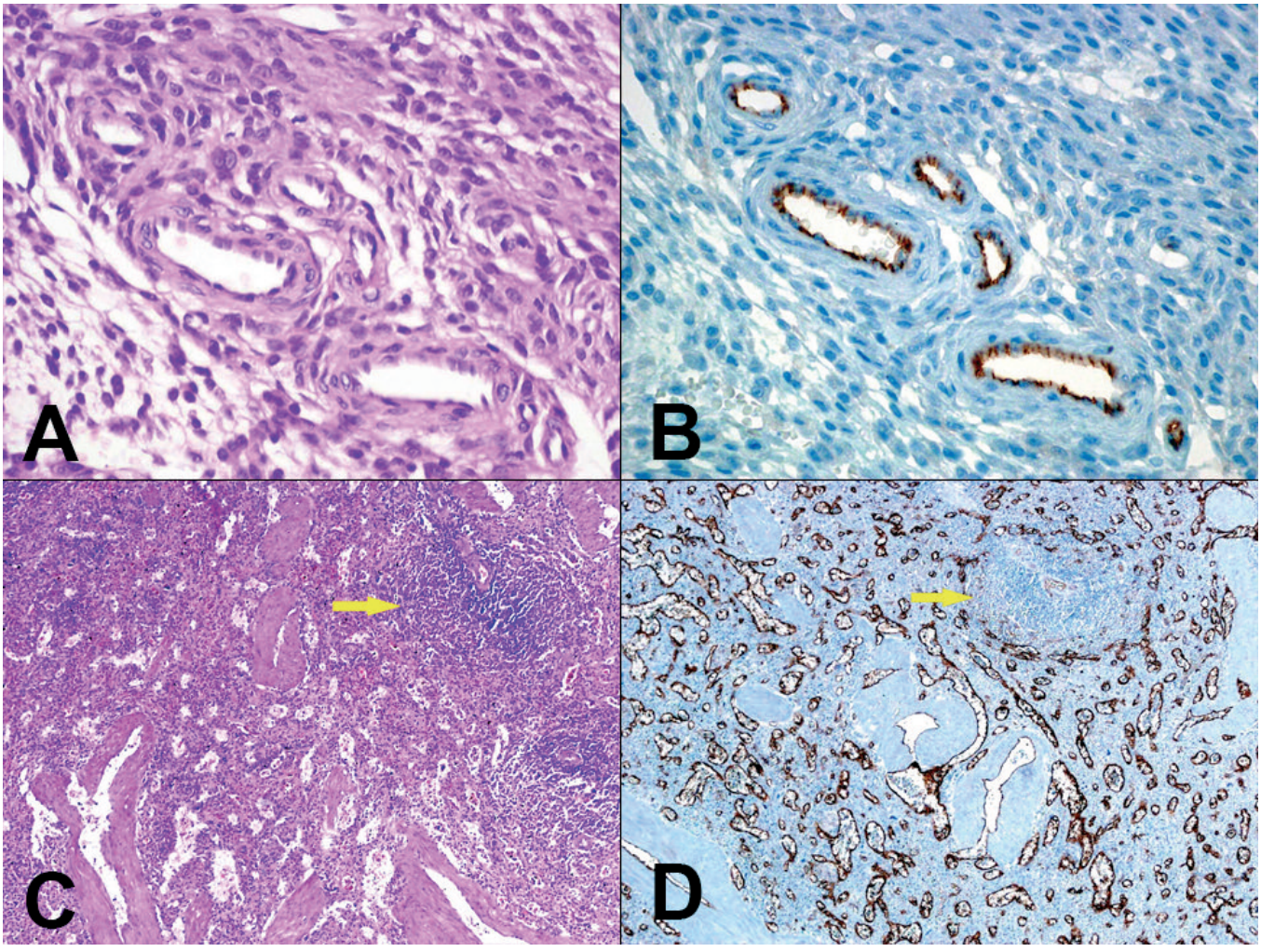

Fig 3. Canine hemangiopericytoma and spleen. **A.** Canine hemangiopericytoma from the skin of a 9 year old, female Hungarian Vizsla. The proliferating spindle shaped cells with elongated nuclei encircle small vessels. HE. **B.** The hemangiopericytoma cells located around the central collapsed vessels proved to be negative for claudin-5, while the endothelial cells showed an intense positive immunoreactivity. **C.** Normal canine spleen from a 6 year old mixed breed male dog. The yellow arrow points at a periarterial lymphatic sheath with central artery. HE. **D.** The endothelial cells of the sinusoids, trabecular and central arteries, and sheathed capillares of the normal canine spleen showed intense immunoreactivity for claudin-5. Periarterial lymphatic sheath with central artery (yellow arrow). A, B, x 60; C, D, x 10.

Immunohistochemical analysis of non-claudin-5 antibodies

In the HSAs, hemangiomas, and cutaneous lymphangiomas the CD31 reacted specifically with endothelial cells. The immunreactivity of CD31 was strong. Immunohistochemically 18/26 HSA samples were positive, however, 8/26 from total HSAs were negative for von Willebrand's factor (vWf). All hemangiomas, and cutaneous lymphangiomas were stained for vWf. Vimentin was identified in HSA-hemangioma - cutaneous lymphangioma cells, as well as other mesenchymal components of the sections. The cytoplasm of the neoplastic endothelial cells showed patchy staining for vimentin. The HSA cells were negative for α-SMA, NSE, and S-100. The neoplastic cells of the hemangiopericytomas were positive for vimentin, and α-SMA. In the hemangiopericytomas samples the differentiated endothelial cells of the central collapsed capillaries showed intense immunoreactivity for CD31. The malignant mesenchymal tumours including fibrosarcomas, myxosarcomas, leiomyosarcomas, cardiac rhabdomyosarcomas, malignant peripheral nerve sheath tumours, synovial sarcomas, osteosarcomas and chondrosarcomas were positive for vimentin. The fibrosarcomas cells were negative for

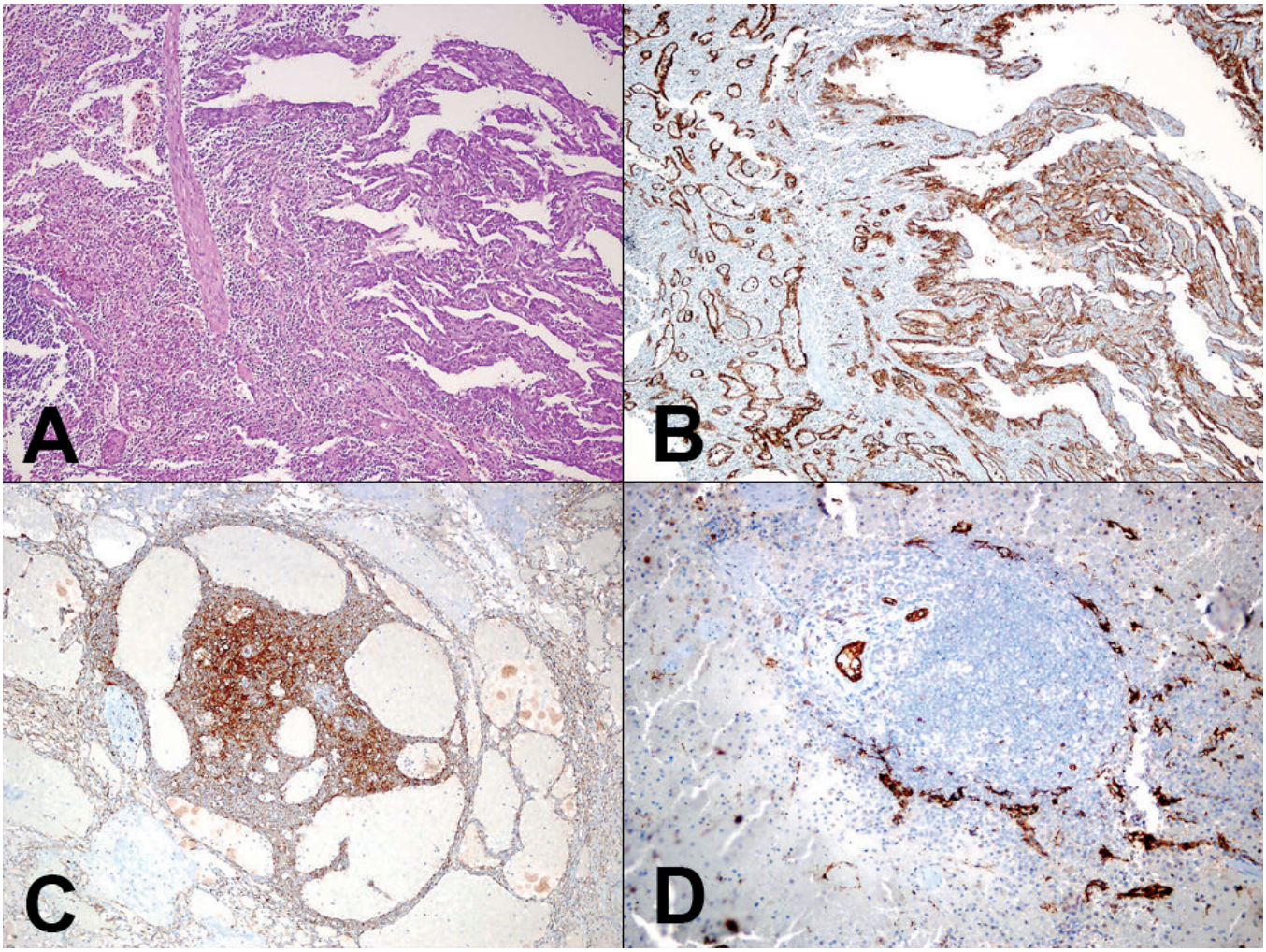

Fig 4. Splenic HSA and hematomas. **A.** Canine splenic HSA from an 11 year old mixed breed male dog. At the right side of the picture HSA cells can be seen, while the left side of the picture is representative of the normal splenic parenchyma. HE. **B.** Strong membrane positivity for claudin-5 in the non-neoplastic endothelial cells of the sinusoids (internal positive control) of the red pulp (left side of the picture) and strong positive HSAs cells (right side). The picture emphasizes the evident differences between the histological features of the non-neoplastic and neoplastic proliferating endothelial. **C.** Canine splenic hematoma induced by HSA from a 10 year old German Shepherd male dog, with a strong membrane positivity for claudin-5 in the neoplastic endothelial cells. **D.** Canine acute splenic hematoma induced by lymphoid hyperplastic nodules from an 11 year old mixed breed female dog. In the picture the hyperplastic lymphoid nodule and strong membrane-bound positivity of endothelial cells of capillares at the marginale zone for claudin-5 are seen. A-C, x 10; D, x 20.

Table 1. The specifity of claudin-5 in neoplastic cells in different canine tumours.

Sample	Number	Biopsy	Necropsy	vWf	CD31	Claudin-5
1. HSAs capillary	4	2	2	Pos=4	Pos=4	Pos=4
2. HSAs cavernous	11	9	2	Pos=8; Neg=3	Pos=11	Pos=11
3. HSAs solid	11	5	6	Pos=6; Neg=5	Pos=11	Pos=11
4. Hemangiomas (5 cavernous, 1 capillary)	6	6	-	Pos=6	Pos=6	Pos=6
5. Lymphangiomas (cavernous)	3	3	-	Pos=3	Pos=3	Pos=3
6. Hemangiopericytomas	7	7	-	Neg	Neg	Neg
7. Fibrosarcomas	11	11	-	Neg	Neg	Neg
8. Myxosarcomas	3	3	-	Neg	Neg	Neg
9. Leiomyosarcomas	3	-	3	Neg	Neg	Neg
10. Cardiac rhabdomyosarcomas	3	-	3	Neg	Neg	Neg
11. MPNSTs	3	2	1	Neg	Neg	Neg
12. Spindle cell melanomas	4	4	-	Neg	Neg	Neg
13. Synovial sarcomas	4	2	2	Neg	Neg	Neg
14. Osteosarcomas	5	-	5	Neg	Neg	Neg
15. Chondrosarcomas	4	4	-	Neg	Neg	Neg
16. Benign fibroblast proliferations	5	5	-	Neg	Neg	Neg
17. Vaginal leiomyomas	5	1	4	Neg	Neg	Neg
Totally	92	54	28			

Table 2. Result of immunostaining for CD31, and claudin-5 in canine capillary, cavernous and solid type HSAs.

Antibodies	Capillary and cavernous types HSA	Solid type HSA
CD31	4,88 (4,7 - 5)*	4,44 (4,1-4,7)**
Claudin-5	4,96 (4,9 - 5)**	4,84 (4,7 - 5)**

*: Average score for CD31 positivity; **: Average score for claudin-5 positivity.

CD31. The fibrosarcomas, leiomyosarcomas, cardiac rhabdomyosarcomas, synovial sarcomas, and osteosarcomas did not show positive immunoreactivity for NSE, and S-100. The chondrosarcomas cells were positive for S-100, and negative for NSE. The leiomyosarcomas, and cardiac rhabdomyosarcomas neoplastic cells showed a positive immunoreactivity for α-SMA, and desmin. The malignant peripheral nerve sheath tumour cells were positive for NSE, and S-100. The benign fibroblast proliferations, and vaginal leiomyomas cells showed a positive immunoreactivity for vimentin, but these tumors were negative for NSE, and S-100. The α-SMA was expressed in the leiomyoma cells. The spindle cell melanoma cells were positive for S-100.

Claudin-5 protein is a stronger marker than CD31, and vWf for detection of HSAs in canines

It was reported in previous HSA-studies that immunohistochemical detection of the CD31 antigen had a higher value than vWf antigen (Ferrer et al., 1995). In the present study 69.23 % of HSAs were positive, and 30.77 % of HSAs were negative for vWf , and all expressed the CD31 and claudin-5 antigens. Claudin-5 staining seemed to be a stronger endothelial marker than vWF for the diagnosis of HSAs in canines (Table 1). We compared claudin-5 and CD31 staining within cavernous, capillary and solid types of HSA. In both types of HSAs the claudin-5 immunstainings were more homogenous, and stronger, than the CD31 immunreactivity.

The percentage of CD31 positive HSA cells was 70-100% (average score 4.88), while the percentage of claudin-5 positive tumour cells was 90-100% (average score 4.96) in cases of the cavernous and capillary type HSAs. In cases of solid type HSAs the percentage of CD31 positive HSA cells was 40-100% (average score 4.44), while the percentage of claudin-5 positive tumour cells was 90-100% (average score 4.84) (Table 2). Both endothelial markers showed membrane staining, but in all canine HSA samples the claudin-5 immunreactivity was stronger than the CD31 intensity. The difference between the two methods was significant in both cases ($P<0.001$ and $P=0.005$ respectively).

We analysed claudin-5 protein, and CD31 staining within four canine normal spleen samples. CD31 was expressed on all continous endothelia, including those of trabecular arteries and central arteries, but it was not expressed on discontinous endothelium of the sinusoids of the red pulp and on the endothelial cells of the capillaries. In addition, CD31 was expressed on the surface of megakaryocytes, macrophages and some subsets of lymphocytes. These cells showed predominantly staining of the cell membrane, with weaker cytoplasmic staining. The claudin-5 protein was expressed on all continous endothelia of arteries, capillaries and on all discontinous endothelia of the sinusoids of the canine spleen, but it was not expressed on the megakaryocytes, macrophages, or lymphocytes.

We analysed 10 canine HSA samples (5 cavernous, 4 solid, 1 capillary types; 5 skin, 5 spleen biopsy samples from different veterinary clinics) which were fixed in 15% non-neutral, non-buffered formalin for 1-2 weeks at room temperature. All overfixed HSA samples showed a strong membrane-bound positivity for claudin-5 protein, but 8 HSA samples from 10 were negative for CD31, 1 HSA sample was positive and 1 HSA sample showed weak, scattered positivity for PECAM-1.

Discussion

Immunohistochemistry has become a practical and widely used tool for diagnosis in human pathology since the 70's. However, its application in veterinary diagnostic pathology has not been so common, especially due to the lack of specific antibodies. To overcome this drawback, antibodies which present cross reactivity with human and animal antigens have been applied (Frost et al., 2000; Rhind, 2002). Veterinary medicine has progressively improved its therapeutic spectrum, especially in oncology of canines, demanding a more accurate diagnosis. This fact has come to the attention of veterinary pathologists to improve the diagnostic value of immunohistochemistry in their daily routine, following the development of human diagnostic pathology.

HSA occurs most frequently in older dogs, most commonly in the spleen, right atrium, or subcutis (Withrow and MacEwen, 2001). Von Beust et al. (1988) analysed 36 canine HSAs and 47 canine hemangiomas for the factor VIII-related antigen. The primary antibody was rabbit anti-human (r/h) F VIII Rag antiserum. All hemangiomas and 89% of 36 HSAs were stained for F VIII Rag. Four out of 36 HSAs were negative in equivocal staining with vWf (Von Beust et al., 1988). Ferrer et al. (1995) reported that eleven of 15 HSAs were positive for vWf and all expressed the CD31 antigen. The result of this study showed that immunohistochemical detection of the CD31 antigen had a higher value than vWf antigen, as was reported in previous studies (Ferrer et al., 1995). Moore et al. (1989) reported that canine HSAs are positive for vimentin, but not for other intermediate filaments (Moore et al., 1989). Several others case reports described the vimentin positivity in the HSA tumor cells (Hayden et al., 1992; Wang and Su, 2001). In early studies the cell surface glycoconjugate expression of endothelial cells in canine cutaneous hemangiomas and HSAs was compared to normal cutaneous endothelial cells using eight different lectins in an indirect immunoperoxidase technique (Augustin-Voss et al., 1990). Fosmire et al. (2004) described the characteristics of eight independent endothelial cell lines derived from explants of canine HSA. Their result suggested that expression of CD117 (c-Kit) may offer a sensitive way to distinguish benign canine splenic hematoma (negative) from malignant canine HSA (positive), at least in the spleen. Their results showed that HSA cell lines retain expression of endothelial-associated antigens, including CD31, CD146, CD105 and CD51/CD61 (Fosmire et al., 2004). Yonemaru et al. (2006) investigated by immunohistochemical analysis the expression of vascular endothelial growth factor (VEGF), its receptor (flt-1 and flk-1), and basic fibroblast factor (bFGF) in canine HSAs and hemangiomas. VEGF, bFGF, flt-1 and flk-1 were immunohistochemically detected in the neoplastic cells in HSA, and the staining intensity (cytoplasmic immunoreactivity) was stronger in HSAs than in hemangiomas. Their results suggested that the expression of both the growth factor and their receptor might be associated with the malignant proliferation of HSAs. For evaluation of the cell growth fraction, immunolabeling of Ki-67 was performed. The Ki-67 proliferative index of the HSA samples ranged from 8.4% to 57.4% (Yonemaru et al., 2006).

Severe malignant spindle cell tumour, including fibro-, myxo-, leiomyo-, rhabdomyo-, neurofibro-, and other poorly differentiated sarcomas with hemorrhage, or massive neovascularizations may resemble hemangiosarcoma, but only genuine endothelial tumours form the vascular channels themselves. In all others cases the blood vessels have a detectable endothelial lining that is distinct from the tumour cells. The most frequently used immunomarkers for animals in the routine diagnosis are polyclonal, as they contain antibodies to a wider range of epitopes, increasing sensitivity of the method, sometimes in detriment of specificity (Frost et al., 2000; Rhind, 2000; Shi et al., 2001; Ruiz et al., 2005). In our study, we detected that the anti-human monoclonal anti-claudin-5 antibody showed cross reactivity with canine antigen, and first in veterinary medicine the claudin-5 immunohistochemistry was used to distinguish between the solid HSA-like spindle cell canine malignant neoplasias including fibro-, myxo-, leiomyo-, rhabdomyo-, neurofibro-, synovial-, osteo-, chondrosarcomas and HSAs. The canine non-vascular mesenchymal tumours were negative except for the stromal vessels, which showed strong positivity, and later were used as internal positive controls. The claudin-5 is a vascular-specific claudin molecule, which is a transmembrane protein reported to be primarily present in tight junctions of endothelia (Morita et al., 1999; Amasheh et al., 2005). Several human studies analyzed the role of the claudin-5 in different pathological processes (Nitta et al., 2003; Kanda et al., 2004; Martin et al., 2006). Claudin-5 expression has been detected in the heart, brain, lungs, kidney, testis, and intestine of mouse embryos (Morita et al., 1999), in the liver, stomach, small and large intestines of rats (Rahner et al., 2001), in the retinal pigment epithelium of the chick eye, and airway of human (Kojima et al., 2002). Claudin-5 localization was also reported in the endothelium of the microvessels in the rat testis, where it forms blood-testis barrier (Kamimura et al., 2002). In our earlier studies we have investigated the microvessel density (MVD) in canine mammary tumours by quantitative CLDN-5 molecule immunohistochemistry

(Jakab et al., 2008b), and claudin proteins expression and localization in the normal canine mammary gland (Jakab et al., 2008a).

Since claudin-5 immunohistochemistry proved to be positive in all lymphangioma samples, further studies are needed to investigate the claudin-5 molecule expression in canine lymphangiosarcomas. However, lymphangiosarcomas are distinguished from HSAs only by the absence of blood within the vascular lumina.

The most common diseases or conditions resulting in nodular splenomegaly with bloody consistency (splenic masses) in canine are: splenic hematomas, incompletely contracted areas of the spleen, vascular neoplasms, and acute splenic infarcts (McGavin and Zachary, 2007). During the macroscopic pathology and routine histolopathological analysis it is difficult to differentiate the canine splenic hematomas induced by lymphoid hyperplastic nodules of the white pulp, and the splenic hematoma induced by splenic hemangiomas, or HSAs. In our study we investigated 12 splenic hematomas induced by splenic HSAs, 3 splenic hematomas induced by splenic fibrosarcomas, 5 acute intrapulpar hematomas induced by lymphoid hyperplastic nodules, 5 fibroblastic organisations of splenic hematomas without tumoural lesions and 4 normal spleen samples. Using the claudin-5 immunohistochemistry we were able to unequivocally differentiate the splenic vascular tumours with hemorrhages from non-tumoural hematomas in the spleen of canines. In the HSA spleen samples we detected 4 cavernous, and 8 solid forms. The HSA cells showed a strong positivity for claudin-5 TJ-protein. In the cases of the splenic hematomas induced by fibrosarcomas the neoplastic cells were negative for claudin-5 but the endothelial cells of the nearby, compressed red pulp and the intratumoural microvessels showed a membrane-bound positivity for this protein. In the acute hematoma without splenic neoplasia samples we did not detect the irregeular proliferation of the HSA cells, only some preexisting sinusoids and blood accumulations. In the samples of organization we detected strong immunoreactivity of endothelial cells of the original, regular sinusoids of the red pulp, which were compressed by the granulation tissues, and the angiogenetic new vessels. We used the endothelia of the trabecular, central arteriies and sinusoids of the red pulp as internal positive controls. The original endothelial cell population of the intact spleen can be considered an internal positive control during the immunohistochemical investigations.

CD31 is a single chain type 1 transmembrane protein with a molecular mass of approximately 135 kDa, belonging to the immunoglobulin superfamily. A previous human study described that the CD31 is expressed on all continuous endothelia, including those of arteies, arterioles, venules, veins, and non-sinusoidal capillaries, but it is not expressed on discontinuous endothelium of the sinusoids of the splenic red pulp. In addition, this vascular marker is expressed on the surface and weaker in the cytoplams of human megakaryocytes, platelets, myeloid cells, natural killer cells, and some subsets of T-lymphocytes, as well as on B-cell precursors (Muller, 1997). In our study we described that the CD31 was expressed on all continous endothelia, including those of trabecular arteries and central arteries, but it was not expressed on discontinous endothelium of the sinusoids of the red pulp or on the endothelial cells of the capillaries in canine inact spleen. In addition, CD31 was expressed on the surfaces of megakaryocytes, macrophages and some subsets of lymphocytes. These cells showed predominantly staining of the cell membrane, with weaker cytoplasmic staining. The claudin-5 protein, as a harder vascular marker, was expressed on all continous endothelia of arteries, capillaries and on all discontinous endothelia of the sinusoids of the canine spleen, but it was not expressed on the megakaryocytes, macrophages, and lymphocytes.
Immunohistochemistry is based on the binding of antibodies to a specific antigen in tissue sections. The most common immunoglobulin used in immunohistochemistry is IgG; IgM is less commonly used (Frost et al., 2000; Ramos-Vara, 2005; Rhind, 2002; Ruiz et al., 2005). Formaldehyde is the gold standard of fixatives for routine histology and immunohistochemistry. Formaldehyde preserves mainly peptides and the general structure of cellular organelles (Ramos-Vara, 2005). It also interacts with nucleic acids but has little effect on carbohydrates. In solution, formaldehyde is capable of binding the following amino acids: lysine, tyrosine, asparagines, histidine, arginine, cysteine, and glutamine (Frost et al., 2000; Shi et al., 2000). The basic mechanism of fixation with formaldehyde is the formation of additional products between the formalin and uncharged reactive amino gruops, forming cross-links (Frost et al., 2000; Rhind, 2002; Ramos-Vara, 2005; Ruiz et al., 2005). The final result of formaldehyde fixation is a profound change in the conformation of macromolecules, which could make the recognition of proteins and antigens by antibodies impossible or at best difficult. Overfixation can produce false negative results in immunohistochemistry from excessive cross-links and is deleterious for the immunreactivity of antigenes (Frost et al., 2000; Montero, 2003). The pH of a fixative buffer dramatically influences the degree of cross-links. The effect of prolonged fixation with formaldehyde on an antigen located in different cell compartments can vary. The duration of formalin fixation can alter immunohistochemical reaction, resulting in failure to detect an antigen, weak reaction (Frost et al., 2000; Montero, 2003; Ramos-Vara, 2005).

At veterinary clinics the irregular formalin fixation of the different biopsies from domestic animals is a frequently occuring problem. The veterinary doctors usually use non-buffered, overconcentrated formaldehyde solution, with abnormally long duration (1-2 weeks). The overfixation frequently causes false negative results in immunohistochemistry from

excessive cross-links in different biopsy samples from dogs and cats. In addition, the long duration of formalin conservation alters the immunohistochemical results. In our work we investigated 10 overfixed canine HSA biopsy samples from different small animal clinics. All HSA samples showed a strong membrane-bound positivity for claudin-5 protein, but 8 out of 10 HSA samples were negative for CD31, 1 HSA sample was positive and 1 HSA sample showed weak, scattered positivity for CD31. All canine HSA samples that were fixed in 8% neutral buffered formalin for 24 hours at room temperature showed positive immunhistochemical reaction for CD31. This means that the claudin-5 protein is a better marker for immunhistochemical investigation of irregular formalin overfixed canine HSA samples than the CD31 vascular marker.

In the present study we compared the immunoreactivity of positive neoplastic endothelial cells of endothelial markers including claudin-5 protein, CD31, and vWF within HSAs. We established, as in previous studies (Ferrer et al., 1995) that CD31 is a better endothelial marker than vWF for detection of malignant endothelial cells in canine HSAs, but claudin-5 is a stronger vascular marker than CD31.

In the present study it was confirmed that anti-claudin-5 antibody produced for use in human histopathology might be applied in veterinary pathology, especially in canine pathology. In summary, our results show that the claudin-5 protein could be used as a new marker with greater sensitivity as compared to vWf, and CD31, and could also be of a diagnostic value in the differential diagnosis of canine HSAs from other sarcomas with hemorrhages or increased vascularisation. In the non-vascular canine tumours the endothelial cells of intra-, and peritumoural vessels stained very strongly for claudin-5, which was easy to use as an internal positive control. Moreover, claudin-5 can help with other immunhistochemical markers, to differentiate between splenic hematomas induced by splenic hemangiomas, or HSAs, and the canine splenic hematomas induced by non-neoplastic and non-vascular neoplastic splenic lesions in canines. The result of this study showed that immunohistochemical detection of the claudin-5 protein had a higher sensitivity, especially regarding solid type, poorly differentiated tumours than CD31 and vWf antigen in case of canine HSAs.

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