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NEOPLASTIC DISEASE

Immunohistochemical Detection of Urokinase Plasminogen Activator and Urokinase Plasminogen Activator Receptor in Canine Vascular Endothelial Tumours

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Summary

Immunohistochemistry was used to assess the expression of urokinase plasminogen activator (uPA) and uPA receptor (uPAR) in 57 canine primary haemangiosarcomas (HSAs), 26 canine cutaneous haemangiomas (HAs) and in control sections of canine cutaneous granulation tissue. The correlation between uPA/uPAR expression and the Ki67 labelling index (LI) was estimated in the HSA and HA tissues. uPA was expressed by 73.2% and 75.0% of splenic HSAs and non-splenic HSAs, respectively. All HSA tissues tested expressed uPAR. Expression of both molecules was significantly higher in HSAs than in cutaneous HAs (3.8% for uPA and 30.7% for uPAR). The average Ki67 LI of the $uPA^+/uPAR^+$ HSAs was significantly higher than that of $uPA^-/uPAR^+$ HSAs and HA tissues (mean ± SDs 32.8 ± 15.3, 15.2 ± 7.2 and 2.1 ± 0.7, respectively; $P < 0.05$). These results suggest that uPA and uPAR play a significant role in the malignant proliferation of canine HSA, regardless of the primary origin of the tumour.

Keywords: dog; haemangioma; haemangiosarcoma; urokinase plasminogen activator

The serine protease urokinase plasminogen activator (uPA) and the uPA membrane receptor (uPAR; also known as CD87) are involved in many physiological and pathological processes, including angiogenesis and the growth and spread of tumours (Raghu *et al.*, 2010). Interaction of uPA with uPAR facilitates extravasation and intravasation of cancer cells by regulating local proteolysis and attachment of the cells to components of the extracellular matrix (ECM). Moreover, the uPA/uPAR system is also implicated in proliferation of some tumour cells and migration of tumour and endothelial cells (ECs) (Alexander *et al.*, 2012; Uhrin and Breuss, 2013). uPAR reacts with neighbouring receptors, such as low-density lipoprotein receptor-related protein, integrins, epidermal growth factor receptor or platelet-derived growth factor receptor. It may also affect the proliferation and survival of angiogenic ECs (Prager *et al.*, 2004). The neoplastic endothelial cells in haemangiosarcoma (HSA) are known to secrete growth factors, their receptors and cytokines, all of which promote angiogenesis (Clifford *et al.*, 2001; Yu *et al.*, 2001; Yonemaru *et al.*, 2006; Asa *et al.*, 2012; Kim *et al.*, 2014) and are similar to those molecules associated with the activity of non-neoplastic ECs during angiogenesis. The aim of the present study was to investigate the expression patterns of uPA and uPAR in canine splenic and non-splenic HSAs, cutaneous haemangioma (HA) and canine granulation tissue as models of

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pathophysiological angiogenesis. The correlation between uPA/uPAR expression and the Ki67 labelling index (LI) was also determined in the HSAs and HAs.

Archival formalin-fixed and paraffin wax-embedded tissue samples from the Department of Veterinary Pathology, Gifu University, were selected for this study. Primary HSAs from a total of 57 dogs were examined. The HSA tissues were divided into two groups according to their primary locations: splenic HSAs ($n = 41$) and non-splenic HSAs ($n = 16$). The primary sites of the non-splenic HSAs were the skin ($n = 8$), liver ($n = 3$), intra-abdominal masses of unknown origin ($n = 2$) and the kidney, adrenal gland and testis ($n = 1$ each). Twenty-six cases of cutaneous HA were examined as well as cutaneous granulation tissue from five dogs. The diagnosis of EC neoplasia had been confirmed in all cases of HSA and HA by immunohistochemical expression of von Willebrand factor and CD31.

All tissues were subjected to immunohistochemistry (IHC) with the following primary antibodies: goat polyclonal anti-uPA (M-20, sc-6831; Santa Cruz Biotechnology, Santa Cruz, California, USA) at a dilution of 1 in 2,000; rabbit polyclonal anti-uPAR (Biorbyt LLC., San Francisco, California, USA) at a dilution of 1 in 500; and mouse anti-Ki67 antigen (clone MIB-1, M7240; Dako, Carpinteria, California, USA) at a dilution of 1 in 50. Secondary identification of these reagents was performed using the EnVision + System-HRP labelled polymer (Dako) and labelling was 'visualized' with the Liquid DAB + Substrate Chromogen System (Dako). Antigen retrieval was performed by immersing the sections in target retrieval solution (pH 6.0, Dako) and heating them at 121°C for 5 min in an autoclave. Normal canine kidney and skin tissues were employed as positive controls, as normal renal tubular cells and epidermal keratinocytes are known to express both uPA and uPAR. Negative controls included sections that were treated with phosphate buffered saline instead of the primary antibodies.

The percentage of neoplastic cells that were positive for uPA and uPAR was determined by counting 1,000 cells from each of 10 high-power (×400) fields. Tumours were considered negative when <10% of the neoplastic cells expressed both uPA and uPAR. Positive labelling was scored as follows: 1+, 10–25% positive cells; 2+, 26–75% positive cells; and 3+, >75% positive cells (Nakanishi *et al.*, 1998). The Ki67 LI was determined as the percentage of positive cells in a minimum of 1,000 neoplastic cells in 10 high-power fields. The data were analyzed by chi-square test, the Kruskal–Wallis test and the Steel–Dwass method. $P < 0.05$ was considered significant for all tests.

In normal skin, macrophages, mast cells, smooth muscle cells and fibroblasts exhibited varying degrees of uPA expression. uPAR immunoreactivity was also detected in macrophages and fibroblasts, but the number of uPAR-positive cells was less than the number of uPA-positive cells. In vascular walls, smooth muscle cells expressed both uPA and uPAR; however,

Table 1
Summary of immunohistochemical studies of splenic and non-splenic haemangiosarcomas and cutaneous haemangiomas

Score	*Haemangiosarcomas* (n = *57*)				*Haemangiomas* (n = *26*)	
	Splenic (n = *41*)		*Non-splenic* (n = *16*)			
	uPA	*uPAR*	*uPA*	*uPAR*	*uPA*	*uPAR*
−	11	0	4	0	25	18
1+	16	8	6	6	1	4
2+	7	15	4	5	0	2
3+	7	18	2	5	0	2
+ cases (%)	73.2 ($n = 30$)*	100 ($n = 41$)*	75.0 ($n = 12$)*	100 ($n = 16$)*	3.8 ($n = 1$)	30.7 ($n = 8$)
uPA/uPAR expression	*Average Ki67 LI (% ± SD)*					
+/+	36.3 ± 13.9†		23.9 ± 15.8†		2.1 ± 0.7	
−/+	17.0 ± 6.5		10.4 ± 7.6			
+/−	0		0			
−/−	0		0			

Tissues were subject to IHC to determine expression of urokinase plasminogen activator (uPA), uPA receptor (uPAR) and Ki67. Scores for uPA and uPAR expression as well as the Ki67 labelling index (LI) are shown.
Scores are: −, <10% of tumour cells labelled; 1+, 10–25% of tumour cells labelled; 2+, 25–75% of tumour cells labelled; 3+, >75% of tumour cells labelled.
*Significantly higher than that of HA ($P < 0.05$).
†Significantly higher than for $uPA^-/uPAR^+$ HSAs and HAs ($P < 0.05$).

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