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Immunohistochemical Detection of Retinoid Receptors in Tumors from 30 Dogs Diagnosed with Cutaneous Lymphoma

C.H. de Mello Souza, V.E.O. Valli, K.A. Selting, M. Kiupel, and B.E. Kitchell

Background: Retinoids exert their effects by binding to retinoid receptors. Two types of retinoid receptors have been described: retinoic acid receptor (RAR) and retinoid X receptor (RXR), and their subtypes α, β, and γ. The expression of subtypes varies depending on the disease process. This study intended to detect the pattern of retinoid receptor expression in cutaneous lymphomas in dogs.

Hypothesis: Cutaneous lymphomas in dogs have variable expression of retinoid and retinoid X receptors.

Animals: Biopsy specimens from 30 dogs with cutaneous lymphoma.

Methods: Tissues of dogs with cutaneous lymphoma were evaluated by immunohistochemistry for expression of retinoid receptors. The tissues were tested for the presence of 3 RAR and RXR subtypes (α, β, and γ). Lymphoma immunophenotype was determined by the use of the immunohistochemical markers CD79a (B-cell) and CD3 (T-cell) in all cases.

Results: Twenty-nine of 30 dogs were CD3 positive. The retinoid receptors expressed with the greatest frequency were RARβ (87% of cases), and RXRα and RXRγ (77% of cases). The expression of RARγ was not observed.

Conclusions and Clinical Relevance: Retinoid and rexinoid receptor binding drugs may have an impact on the treatment of dogs with cutaneous lymphoma.

Key words: Mycosis fungoides; RAR; Retinoic acid; Rexinoids; RXR.

Retinoids are natural or synthetic derivatives of vitamin A that exert profound effects on the growth and differentiation of many cell types both in vivo and in vitro. Retinoids are essential for epithelial differentiation; they have been demonstrated to induce growth inhibition and cell cycle arrest in cancer cells by induction of terminal differentiation and apoptosis.[1] These effects occur through interaction with specific retinoid nuclear receptors that are part of the steroid superfamily of nuclear receptors.[2,3] Retinoid receptors function as ligand-dependent transcription factors, and retinoid binding and activation of the receptor is followed by transcription of responsive genes. Retinoid receptors are divided into 2 families: the retinoic acid receptors (RARs); and the retinoid X receptors (RXRs, also called rexinoids). Each family has 3 receptor subtypes (α, β, and γ), with multiple isoforms and specific genes that encode each of them. In the physiologic setting, the natural diet-derived retinoids (all-trans retinoic acid [ATRA] and 9-cis-retinoic acid [9-cis-RA]), have different binding affinities to the receptors. Specifically, ATRA and 9-cis-RA bind RAR, but only 9-cis-RA binds RXR.[2–5] Because both therapeutic and adverse effects vary depending on the class and subtype of receptor bound, a variety of new synthetic retinoids with exquisite isoform specificity are under investigation.[6]

Abbreviations:

ATRA	all-trans retinoic acid
CTCL	cutaneous T-cell lymphoma
MCT	mast cell tumors
MF	mycosis fungoides
RAR	retinoic acid receptor
RXR	retinoid X receptor
SCC	squamous cell carcinoma
RARα	sc-551 and blocking peptide (BP) sc-551P
RARβ	sc-552 and BP sc-552P
RARγ	sc-7387 and BP sc-7387P
RXRα	sc-553 and BP sc-553P
RXRβ	sc-831 and BP sc-831
RXRγ	sc-555 and BP sc-555P.

Retinoids suppress premalignant lesions such as oral leukoplakia, cervical dysplasia and xeroderma pigmentosum in people, and actinic keratosis in dogs.[1,2,7] Retinoids have been used successfully in people as single agent therapy in the treatment of promyelocytic leukemia, juvenile chronic myelogenous leukemia, and cutaneous lymphoma.[2,3,8–11] When administered in conjunction with interferon-α2A, retinoids aid in the treatment of human squamous cell carcinoma (SCC) of the skin and uterine cervix, and renal carcinoma. Administration of retinoids can inhibit the development of second primary cancers after treatment of SCC of the head and neck, lung tumors, and hepatic carcinomas.[2,8–11] Retinoic acid induces differentiation of thyroid

From the College of Veterinary Medicine, Veterinary Medical Teaching Hospital, University of Missouri-Columbia, Columbia, MO (de Mello Souza, Selting); Veterinary Diagnostics, Davis, CA (Valli); Department of Pathobiology and Diagnostic Investigation, Diagnostic Center for Population and Animal Health, Michigan State University, East Lansing, MI (Kiupel); and the Center for Comparative Oncology, D208 Veterinary Medicine Center, College of Veterinary Medicine, Michigan State University, East Lansing, MI (Kitchell). Valli is presently affiliated with Veterinary Diagnostics, Davis, CA. Kitchell is presently affiliated with the Center for Comparative Oncology, D208 Veterinary Medicine Center, College of Veterinary Medicine, Michigan State University, East Lansing, MI.
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Corresponding author: Carlos H. de M. Souza, College of Veterinary Medicine, Veterinary Medical Teaching Hospital, University of Missouri-Columbia, Clydesdale Hall, 900 East Campus Drive, Columbia, MO 65211; e-mail: souzac@missouri.edu.
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carcinomas, leading to increased radiation sensitivity to I^{131}.[12,13]

In dogs, retinoids have been used in dermatology as a differentiation agent for cases affected by actinic keratosis, sebaceous adenitis, and benign pilomatrixomas.[7,14,15] Few studies have demonstrated the effects of retinoids in canine cancer. Fourteen dogs with cutaneous lymphoma were treated with isotretinoin or etretinate, with reported clinical response rate of 42%.[16] Natural retinoids (ATRA and 9-cis-RA) induce differentiation, growth inhibition, and apoptosis in canine osteosarcoma cells.[17–21] In addition, mast cell tumor (MCT) cell lines express retinoid receptors and that the level of expression of RARα mRNA correlates well with growth inhibition caused by ATRA.[22,23] With development of synthetic retinoids, specific receptor targeting has led to superior results in both cancer prevention and treatment in human medicine. Additional understanding of the pattern of retinoid receptor expression suggests that specific retinoid receptor interacting agents may enhance responses in canine cancer patients. Retinoid receptor expression has been evaluated in canine nodal lymphomas, benign lymphoid hyperplasia, and normal lymph nodes. This study showed that nonneoplastic lymphoid tissue did not express retinoid receptors, while lymphomas had strong expression.[24]

Materials and Methods

The choice of antibodies used in our study was based on a previous work by Mori et al,[25] in which a Cy3-labeled IgG immunoflorescence was used to detect retinoid receptor expression in murine tissues. In order to confirm any similarities in the staining pattern of mice and dogs, we developed a protocol for immunohistochemistry and compared the pattern of retinoid receptor expression (RARα, β, and γ and RXRα, β, and γ) in different regions of the retinas of neonatal mice and pups as fully described below. The retinas of murine pups from several litters were formalin fixed and trimmed to serve as positive controls and as a standard for retinal region staining. The retinas of canine pups from several litters were prepared in the exact same manner. Slides were incubated for 30 minutes and trial dilutions of the primary antibodies were performed at 1 : 10, 1 : 50, and 1 : 100. Formalin fixed, paraffin embedded 3 μm in thickness unstained slides were prepared from paraffin blocks for immunostaining with all markers. Heat-induced antigen retrieval was used before antibody staining by heating the slides to 125°C for 30 seconds, followed by 95°C for 10 seconds in citrate buffer at pH 6. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 15 minutes. Nonspecific immunoglobulin binding was blocked by incubation of slides for 10 minutes with a protein blocking agent and allowed to react for 30 minutes at room temperature before application of the primary antibody. Secondary antibody was then applied and slides were incubated for 20 minutes. The sections were stained in a Biogenix autostainer.[a] The antibodies used were: RARα C-20 and RARβ C-19 (rabbit polyclonal); RARγ G-1 (mouse monoclonal); RXRα D-20, RXRβ C-20, and RXRγ Y-20 (rabbit polyclonal).[b] For the negative controls, specific blocking peptides were added to each antibody (25 μL of blocking peptide to 5 μL of antibody) and incubated for 2 hours before dilution.[b] The Supersensitive kit (streptavidin-biotin system) was used for all antibodies.[a] Immunoreaction was visualized with 3,39-diaminobenzidine substrate. Final dilutions were RARα (1 : 100), RARβ (1 : 100), RARγ (1 : 75), RXRα (1 : 100), RXRβ (1 : 100), and RXRγ (1 : 100). Finally, slides were counterstained with hematoxylin for 1 minute, rinsed in water, dehydrated, and mounted. Our study showed that the distribution pattern of retinoid receptors in different areas of the retina of the mouse paralleled the descriptions of Mori et al.[25] In addition, receptor localization in the retinas of pups and neonatal mice was similar, considering the anatomical differences between the 2 species. Briefly, consonant to the data shown by Mori et al,[25] our pilot study showed that strong staining compared in mice and pups in the following regions: the neural retina was positive for RARα, RARβ, RXRα, RXRβ, and RXRγ; the retinal pigment epithelium was positive for RARα, RARβ, RXRα, RXRβ; the mesenchyme was positive for RARγ and RXRγ (Fig. 1).

For this retrospective study, hematoxylin and eosin-stained slides and paraffin blocks from 30 consecutive cases diagnosed between 1998 and 2001 with cutaneous lymphoma were retrieved from the archives of IPEV (Toledo-Piza, Rio de Janeiro, Brazil) and the pathology laboratory of the Veterinary Teaching Hospital of the University of Illinois. Immunophenotyping for B and T-lymphocytes was performed. Antibodies used for immunohistochemistry included the T-cell marker CD3 (polyclonal-mouse anti-human) and the B-cell marker CD79a (monoclonal-mouse anti-human).[c] The primary antibodies and the protocol used for detection of retinoid receptors were the same as in our initial study described above. One pathologist reviewed all the slides (VEOV) in order to confirm the diagnosis of cutaneous lymphoma. Formalin fixed, paraffin embedded 3 μm in thickness unstained slides (15 from each case) were prepared from all blocks for immunostaining for all markers. Immunohistochemical analysis was performed by routine Biotin-streptavidin-immunoperoxidase amplified detection system as described previously, with minor changes as described above.[26,27] For CD3 and CD79a, normal lymph nodes were used as controls. For retinoid receptors, neonatal murine eyes were used as positive controls due to limited availability of retinas from pups and based on the results of our protocol development. Blocking peptides specific to each retinoid receptor were used as described above for negative controls. The immunostaining for retinoid receptors had to be strong in the nucleus of the cell in order for the sample to be considered positive.

Results

The majority of animals in our population were of mixed breeds (17/30 cases). Great Danes (3 cases) and German Shepherd, Rottweiler, and Cocker Spaniel (2 cases each) were the pure breeds affected most frequently. The mean and median age for dogs affected was 8 years (range from 3 to 12 years). The majority of cases (25/30) were diagnosed as nonepitheliotropic lymphoma. Epitheliotropic lymphoma was diagnosed in the remaining 5 cases.

CD3+ T-cell lymphoma was diagnosed in 29 cases. Only one case of CD79a+ B-cell lymphoma (nonepitheliotropic) was detected. Strong immunoreactivity for retinoid receptors was detected in 29 cases (Fig. 2a). One case was negative for all receptors. The number of receptor subtypes determined to be positive per tumor were: 1 receptor (7%), 2 receptors (17%), 3 receptors (48%), 4 receptors (14%), and 5 receptors (14%). From the cases that expressed 3 receptors, 64% expressed a combination of RARβ, RXRα, and RXRγ. RARβ was most commonly expressed (87% of cases) followed by RXRα and RXRγ (77% each), RXRβ (70%), and RARα (37%). None of the cases tested was positive for RARγ (Table 1). Overall, retinoid receptor expression in positive cases occurred in the majority of the cells (>60%). Most cases had

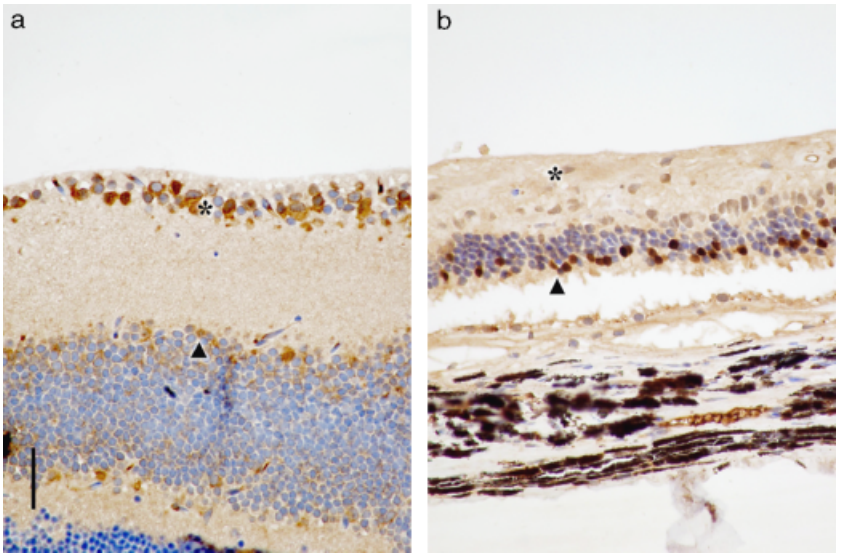

Fig 1. Mouse (**a**) and dog (**b**) retinas (positive controls). Immunohistochemistry showing positivity for RXR-α at the ganglion cell layer (∗) and inner nuclear layer (▲). Bar=80 μm.

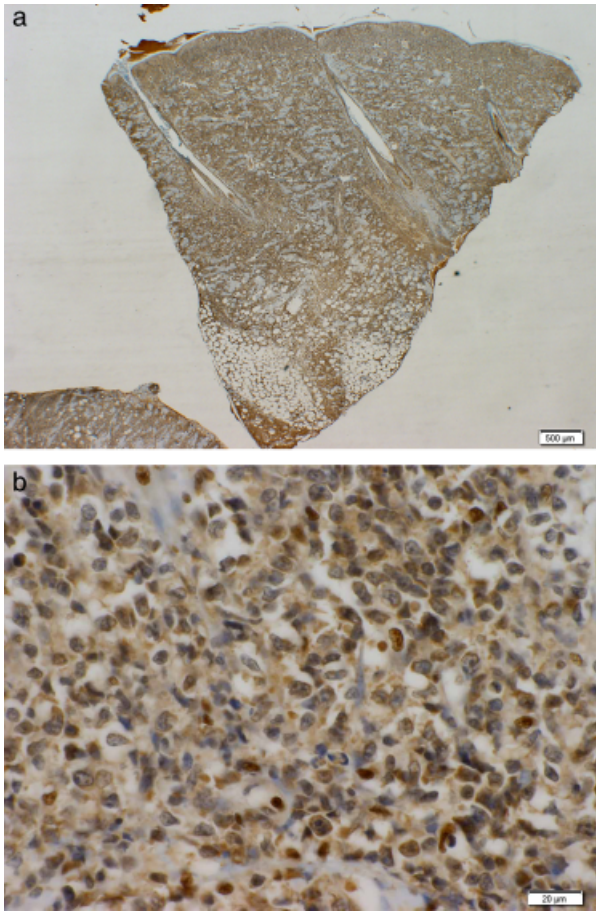

Fig 2. Dog skin CTCL. RXRα stain. (**a**) Note that the stain closely follows the infiltration pattern of lymphocytes sparing areas of fat at the bottom. (**b**) Detail of Fig. 1a. High proportion of nuclei show positive staining for RXRα. Some also show mild cytoplasmic staining.

massive infiltration of neoplastic cells and normal elements of the skin were not seen. When normal epithelium was present, well-differentiated cells were mostly negative. Retinoid receptor staining was strong in the nucleus of the cells, but occasionally, mild cytoplasmic staining was also seen. Staining restricted to the cytoplasm was not seen in any case (Fig. 2b).

Discussion

The majority of the cutaneous lymphomas in this study had strong retinoid receptor expression and as expected, most of them were T-cell lymphomas. The pathologic diagnosis of cutaneous lymphoma comprises a variety of different histologic criteria, and at least in humans, a large number of well-characterized clinical entities. Both B- and T-cell lymphomas occur in the skin, and cutaneous T-cell lymphomas (CTCL) are by far the most common cell type reported.[28–33] Twenty-nine of 30 of our cases stained positive for CD3. The majority of our cases were diagnosed as nonepitheliotropic lymphoma. Mycosis fungoides (MF) has been described as

Table 1. Frequency of retinoid receptor expression.

Diagnosis (# of cases)	Retinoid Receptor					
	RARα	RARβ	RARγ	RXRα	RXRβ	RXRγ
Mf (5)[a]	3	4	0	4	4	4
CTCL (24)[b]	8	21	0	18	16	18
CBCL (1)[c]	0	1	0	1	1	1
N/%	11/37	26/87	0/0	23/77	21/70	23/77

[a]Mycosis fungoides/epitheliotropic lymphoma.
[b]Cutaneous T-cell lymphoma/nonepitheliotropic lymphoma.
[c]Cutaneous B-cell lymphoma.

the most common form of cutaneous lymphoma in humans and dogs, and in some reports the term is used as a synonym for cutaneous lymphoma.[34] In our report, only cases that demonstrated clear epitheliotropism (subepidermal and peri-adnexal infiltration by neoplastic lymphocytes) were diagnosed as MF. The presence of Pautrier microabcesses (small interepidermal clusters of neoplastic cells surrounded by a clear halo) was also required for the diagnosis of MF.[30,35,36]

Only 1 case series demonstrated a greater frequency of epitheliotropic over nonepitheliotropic lymphomas.[35] A feature that seems to distinguish canine from human MF is adnexal gland tropism, which is observed more commonly in dogs. In our case series, periadnexal neoplastic lymphocytes were commonly found in the MF cases. Nonepitheliotropic lymphomas do not show this pattern of tumor cell distribution.[35,36] Although most tumors were diagnosed in advanced stages, and while epitheliotropism was not a distinct feature, it has been previously reported that epitheliotropism remains even in dogs with advanced local tumor stages of disease. [35] In contrary, epitheliotropism has also been reported to be more or less obvious depending on stage of disease.[33] Because the majority of cases came from Brazil, a regional difference in tumor distribution is possible. A regional difference in disease type has not been reported.

Most patients affected by cutaneous lymphoma are older. The mean age at presentation in our study was 8 years. The majority of dogs included in our study were mixed breeds. Although breed predisposition has been suggested (English Cocker Spaniel, Poodle, and Boxer), only 1 study found Weimaraners and Airedales to be predisposed to cutaneous lymphoma when compared with that study's hospital population.[28,35–38]

Retinoid receptor expression was demonstrated in 29/30 cases and only 1 receptor (RARγ) was not positive in any case. Previous optimization of the IHC steps for all the receptors including RARγ (positive in both mice and dog retina) suggests that canine cutaneous lymphomas do not express RARγ. Despite this fact, a methodology error such as unsuccessful antigen retrieval in slides stained by RARγ antibody cannot be completely ruled out. In all positive cases, the staining in the nucleus was strong and none of the cases had the staining restricted to the cytoplasm. In addition to the nuclear staining, mild cytoplasmic staining was seen occasionally. Retinoid receptors are in general located in the nucleus, but aberrant subcellular staining has been reported in oral SCC, thyroid, and renal carcinoma and staining location has been associated with prognosis and survival.[39–41] The importance of retinoid receptor subtype in cancer is underscored by studies demonstrating that variation in the expression of some of them is associated with malignant progression, stage of disease, biologic behavior, response to treatment, and overall survival. This has been demonstrated in a variety of carcinomas, such as lung, head and neck, ovary and thyroid, and cutaneous lymphomas in people.[39–45] A recent study showed that retinoid receptor expression is negative in normal canine lymph nodes. In that same study, strong retinoid and rexinoid receptor expression was detected in B- and T-cell canine lymphomas.[24] It seems reasonable to expect that retinoid receptor expression is either the cause or the consequence of lymphocyte malignant transformation and these receptors may serve as new diagnostic and therapeutic intervention targets. MCT cell lines from dogs expressed RAR and RXR mRNA and in addition, higher levels of RARα were associated with greater growth inhibitory effect of ATRA.[22,23] As shown in Table 1, RARβ, RXRα, and RXRγ were most frequently expressed in cutaneous lymphomas in dogs. This combination may have implications in relation to tumor behavior, response to therapy, and overall survival and it should be further investigated. A recent study revealed that the combination of positive RARα and negative RARβ had a 91% predictive value for benign thyroid lesions.[41] In people, the use of specific ligands for RXR, such as the drug bexarotene (Targretin), has been associated with improved responses in CTCL, and clinical benefits from this drug have also been observed in metastatic breast cancer trials.[46–50] The same may be proven true in canine CTCL and other forms of canine cancer. Furthermore, the use of specific retinoid-receptor binding drugs, such as the experimental RARβ synthetic analog fenretinide, has been shown to be more effective and less toxic than isotretinoin (Accutane) in humans.[51] Detection of patterns of retinoid expression in cutaneous lymphoma may lead to a more firm rationale for the use of classic and novel retinoid analogs in veterinary medicine. In addition, patterns of expression can be associated with tumor behavior and response to therapy as shown in some cancers in people and this should be further investigated.

Limitations of the current study include the fact that IHC does not detect receptor activity, but only the presence of the receptor itself. The majority of our cases were classified as nonepitheliotropic LSA. For this reason, the retinoid receptor pattern detected in our study may vary when larger number of cases of *M. fungoides* is analyzed.

Conclusions

In this study, we demonstrated that canine cutaneous lymphoma has variable expression of RAR and RXR receptors, as hypothesized. The presence of these receptors paves the way for further studies. In future studies, the presence of specific retinoid receptors and combination of receptors should be evaluated to detect response to retinoid therapy, correlations with response to chemotherapy, and any influence on prognosis. The high expression of the RARβ, and RXRα and RXRγ isoforms, indicates that these forms in particular may be suitable targets for therapy of canine cutaneous lymphoma.

Footnotes

[a] Biogenex, San Ramon, CA
[b] Santa Cruz Biotechnology Inc, 2161 Delaware Ave, Santa Cruz, CA
[c] Dako-Cytomation, Carpinteria, CA

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Conflicts of interest: None.

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