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Short communication

Evaluation of CD25-positive cells in relation to the subtypes and prognoses in various lymphoid tumours in dogs

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ABSTRACT

Interleukin-2 receptor alpha chain (CD25) expression has been reported in human lymphoid tumours and suggested to correlate with the prognosis. In this study, we detected CD25-positive cells in various types of lymphoid tumours in dogs. Immunohistochemical analyses of the tissues from diffuse large B-cell lymphoma (DLBCL) ($n=6$), T-zone lymphoma (TZL) ($n=5$), and follicular lymphoma (FL) ($n=2$) revealed that cells strongly positive for CD25 were observed generally in accordance with lymphoma cell localization. CD25-positive cells were consistently detected in TZL and FL cases; however, the number of CD25-positive cells was variable among DLBCL cases. Furthermore, we evaluated the rate of CD25-positive cells by flow cytometric analysis in 29 dogs with lymphoid malignancies, including high-grade B-cell lymphoma ($n=17$), TZL ($n=5$), FL ($n=2$), cutaneous lymphoma ($n=2$), and acute lymphoblastic leukaemia (ALL) ($n=3$). CD25-positivity in the lymph node cells was significantly higher in dogs with high-grade B-cell lymphoma (mean ± SD, 49.6 ± 31.3%) or TZL (mean ± SD, 80.2 ± 10.0%) than that in healthy dogs (mean ± SD, 9.8 ± 2.8%). In prognostic analysis of 15 cases with high-grade B-cell lymphoma, the progression-free survival was significantly shorter in CD25-high group than that in CD25-low group. The results obtained in this study are useful for subtype differentiation and prognostic analysis of canine lymphomas and future development of molecular-targeted therapy directed at CD25.

1. Introduction

Interleukin (IL)-2 receptor alpha chain (CD25) is a component of IL-2 receptor in conjunction with two other subunits, beta and gamma chains. Heterotrimerization of these subunits leads to high-affinity binding to IL-2, which induces proliferation and maturation of lymphocytes (Lowenthal et al., 1985) .

In human medicine, CD25 expression has been reported in activated normal T cells and B- and T-cell neoplasms, whereas only <5% of unstimulated peripheral blood T cells were CD25-positive (Robb et al., 1981; Sheibani et al., 1987). Particularly intense expression of CD25 was observed in blast cells in adult T-cell leukaemia (ATL) or hairy-cell leukaemia patients (Sheibani et al., 1987; Horiuchi et al., 1997). Expression of CD25 has also been demonstrated in B-cell chronic lymphocytic leukaemia, acute lymphoblastic leukaemia (ALL) (Burton and Kay, 1994; Nakase et al., 1994a,b), Hodgkin's lymphoma, and non-Hodgkin's lymphoma (Tesch et al., 1993; Nakase et al., 1994a). Recently, it was reported that CD25-positivity was associated with poor response rates and inferior progression-free survival (PFS) in diffuse large B-cell lymphoma (DLBCL) or follicular lymphoma (FL), and expected to constitute a new prognostic marker (Fujiwara et al., 2013, 2014).

Similar to humans, canine CD25 is known as an important marker of activated lymphocytes (Helfand et al., 1992; Galkowska et al., 1996). Although messenger RNA (mRNA) expression of CD25 has also been reported in the cells derived from canine lymphoma and leukaemia patients (Dickerson et al., 2002), clinical significance of CD25-positivity has not been explored in lymphoid tumours in dogs.

In the present study, we detected CD25-positive cells in various subtypes of canine lymphoma and ALL by using immunohistochemistry (IHC) and flow cytometry (FCM). Furthermore, we

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analysed the relationship between CD25-positivity and the prognoses for dogs with high-grade B-cell lymphoma.

2. Materials and methods

2.1. Samples for IHC

Thirteen dogs with enlarged peripheral lymph nodes who were referred to the Veterinary Medical Center of the University of Tokyo, or whose tissue samples were subjected to histopathological examination at NORTH LAB Ltd. were diagnosed as DLBCL ($n = 6$), TZL ($n = 5$), and FL ($n = 2$) based on the histopathological findings of the biopsy specimens by referring World Health Organization (WHO) histological classification system for haematopoietic tumours of domestic animals (Valli et al., 2011). These tissue samples were further subjected to IHC to examine the presence of CD25-positive cells. As controls, lymph node specimens from seven healthy dogs and six dogs with reactive lymphadenopathy kept for experimental purposes were also subjected to this analysis. The procedures were conducted in accordance with the guidelines of the Animal Care Committee of the Graduate School of Agricultural and Life Sciences, The University of Tokyo.

2.2. IHC

Sections of formalin-fixed paraffin-embedded tissues (4 μm) were prepared using the following protocol. The sections were de-waxed, rehydrated, and subjected to antigen retrieval in 1% citrate buffer solution (pH 6.0) in an autoclave at 120 °C for 15 min. Endogenous peroxidase was inactivated by 1% hydrogen peroxide in methanol for 30 min, and blocking was processed by 8% skim milk in Tris-buffered saline (TBS) for 40 min. The slides were then incubated overnight at 4 °C with the panel of anti-CD3 (polyclonal rabbit anti-human A0452; DAKO, Glostrup, Denmark; 1:50 dilution), anti-CD20 (polyclonal rabbit anti-human RB-9013-P; Thermo Fisher Scientific, Waltham, MA, USA; 1:400 dilution), or anti-CD25 (monoclonal mouse anti-human, clone 4C9; Thermo Fisher Scientific; 1:40 dilution). After washing for 15 min in TBS, slides underwent reactions with EnVision + Dual Link System-HRP (DAKO) for 45 min at room temperature, were washed for 15 min, and visualized using 3,3′-diaminobenzidine (DAB) as a chromogen. Slides were counterstained in Mayer's hematoxylin.

In six DLBCL cases, the number of CD25-positive cells was counted in 1,000 tumour cells and the percentages of CD25-positive cells were calculated.

2.3. Samples for FCM analysis

Neoplastic cells were obtained from 29 dogs with lymphoid malignancies which were referred to the Veterinary Medical Center of the University of Tokyo. Samples were obtained from peripheral lymph nodes in 24 nodal lymphoma cases, cutaneous masses in two cutaneous lymphoma cases, and peripheral blood separated by Ficoll-Paque gradient centrifugation (Ficoll-Paque PLUS; GE Healthcare, Amersham Place, England) from three ALL cases at the initial diagnoses. Diagnosis was made based on clinical data and cytological/histological examination of lymph-node or bone marrow in dogs with lymphoma and leukaemia, respectively. PCR for antigen-receptor gene rearrangement (PARR) was performed to determine the clonality and cell lineage of 26 lymphoma and three ALL cases. DNA samples of all cases were extracted from fresh samples from the lymph node, peripheral blood, or bone marrow. Primers and protocols for PCR analyses were described previously (Burnett et al., 2003; Valli et al., 2006). Twenty six dogs with lymphomas were further classified into subtypes of high-grade B-cell lymphoma ($n = 17$), TZL ($n = 5$), FL ($n = 2$), or cutaneous lymphomas ($n = 2$) according to the cytology and/or histopathology (Fournel-Fleury et al., 1997, 2002; Valli et al., 2006, 2011). For comparison, samples were also obtained from peripheral lymph nodes from seven healthy dogs and six dogs with reactive lymphadenopathy.

2.4. FCM

Single-color FCM was performed for the cell samples obtained from dogs with lymphoid malignancies. Cell suspensions were washed with staining medium (phosphate-buffered saline supplemented with 5% foetal calf serum) and stained with fluorescein isothyocyanate (FITC)-conjugated anti-canine CD25 mAb (P4A10; eBioscience, San Diego, CA, USA) for 30 min at 4 °C. Cells were washed twice and analysed with a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA, USA). An FITC-conjugated mouse IgG_1 was used to analyse the background fluorescence intensity during each analysis. Lymphoma or leukaemic cells were gated from the forward- and side-scatter properties. A minimum of 10,000 events in the gated region was collected for each sample. The obtained data were analysed using FlowJo software (Tree Star, Ashland, OR, USA). The percentages of CD25-positive cells were calculated from the isotype-matched control for each sample.

2.5. Statistical analysis

The Mann–Whitney *U* test was used to compare the prognosis in dogs with DLBCL showing different CD25-positivity. PFS and overall survival (OS) were calculated according to the criteria proposed by the veterinary cooperative oncology group (Vail et al., 2010). PFS was calculated from the date of initial treatment to disease progression or death from any cause. OS was calculated from the date of initial treatment to death from any cause. Dogs that were still alive or lost to follow-up were censored at the date on which they were last known to be alive. PFS and OS were estimated by the Kaplan–Meier method, and the groups were compared by the log-rank test. The *p* values were two-sided and regarded as significant if $p < 0.05$. Data were analysed using StatMate software version IV for windows (ATMS, Tokyo, Japan).

3. Results and discussion

First we performed immunohistochemical analyses for six dogs with DLBCL, five dogs with TZL, two dogs with FL, seven healthy dogs, and six dogs with reactive lymphadenopathy to detect CD25-postive cells in the lymph node tissues. In seven healthy dogs and six dogs with reactive lymphadenopathy, reactivity to anti-CD25 antibody was generally weak in both CD3-positive and CD20-positive cells, regardless of lymph node regions (Fig. 1A, B). Less than 10% of the cells in the entire lymph node field were CD25-positive in these dog groups.

In the lymph nodes from five dogs with TZL, neoplastic T cells, which are small or intermediate in size, expanded in the paracortex, pressing the atrophic germinal centres against the outer sinus. Majority of the neoplastic CD3-positive cells were shown to be strongly immunopositive for CD25 (Fig. 2A–D). In the two FL cases, the majority of CD20-positive centrocytes in the follicles were shown to be strongly immunopositive for CD25 (Fig. 2E–H). In all six cases with DLBCL, neoplastic large B cells with uniformly large nuclei and scanty cytoplasm diffusely proliferated in the lymph nodes as a mixture of centroblastic cells with multiple nucleoli and immunoblastic cells with a single central prominent nucleolus. Unlike in TZL and FL, CD25-positivity in the lymph nodes of DLBCL was variable (Fig. 2I and J). The percentages of CD25-positive cells in lymph node of six cases with DLBCL were 0.6, 4.1, 42.9, 53.8, 67.3, and 78.0% (range: 0.6–78.0%). Morphological features