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Development and characterization of 5 canine B-cell lymphoma cell lines

Allison L. Zwingenberger[a,*], William Vernau[b], Changying Shi[a], Wensheng Yan[a], Xinbin Chen[a], Ira K. Gordon[c], Michael S. Kent[a]

[a] Department of Surgical and Radiological Sciences, School of Veterinary Medicine, University of California, Davis, CA, USA
[b] Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, CA, USA
[c] Radiation Oncology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

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ABSTRACT

Canine and human lymphoma share similar characteristics in disease development and response to therapy. Translational research can be furthered using tools such as canine cell lines to model therapeutic compounds and strategies. We developed 5 B-cell lymphoma cell lines from dogs with confirmed large B-cell lymphoma. These cell lines were CD3, CD18, CD20, and CD90 positive with variable CD79a, CD1c and CD34 expression. All cell lines were tumorigenic in Nu/nu mice and were wild type for p53. Canine lymphoma cell lines serve as an important resource for translational lymphoma research.

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1. Introduction

Lymphoma is the most common hematological cancer in dogs with an estimated incidence of 1/100,000 dogs per year, with non-Hodgkin's diffuse large B-cell lymphoma representing the most common type [1]. Dogs are emerging as important models of human cancer because of the shared genome and polygenetic development of spontaneous cancers [2–4]. In fact, the canine genome, recently sequenced, is reported to be more homologous to humans than that of mice [5]. Dogs are affected by naturally occurring lymphoma that closely resembles the human disease with respect to the World Health Organization's (WHO) classification of lymphoma types, incidence rate, response to chemotherapy, and survival time [1,6,7]. Canine hematologic malignancies have also been shown to have evolutionarily conserved chromosome aberrations that are similar to equivalent diseases in humans, and also to have similar effects in both species [3]. Additionally, due to intense selection pressure, the genome of some pure bred dogs is more homogeneous than the outbred genome of most human populations. This homogeneity can facilitate the identification of key molecular events involved in lymphomagenesis, which can then be interrogated in humans. Canine lymphoma has been used to model the P53 pathway, and has been shown to have increased expression of RNPC1 with downregulation of P53, which may contribute to development of lymphoma [8]. Companion animals also have accelerated aging compared to humans, and are kept into old age where development of spontaneous cancers is more common [4]. The study of this naturally occurring tumor in dogs, is therefore likely to provide additional and useful information regarding lymphoma in people upon which to base human clinical trials.

While several canine cell lines have been generated and at least partially characterized, only one B-cell lymphoma line has been established and well characterized [9], likely due to the known difficulty in establishing lymphoid cell lines [10]. Because canine lymphoma appears to be a useful model of human non-Hodgkin's lymphoma, development of canine cell lines will provide greater insight into the molecular mechanisms of the disease and will allow *in vitro* testing of promising agents and therapies.

Therefore, the purpose of this study was to develop and characterize 5 distinct canine cell lines in order to provide more tools for research into both canine and human lymphoma.

2. Materials and methods

2.1. Canine lymphoma sample collection

Fresh tissue samples were obtained from a series of dogs presenting to the William R. Pritchard Veterinary Medical Teaching hospital for the diagnosis and/or treatment of lymphoma. Owner consent was obtained, and the procedure was approved by the institution's Animal Care and Use Committee.

Dogs were staged according to the WHO's classification of lymphoma in domestic animals. A 23-gauge needle was inserted into the lymph node and redirected through the node 2–3 times. The needle was attached to a 12 mL syringe, and the cells of the needle were expelled into a tube containing 1 mL 1× TBS buffer. A 2nd

* Corresponding author at: Department of Surgical and Radiological Sciences, School of Veterinary Medicine, University of California, 1 Shields Avenue, Davis, CA, 95616, USA. Tel.: +1 530 752 6638; fax: +1 530 752 6638.
E-mail address: azwingen@ucdavis.edu (A.L. Zwingenberger).

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needle was introduced into the same lymph node, the sampling procedure was repeated, and the two samples were pooled.

2.2. Preparation of primary canine lymphoma cell cultures

The canine lymph node aspirated cells were suspended in TBS buffer and washed twice. 3 mL of red blood cell lysis buffer (155 mM ammonium chloride, 12 mM potassium bicarbonate in purified water (Millipore Corporation, Billerica, MA), pH 7.2) were added, and the sample was incubated for 5 min at room temperature until clear. The sample was then centrifuged for 5 min at 220 × *g*. The supernatant was removed, and the cells were washed in RPMI-1640 medium (ATCC, Cat. 30-2001).

2.3. Establishment of canine lymphoma cell lines

Cells were cultured in T25 flasks in RPMI-1640 medium supplemented with 20% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin, and L-glutamine. The cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2. For culture maintenance, a small volume of fresh growth medium was added once every 2–3 days, and the medium was thoroughly changed after centrifugation of the culture cells weekly. For the first 4 weeks, both adherent and non-adherent cells were passaged together weekly. After 4 weeks, the non-adherent cell population grew as a suspension culture consisting primarily of single cells and small aggregates of cells. After cell growth became apparent, the cells were passaged at 4 or 5 day intervals by adjusting the cell density to 2×10^5–1×10^6 cells/mL. For storage, aliquots of cells were kept at a concentration of 1–5 × 10^6 cells/mL in 90% RPMI 1640 (supplemented with 10% FBS) and 10% DMSO and stored in liquid nitrogen. The cell lines were subsequently maintained in continuous cultures for over 1 year.

For generation of growth curves, cells were plated to achieve a plating density of 5–8 × 10^4 cells suspended in 1 mL of culture medium per well of a 48-well plate. Cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cells in triplicate wells were counted every 24 h. The values obtained were plotted on a log-linear scale. The population-doubling time was determined from the exponential phase of the growth curve.

2.4. Flow cytometry

Lymph node aspirates were analyzed within 24 h of collection and were unfixed. For monoclonal antibody (MAb) binding to the indicated antibodies in Table 1, aliquots of approximately 1×10^6 cells were incubated with 25 μL of MAb tissue culture fluid supernatant for 30 min at room temperature. Cells were then spun and washed twice with flow buffer (1 mM Mg^{2+} in 1× TBS, 1% horse serum). For indirect immunofluorescence, these cells were detected by incubation with 50 μl of a 1:100 dilution of FITC-conjugated horse anti-mouse IgG (Vector Laboratories, Carpenteria, CA) for 15 min. Cells were then spun, washed and resuspended in 500 μL of flow buffer. A negative control consisting of an isotype matched irrelevant MAb was included. Fluorescence was measured in 10,000 cells using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Data was analyzed with commercial software (Flowjo, Treestar Inc., Ashland, OR).

For the original patient samples, single- and double-labeled tubes of MAb to CD3, CD21 and CD49d were used followed by the FITC-conjugated secondary of horse anti-mouse IgG (Vector Laboratories, Carpenteria, CA) were prepared and a maximum of 20,000 cells were analyzed with the flow cytometer. Cell line preparations were prepared in single-labeled tubes. Monoclonal antibodies used in immunophenotyping of the cell lines are shown in Table 1.

2.5. Immunocytochemistry and immunohistochemistry

The expression of antigens was assessed on one original patient sample, cytospin smears of lymphoma cell lines, and xenograft tissue, as previously described [11]. To address the complication of using murine monoclonal antibodies in mouse tissue, the Dako ARK method was used for assessing the xenograft tissue in mice (Dako, Carpenteria, USA).

2.6. PCR test for clonality

PCR assays for clonality assessment were performed on DNA extracted from original patient samples (aspirate smears or blood from leukemia patients), cytospin smears of lymphoma cell lines, and xenograft tissue as described previously [12]. Two additional primer sets, directed against 2 additional canine Ig loci, FR2 and Kde, were also run [13]. All assays included a negative control (10 μL water instead of gDNA template), polyclonal control, and appropriate clonal controls. DNA samples were prepared using the QIAamp DNA mini kit (Qiagen Inc., Valencia, CA). For amplification (in triplicate), approximately 100 ng of DNA was amplified with 200–400 nM of each primer in a 50 μL reaction volume, including 1× reaction buffer, 0.5 mM $MgCl_2$, 50 mM each dNTP and 0.25 μL Qiagen HotStart Taq Polymerase. PCR products were analyzed on an eGene capillary electrophoresis machine (HDA-GT12 system, eGene, Inc., Irvine, CA). A reaction was considered clonal if one or more reproducible, dominant and discrete bands were present after electrophoresis. A reaction was considered negative if no bands, a diffuse smear or a ladder of faint bands was observed.

Table 1
Antigen, clone number and targets of antibodies used to immunophenotype the canine lymphoma cell lines by flow cytometry and immunocytochemistry.

Reagent	Clone #	Source
CD1a[c] allotype	Ca9.AG5	P.F. Moore, UC Davis
CD1c[a]	Ca13.9H11	P.F. Moore, UC Davis
CD3[b]	CD3-12	Serotec, Oxford, UK
CD3[c]	Ca17.2A12	P.F. Moore, UC Davis
CD4[c]	Ca13. 1E4	P.F. Moore, UC Davis
CD5[c]	YKIX.322	Serotec, Oxford, UK
CD11a[c]	Ca11.4D3	P.F. Moore, UC Davis
CD11a[c]	Ca16.1B11	P.F. Moore, UC Davis
CD11b[c]	Ca16.3E10	P.F. Moore, UC Davis
CD11c[c]	Ca11.6A1	P.F. Moore, UC Davis
CD11d[c]	Ca11.8H2	P.F. Moore, UC Davis
CD14[c]	TÜK 4	CALTAG, Burlingame, CA
CD18[c]	Ca1.4E9	P.F. Moore, UC Davis
CD20[b]	RB-9013	Neomarkers, Fremont, CA
CD21[a]	Ca2.1D6	P.F. Moore, UC Davis
CD34[c]	1H6	Peter McSweeney & Richard Nash, Fred Hutchinson Cancer Research Center, Seattle, WA
CD45[a]	Ca12.10C12	P.F. Moore, UC Davis
CD45RA[c]	Ca4.1D3	P.F. Moore, UC Davis
CD49D[c]	Ca4.5B3	P.F. Moore, UC Davis
CD54[c]	CL18.1D8	P.F. Moore, UC Davis
CD79a[b]	HM57	Dako, Carpenteria, CA
CD90[c]	Ca1.4G8	P.F. Moore, UC Davis
Granulocytic (unclustered)[c]	DM5	Brenda Sandmaier Fred Hutchinson Cancer Research Center, Seattle, WA
MHCII[a]	Ca2.1C12	P.F. Moore, UC Davis

[a] Antibody used for flow cytometry and immunocytochemistry.
[b] Antibody used for immunocytochemistry only.
[c] Antibody used for flow cytometry only.

2.7. Tumor cell growth in mice

All mouse studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Animals and were approved by the Animal Care and Use Committee of the National Cancer Institute. 8- to 10-week old immunodeficient athymic NCr-*nu*/*nu* female mice (NCI Animal Production Program, Frederick, MD) were used for all *in vivo* studies. Mice were housed in the animal facility at the National Institutes of Health in cages of 5 or fewer mice and fed animal chow and water *ad libitum*.

Each cell line was tested for viral contaminants prior to injection into mice. When cells of each cell line reached near confluence *in vitro*, a single cell suspension of 10×10^6 cells was implanted on the lateral aspect of the rear leg of 10–14 week old mice. Mice were injected with a tumor cell suspension, and subsequent tumors were passaged into new mice 2–4 times as cell suspensions. When tumors reached 1000 mm^3 or if mice showed signs of systemic illness, mice were euthanized and a full necropsy was performed by a veterinary pathologist. Masses from the inoculation site were excised and divided with 1/3 fixed in 10% neutral buffered formalin for histopathology, 1/3 embedded and frozen in optimal cutting temperature compound for immunohistochemistry, and 1/3 snap frozen for future studies.

2.8. P53 status

mRNA was purified from each cell line using TRIzol Reagent (Invitrogen), and then transcribed using oligo(dT)18 primer (Fermentas) and M-MLV reverse transcriptase (Promega). The upper p53 fragment (nt 18–932) was amplified from cDNA with primers 5′-AAGTCCAGAGCCACCATCC and 5′-TCAAAGCTGTTGCGTCCC, and the lower fragment (nt 727–1362) was amplified with primers 5′-GCCAAGTACCTGGACGACA and 5′-CAGGGAAGGAGGACGAGA. Both fragments were sequenced.

For detection of p53 protein, 3 cell lines were treated with or without 0.1 and 0.15 μg/mL doxorubicin, or 5 or 10 μM nutlin-3 for 18 h. Protein was extracted, and Western blot analysis was done, as previously described [14]. Briefly, cell lysates were made with 2× SDS sample buffer and boiled for 5 min. Proteins were then resolved in SDS-PAGE gels and transferred onto nitrocellulose membranes. Membranes were then subjected to blocking, washing, antibody incubation, and detection by enhanced chemoluminescence. Antibodies against p53 (FL-393), MDM2 (SMP14

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