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The effects of vincristine sulfate on expression of galectin-3, Bcl-2, and carbohydrate structures specific for EEL, GSL-1, and RCA-1 lectins in bitches with naturally occurring canine transmissible venereal tumor

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Abstract: Vincristine sulfate is one of the most effective chemical agents used in canine transmissible venereal tumor (CTVT) chemotherapy. Its therapeutic effectiveness is pronounced and significant at the beginning of treatment. The present study was designed to investigate vincristine sulfate treatment and the relationship between CTVT tumor regression and apoptosis [estimated by TdT-mediated dUTP-biotin nick end labeling (TUNEL)], galectin-3 (H-160), Bcl-2 (N-9), and lectin [EEL (B-135), GSL I (L-1100), and RCA I (BA-0084)] expression using immunohistochemical staining in 20 bitches with naturally occurring CTVTs. Evaluation was semiquantitatively performed by HSCORE values using light microscopy. All observed staining was intracytoplasmic and increased endothelial staining was noted with the Bcl-2 marker in tumor vessels. Apoptosis, TUNEL, and lectin scoring was higher in vincristine sulfate-treated tumors than in untreated tumors, while expression of antiapoptotic factors galectin-3 and Bcl-2 were lower in treated than in untreated CTVTs. These results show that galectin-3, Bcl-2, and lectins (EEL, GSL-1, and RCA-1) are suitable indicators/markers for CTVT tumor regression after treatment with vincristine sulfate.

Key words: Transmissible venereal tumor, Bcl-2, galectin-3, vincristine sulfate, TUNEL, immunohistochemistry, lectins

1. Introduction

Canine transmissible venereal tumors (CTVTs) are most commonly found on the external genital organs. The tumor is spread in healthy dogs by direct contact with injured skin or mucous tissue (1). CTVTs are observed most often in young, stray dogs, particularly sexually active and intact dogs, and have been reported worldwide. Several treatment options are available for the tumor, although chemotherapy is considered the most effective (2). Vincristine sulfate is considered to be an efficient chemotherapeutic agent and complete remission can be expected. Regression of tumors is associated with the presence of large numbers of lymphocytes, plasma cells, and activated macrophages in the regressing tumors, suggesting a role for localized antibody-mediated control of CTVT and making vincristine sulfate a drug of choice. The Bcl-2 gene is a member of a rapidly expanding family of genes that regulates apoptosis and can be functionally characterized as an apoptosis-suppressing factor (3). It has been observed that CTVT samples overexpressed the Bcl-2 protein independent of the stage of tumor development (1). Galectin-3, a nonintegrin β-galactoside-binding lectin, seems to play an important role in cellular adhesion, inflammation, inhibition of apoptosis, and malignant transformation of tissue and cells (4). The present study was conducted to investigate the relationship between CTVT tumor regression by apoptosis in dogs treated with vincristine sulfate and the expression of galectin-3, Bcl-2, and lectins (EEL, GSL-I, RCA-I) as detected by immunohistochemical staining in regressing tumors.

2. Materials and methods

2.1. Tumor material

A total of 20 bitches, 2–12 years old (4.05 ± 0.52 years) and with naturally occurring CTVTs, were investigated at a local kennel (Karacabey, Bursa, Turkey) and the Faculty of Veterinary Medicine of Uludağ University (Bursa,

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Turkey). During the treatment period, the dogs were kept in Karacabey under uniform management conditions and were fed commercial feed and water ad libitum. CTVT was diagnosed and confirmed by clinical signs and cytological procedures. Throughout the treatment period, blood samples were taken from the cephalic vein of all dogs for hematological examination prior to the introduction of chemotherapy. Hematological examination was performed with an Abbott Cell-Dyn 3500 hematological analyzer (GML Inc., USA). Chemotherapy was discontinued when the packed cell volume (PCV) was less than 25% and the leukocyte count was below 3000/µL, when normal values were observed. For chemotherapy, vincristine sulfate (Vincristine DBL, Mayne Pharma Pty Ltd., Australia) was administered weekly as an infusion for 3 min via the intravenous route at a dose of 0.025 mg/kg after dilution with physiologic saline to a total volume of 10 mL. Treatment was continued until the total remission of the tumor or for a maximum period of 7 weeks. Before each vincristine sulfate application, the dogs were reevaluated clinically and macroscopically. Absence of growth in the vagina was considered as the recovery point. The tumor samples were collected prior to the first vincristine treatment (untreated samples) and after treatment with vincristine sulfate (treated samples). Treated tumor biopsy samples were collected 7 days after the first vincristine sulfate application. Both control and treated tumor samples were fixed overnight in 10% neutral phosphate-buffered formalin, dehydrated through a graded ethanol series, and then embedded in paraffin blocks and cut to 4 µm in thickness using a rotary microtome.

2.2. TUNEL for apoptosis

Specimen sections with a thickness of 4 µm were deparaffinized in xylene and rehydrated in graded alcohol (70%–100%). Apoptosis was detected by the TdT-mediated dUTP-biotin nick end labeling (TUNEL) method using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (S7100; Chemicon International, USA). The tissue sections were fixed in 1% paraformaldehyde [in phosphate buffered saline (PBS), pH 7.4] for 10 min at room temperature, washed twice in PBS for 5 min, and refixed in precooled ethanol/acetic acid (2/1) for 5 min at –20 °C. Thereafter, the sections were incubated with 3% H_2O_2 for 5 min at room temperature followed by a wash with PBS (2 × 5 min). Sections were incubated with equilibration buffer for at least 10 min at room temperature and then with working-strength TdT enzyme at 37 °C for 60 min, at which point the sections were agitated for 15 s at room temperature and then incubated for 10 min with working strength stop/wash buffer, followed by a wash with PBS (2 × 1 minute). Sections were then incubated with antidigoxigenin conjugate for 30 min at room temperature followed by a wash with PBS (4 × 2 minutes). Sections were stained with 3,3'-diaminobenzidine (DAB) solution, dehydrated, and mounted.

2.3. Galectin-3 and Bcl-2 immunohistochemistry

Sections of specimens were deparaffinized in xylene and rehydrated in graded alcohol (70%–100%). Slides were subjected to treatment with boiling water with 10% antigen retrieval solution (10 mM citrate buffer pH 6.0, LabVision, USA) followed by a 0.3% methanol-H_2O_2 solution for 30 min. After washing 3 times with PBS, the tissues were treated with 2% bovine albumin serum (BSA, Sigma) for 30 min to prevent nonspecific binding and then were incubated with antibodies to galectin-3 (H-160, Santa Cruz Biotechnology, USA) and Bcl-2 (N-9, Santa Cruz Biotechnology), each diluted to 1:200, for 30 min at room temperature. After incubation for 10 min with biotinylated secondary antibodies, they were incubated with an avidin biotin complex enzyme solution for 45 min, and DAB was applied as the chromogen for 5 min and hematoxylin was used for counter staining. Negative controls were performed by replacing both galectin-3 and Bcl-2 with PBS.

2.4. Histochemistry for lectins

Specimen sections with a thickness of 4 µm were deparaffinized in xylene and rehydrated in graded alcohol (70%–100%). Slices were treated with 0.1% (w/v) trypsin solution for 2 min at 37 °C followed by a 0.3% methanol-H_2O_2 solution for 30 min. After washing 3 times with PBS, the tissues were conjugated with 2% BSA (Sigma) for 30 min to prevent nonspecific binding and then were incubated separately with EEL (B-135), GSL I (L-1100), and RCA I (BA-0084) lectins (Vector, UK), each diluted to 1:200, for 1 h at room temperature. DAB was used as the chromogen and hematoxylin was used for counterstaining. Negative controls were performed by replacing the lectins with PBS.

2.5. Semiquantitative evaluation

Sections were semiquantitatively evaluated for TUNEL, Bcl-2, galectin-3, and lectin (EEL, GSL I, and RCA I) localization using a light microscope and selected areas were photographed. HSCORE values of TUNEL, Bcl-2, galectin-3, and lectin staining were scored in a semiquantitative manner and included both intensity and staining distribution patterns. Three different fields of each slide at 200× magnification were evaluated for immunohistochemical staining. Values were recorded as percentages of positively stained target cells in each of 4 intensity categories as follows: no staining (-); weak staining (+); moderate staining (++); strong staining (+++). For each tissue, an HSCORE value was derived by adding the percentages of cells that stained at each intensity category and multiplying that value by the weighted intensity of the staining using the formula HSCORE = Σ (I × PC), where I is the intensity score and PC is the corresponding percentage of the cells (5,6).

2.6. Statistical analysis

Data were analyzed using the Kolmogorov–Smirnov test and were not distributed normally; therefore, results were presented as medians with minimum–maximum values. For statistical analysis, the Wilcoxon matched-pairs signed-rank test was used with IBM SPSS Statistics 20 (IBM Corp., USA). $P < 0.05$ was accepted as significant.

3. Results

3.1. Clinical results

Clinical examination of the animals revealed serosanguineous-hemorrhagic exudate oozing from the vulva. The masses were located in the posterior vagina and protrusion of these masses was easily noticed upon examination of the vulva. The vulva bulged outwards in most cases because of the masses. Tumors had the characteristic appearance of transmissible venereal tumors (TVTs): cauliflower-shaped nodules of various sizes (0.5–6 cm in diameter), including ulcerated surfaces.

Complete tumor regression was observed in all cases after 2 to 7 injections (mean: 3.65 ± 0.29) of vincristine. In the second week after the first injection of vincristine, bleeding ceased and regression of tumor size was noticed. Tumors were clearly reduced in the third week after the second injection.

Leucopenia (13/20, 65%), neutropenia (3/20, 15%), lymphocytosis (5/20, 25%), thrombocytopenia (7/20, 35%), erythropenia, hemoglobinemia, and decreased PCV (8/20, 40%) values were observed in the vincristine-treated animals. The most common anemia type was normocytic-normochromic, noted in 5 cases, followed by 3 cases of the microcytic-normochromic type. Anorexia (7/20, 35%), diarrhea (3/20, 15%), weight loss (3/20, 15%), and generalized alopecia (8/20, 40%) were observed in some animals.

The cytological examination of the smears confirmed tumor cells as well as vaginal epithelial cells, erythrocytes, bacterial cells (in a few cases), neutrophils, and lymphocytes. TVT cells had round-to-ovoid hyperchromatic nuclei, marginal chromatin, and large, centrally located nucleoli. The cytoplasm was moderate in amount and eosinophilic. Bacteria, neutrophils, and erythrocytes were observed in smear samples taken from tumors with ulceration and superficial hemorrhage.

3.2. Laboratory results

TUNEL, Bcl-2, galectin-3, and lectin (EEL, GSL, RCA-1) staining shown by the corresponding HSCORE values in the treatment and control groups are given in the Table.

3.2.1. Apoptosis

The HSCORE value was higher in treated tumor cells than in untreated cells (Figure 1). Staining was intracytoplasmic and positive-stained cells were distributed randomly (Figures 2a and 2b) in both groups. Although the TUNEL HSCORE value of the treated group was 2.09-fold higher than in untreated tumors, there were no statistically significant differences between the groups ($P < 0.675$; Table).

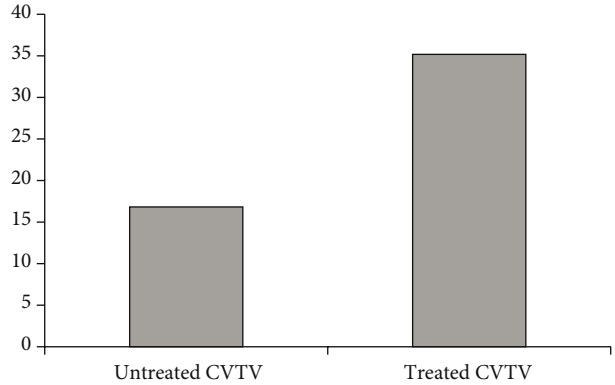

Figure 1. TUNEL HSCORE values in treated and untreated cells.

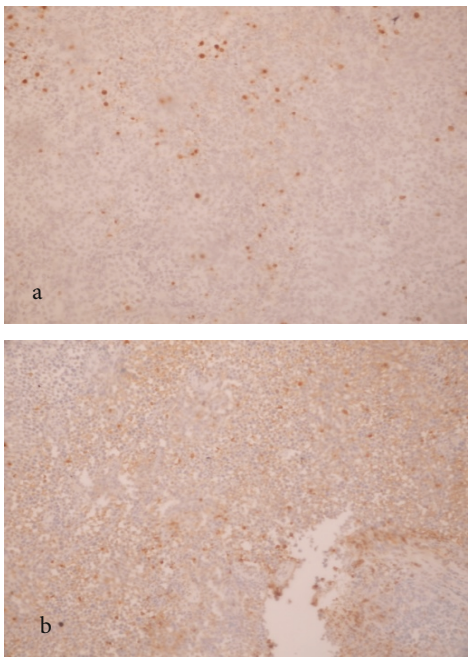

Figure 2. Intracytoplasmic stained positive cells showing lower scoring after treatment **(b)** compared with untreated tumor cells **(a)**. IHC 10× original magnification, DAB chromogen.

3.2.2. Galectin-3

Galectin-3 was significantly decreased (HSCORE median: 42.5 vs. 4) in cells from treated TVT tumors compared to untreated tumors (Figure 3). Expression was found predominantly in the cytoplasm in both groups, with frequent localization at the superficial surface of TVT tumoral cells. Galectin-3 labeling was stronger in untreated tumor cells compared to treated tumor cells (Figures 4a and 4b).

Table. Medians with minimum–maximum values of HSCOREs of TUNEL, Bcl-2, galectin-3, and lectin staining in tumor samples prior to the first vincristine treatments (controls) and tumor tissue after first week vincristine treatments. Bold numbers in P-value column are ≤0.05.

	N	Before	After	P-value
TUNEL	39	6 (0–210)	6 (1–270)	0.675
BCL-2	18	90 (60–240)	50 (20–180)	**0.030**
GAL-3	18	42.5 (5–120)	4 (1–100)	**0.007**
EEL	18	0 (0–80)	15 (0–300)	**0.030**
GSL	18	105 (20–240)	180 (30–300)	**0.002**
RCA	18	50 (5–300)	65 (20–210)	0.678

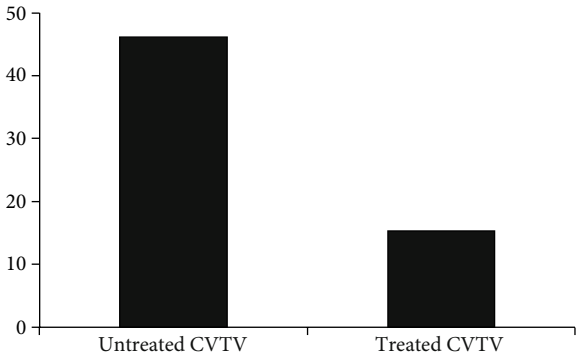

Figure 3. Decreased galectin-3 in treated TVT tumoral cells.

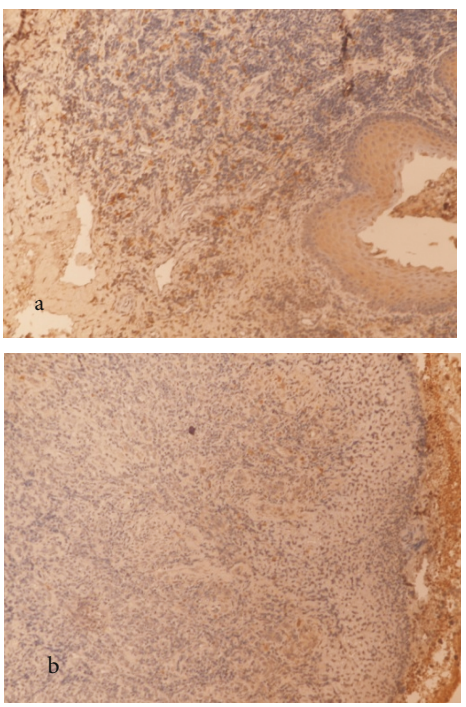

Figure 4. Strong staining with galectin-3 in untreated tumor cells **(a)** compared to treated tumor cells **(b)**. IHC 20× original magnification, DAB chromogen.

3.2.3. Bcl-2

The control group had higher HSCORE values than the treated group, reflecting higher Bcl-2 antiapoptosis activity (Figure 5). After the first week of treatment, the Bcl-2 HSCORE was reduced 2.25-fold ($P < 0.03$). Bcl-2 labeling showed an intracytoplasmic pattern in tumor cells. The distribution of Bcl-2 staining varied from limited to widespread in microscopic areas in both groups. Strong positive Bcl-2 immunoreactivity was seen in untreated TVT cells and was also noted in blood vessels in the treated TVT group (Figures 6a and 6b).

3.2.4. Lectins

Lectin HSCORE values are shown in Figure 7. In lectin staining, galactose-specific *Euonymus europaeus* lectin (EEL) was not stained in untreated TVT tumors but was stained remarkably in TVT cells following vincristine sulfate treatment (Figures 8a and 8b). Tumor cells were stained intracytoplasmically with lactose-specific *Ricinus communis* agglutinin-I (RCA-I) (Figures 9a and 9b). After vincristine sulfate treatment, the RCA-I HSCORE value index was increased 1.3-fold. The N-acetylgalactosamine–specific *Griffonia simplicifolia* lectin-I (GSL-I) HSCORE value increased 1.7-fold after vincristine sulfate treatment. The staining pattern was intracytoplasmic and was primarily observed close to the outer edge of the tumor in the untreated group, whereas the distribution of the staining pattern was scattered all around the section (Figures 10a and 10b) in the treated group.

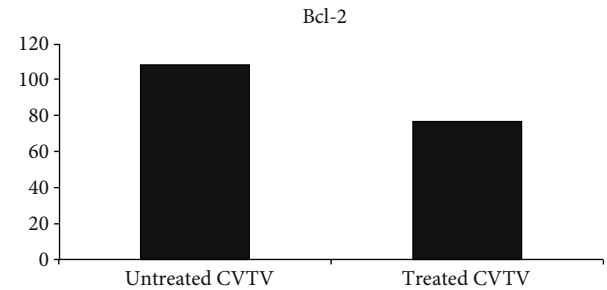

Figure 5. Bcl-2 HSCORE values reflecting antiapoptosis.

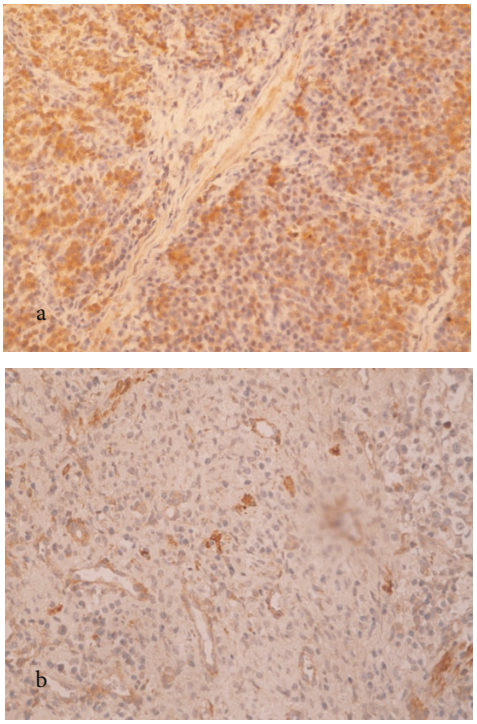

Figure 6. Strong Bcl-2 immunoreactivity in untreated TVTs **(a)** and positive reaction in treated tumor blood vessels **(b)**. IHC 40× original magnification, DAB chromogen.

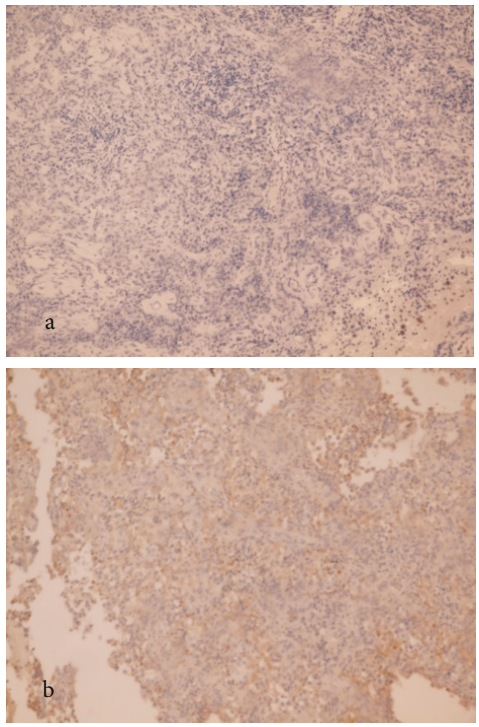

Figure 8. EEL was not stained in untreated TVT tumors **(a)** but was remarkably stained in TVT cells treated by vincristine sulfate **(b)**. IHC 4× original magnification, DAB chromogen.

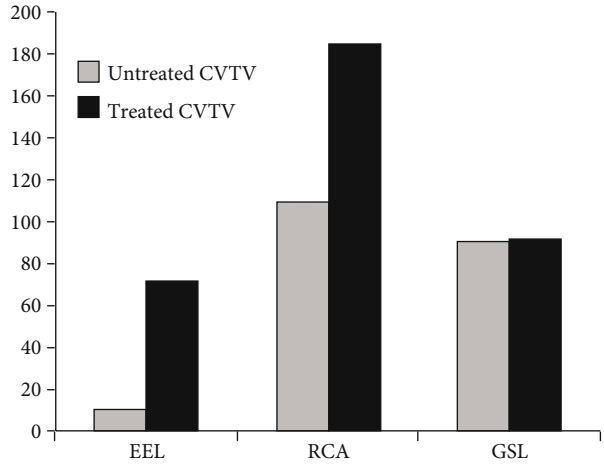

Figure 7. Lectin (EEL, RCA-I, and GSL-I) HSCORE values.

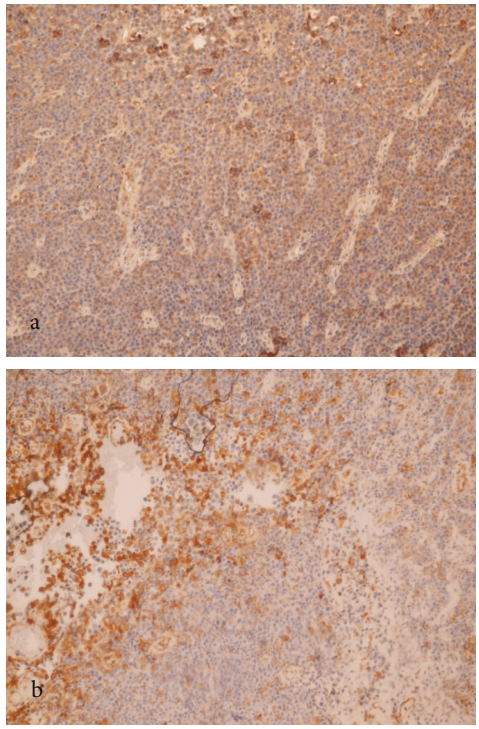

Figure 9. Intracytoplasmic RCA-I staining, where untreated tumor cells **(a)** have higher HSCORE values than do treated tumors **(b)**. IHC 10× original magnification, DAB chromogen.

4. Discussion

CTVT, also known as infectious sarcoma, venereal granuloma, transmissible lymphosarcoma or Sticker tumor, is a mostly benign reticuloendothelial tumor, usually transmitted during coitus, that occurs in young, sexually mature, and intact dogs. It primarily affects the external genitalia, though it is sometimes found in the internal genitalia as well (3). Treatment protocols include

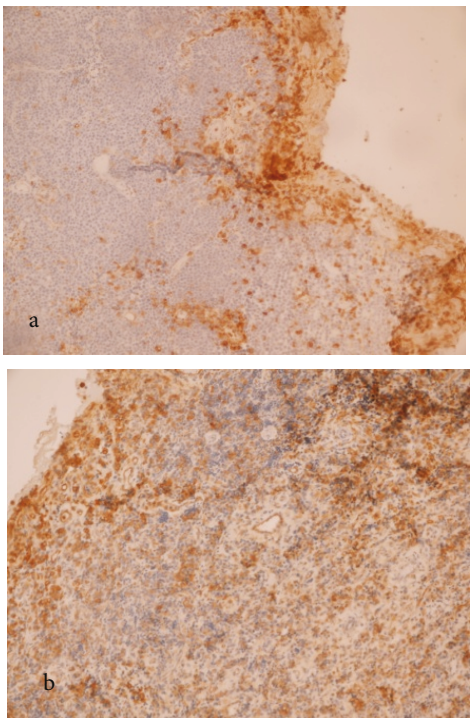

Figure 10. Intracytoplasmic GSL-I staining, where treated tumor cells **(b)** have higher HSCORE values than do untreated tumors **(a)**. IHC 20× original magnification, DAB chromogen.

surgery, radiotherapy, immunotherapy, biotherapy, and chemotherapy. Surgery has been used extensively for the treatment of small, localized CTVTs, although the recurrence rate is as high as 50%–68% in cases of large invasive tumors (3). Contamination of the surgical site with CTVT cells is also a source of recurrence (7). Chemotherapy using vincristine sulfate has been shown to be the most effective and practical therapy. Vincristine is administered weekly at a dose of 0.5 to 0.7 mg/m^2 of body surface area or 0.025 mg/kg, intravenously. The healing of the lesions is gradual, although it is particularly noticeable and significant at the beginning of the treatment (8). Complete regression usually takes 2 to 8 treatments and occurs in more than 90% of the treated cases with vincristine sulfate (6,9,10). The recovery rate is approximately 100% in cases with tumor diagnosis in the initial stages, particularly in cases of less than 1 year of duration (7). In the present cases, tumors were treated with 7 weeks of vincristine sulfate application with complete regression of the tumor from the genital sites. During the regressive stages of the tumor, the host immune response may play a role in the variable duration of the treatment required (from 2 to 8 weeks). Özalp et al. (6) reported that TUNEL reactions in control tissue samples prior to vincristine sulfate applications were clearly less than those of the samples from treated animals and that the differences between samples from the control and 2 treatments were statistically significant. However, there were no differences between 2 treatments within a treated group. In this study, the TUNEL HSCORE was higher in the treatment group than in the untreated group, although no statistical differences between the groups were recorded. The increasing rates in TUNEL HSCORE showed that weekly regular chemotherapy with vincristine sulfate for CTVT treatment stabilized the inhibition of tumor growth (6). This result is in accordance with our study and it was observed that apoptosis began with the first administration of vincristine sulfate.

Bcl-2 and Bax are 2 members of the Bcl-2 gene family that play a prominent role in the regulation of apoptosis. Bcl-2 is an intracytoplasmic and membrane-associated apoptosis suppressor (antiapoptotic protein) gene that is found in human and canine cells, and its overexpression is closely associated with the survival of malignant tumors, in particular their aggressive behavior and poor prognosis (11,12). Apoptosis is triggered by the release of proapoptotic molecules from the mitochondria by multiple mechanisms, including maintenance of mitochondrial membrane integrity and binding to proapoptotic members of the Bcl-2 family (13). It has been observed that hormonal estrogen receptors are significantly associated with overexpression of Bcl-2 and mutant p53 in malignant transformation of breast tumors (14). Jeon and Yoon (11) reported heterogeneously strong expressions of Bcl-2 in dysplastic bile ducts, but negative-to-weak immunoreactivity in normal and hyperplastic small bile ducts. In addition, the expression of Bcl-2 significantly increased by 85% in premenopausal patients compared to postmenopausal breast tumors. Bcl-2 has been shown to repress cell death triggered by a diverse array of stimuli, including chemotherapy and gamma irradiation. Rui et al. (3) stated that upregulated expression of the antiapoptotic Bcl-2 protein in tumor cells differs depending on the malignancy and treatment regimens and might be related to chemotherapy resistance in feline tumors. Red deposits in the cytoplasm of positive cells were demonstrated by immunohistochemical analysis of Bcl-2 in CTVT samples (1). A high concentration of the antiapoptotic protein Bcl-2 might allow cells promoting mutations to survive by evading apoptosis. The Bcl-2 protein is overexpressed in CTVT samples independent of the stage of tumor development and the contribution of Bcl-2 in carcinogenesis remains puzzling because its expression can be associated with resistance to drugs and radiotherapy. The role of Bcl-2 in CTVT may suggest a mechanism for tumor cell survival for a period of time, closely related to exogenous tissue (1). In the present study, the immunohistochemical Bcl-2 expression was higher in untreated tumor cells and was reduced after vincristine treatment. Increased endothelial staining in the treated

group might indicate that CTVT has upregulated synthesis of and resistance to Bcl-2 to protect from apoptosis. It has been suggested that vincristine sulfate downregulates Bcl-2 expression after the first treatment and this downregulation protects from apoptosis. Although there was significant downregulation in Bcl-2 after the initial treatment, there was insignificant upregulation of apoptosis in treated CTVT. Therefore, further studies should be conducted to clarify the mechanisms and routes that downregulate Bcl-2 in CTVT.

Galectin-3, which plays a vital role in cell differentiation, proliferation, adhesion, cell-cell and/or cell-extracellular matrix interactions, cell spread, and tumor cell apoptosis, is a member of a family of multifunctional β-galactoside–binding lectins (15–17). Although galectin-3 is widely expressed in normal and tumor cells (18,19), its overexpression or downregulation is controversial in tumor malignancies (15). The metastatic potential of colon tumor cell lines and malignant nonmedullary thyroid neoplasms were directly related to the expression of galectin-3 (4,20), while human colon cancer (21,22) as well as mammary cancer in human and canine requires downregulation of galectin-3 for progression (16,23). Choi et al. (15) and de Oliveira (16) clearly demonstrated that galectin-3 was overexpressed in adenoma but not in adenocarcinoma and that low expression of galectin-3 was associated with canine mammary tumor progression. In contrast, high expressions of galectin-3 in invasive neoplastic cells in canine gastric carcinoma suggested an important role in metastasis (24). In this study, CTVTs were intracytoplasmically stained with galectin-3 in both groups. Galectin-3 was expressed in the CTVT control group but sharp downregulation was observed in treated cells after vincristine sulfate administration. This decrease is compatible with the result of Choi et al.'s study (15) because CTVT usually remains local and does not metastasize. Vincristine might be responsible for thwarting antiapoptotic effects. It has been speculated that subcellular distribution of galectin-3 might be responsible for increasing apoptosis resistance of tumor cells (16). CTVT cells with decreased staining of galectin-3 may be more susceptible to apoptosis after vincristine sulfate treatment. It has been thought that galectin-3 overexpression is phenotypically associated with malignant transformation and progression toward metastatic potential (4,16). Moreover, galectin-3 is a chemoattractant to endothelial cells, and it stimulates angiogenesis and provides an escape route by which cells can leave the primary tumor and enter into blood circulation (25,26). Because decreased galectin-3 expression would lead to reduced adhesiveness between tumor cells and facilitate cancer cell invasion (16), vincristine sulfate might be helpful for downregulation of galectin-3 to prevent malignant forms and metastases.

Lectins are specific carbohydrate-binding proteins (27). They have a significant role in identifying cellular carbohydrate residues. Different biological functions in animals are attributed to lectins, including regulation of cell adhesion and glycoprotein synthesis. Pluripotent stem cells had the specific glycan structure recognized by EEL multipotent cell types (28). GSL-I has been reported to bind several glycoproteins, including laminin, and is necessary for intercellular communication (29). RCA-1 histochemical staining was found to be negative in the normal central nervous system. However, benign tumors like astrocytoma, medulloblastoma, ependymoma, and oligodendroglioma were stained with RCA-1. Moreover, RCA-1 staining was decreased in malignant tumors such as neurocytoma and malignant astrocytoma (27). In this study, RCA-1 staining scores increased after treatment, as is seen in benign tumors. Increased RCA-1 staining after treatment might be related in that CTVT cells were subject to apoptosis. After treatment, HSCORE values showed increasing rates in all 3 of the lectins examined (EEL, GSL-1, and RCA-1). A significant outcome was observed in this study. Because CTVT cells occurred discretely or in sheets and have loose connective tissue, the tumor can easily spread from site to site and from dog to dog by direct contact with the mass. During the treatment period, minimal tumor spread and fewer malignancies might be related to increased lectins, which regulate cell-to-cell and cell-to-matrix adhesion.

In summary, increased apoptosis and lectin-binding rates and decreased expression of the antiapoptotic factors Bcl-2 and galectin-3 were observed after vincristine sulfate treatment of CTVTs. These results show that the expression of galectin-3, Bcl-2, and certain lectins (EEL, GSL-1, and RCA-1) are related to CTVT regression after treatment with vincristine sulfate.

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