Establishment of Five Canine Lymphoma Cell Lines and Tumor Formation in a Xenotransplantation Model

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(Received 7 October 2012/Accepted 14 November 2012/Published online in J-STAGE 28 November 2012)

ABSTRACT. Five novel, canine lymphoma cell lines (Ema, CLC, CLK, Nody-1 and UL-1) were established from dogs suffering from lymphoma and characterized in vitro and in vivo. All cell lines, except CLC, were characterized with T-cell phenotypes, by flow cytometric analysis and polymerase chain reaction for antigen receptor rearrangement. Cell proliferation rates and transcriptional levels of MYC, PTEN, KIT and FLT3 varied between each cell line. Intraperitoneal xenotransplantation of Ema, CLC, Nody-1 and UL-1 lymphoma cell lines into NOD/SCID mice induced ascites, intraperitoneal tumors and severe infiltration of lymphoma cells into the pancreas and mesentery. Establishment of novel canine lymphoma cell lines with different characteristics is critical for elucidating the pathophysiology of canine lymphoma and improving current therapies.

KEY WORDS: canine lymphoma, cell line, characterization, xenograft model.


Canine lymphoma is a common malignant tumor in dogs and has been recently recognized as an informative animal model to study human lymphoma [5]. Although canine lymphoma is typically treated by chemotherapy, B-cell lymphomas become resistant to chemotherapy after subsequent treatments, and T-cell lymphomas often do not respond well to chemotherapy from the onset of treatment [5]. Many studies have been performed to decipher the pathophysiology of canine lymphoma in order to improve curative treatments. Prognosis depends on many factors, such as histological type and lymphoma stage. Currently, there are several canine lymphoma cell lines available [6, 8, 11]. However, the establishment of additional canine lymphoma cell lines is important to further enhance our understanding of canine lymphoma pathophysiology.

Few studies have reported the xenotransplantation of canine lymphoma cells into immunodeficient mice [2, 4, 7]. Xenotransplantation of tumor cells is a method to elucidate the mechanisms of tumor formation and invasion. Additionally, the effects of newly developed therapies can be evaluated in vivo using established tumor cell lines in xenotransplantation models.

In the present study, we established five canine lymphoma cell lines and investigated the in vitro and in vivo properties of these cell lines.

MATERIALS AND METHODS

Case presentation: Case 1. A six-year-old female English Springer Spaniel Dog was referred to Yamaguchi University Animal Medical Center (YUAMEC), presenting with multiple abdominal lymphadenopathy, pleural effusion and ascites.

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Case 2. An eight-year-old female French Bulldog was referred to YUAMEC with multiple abdominal lymphadenopathy, splenomegaly, pleural effusion and ascites.

Case 3. An eight-year-old female Great Pyrenees Dog was referred to YUAMEC presenting with multiple abdominal lymphadenopathy and ascites.

Case 4. An eight-year-old male Shiba with abdominal mass and ascites, was referred to Iwate University Veterinary Teaching Hospital. All dogs were diagnosed with gastrointestinal lymphoma with cytology of the abdominal lymph node.

Case 5. A six-year-old male Labrador Retriever mix with renal mass, was referred to the University of Tokyo Veterinary Medical Center, and ascites was observed after treatment with chemotherapy. The dog was diagnosed with renal lymphoma with cytological sample of the kidney.

Cell culture of the primary materials: Primary lymphoma cells were obtained from clinical specimens, pleural or ascitic fluid and were cultured within 3 hr post collection. Clinical specimens were also analyzed by flow cytometry and polymerase chain reaction for antigen receptor rearrangement (PARR). In case 3, specimens were separated using the Lymphoprep™ kit (Axis-Shield PoC AS, Oslo, Norway) to avoid the contamination of inflammatory cells. Lymphoma cells were cultured in R10 complete medium [RPMI1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 55 µM 2-mercaptoethanol], except in case 1, in which recombinant human interleukin-2 (IL-2) (100 IU/ml; PeproTech, Rocky Hill, NJ, U.S.A.) was added to the R10 complete medium for the first 21 days. All cells were grown at 37°C in a humidified 5% CO2 incubator. Cells were cultured in fresh R10 complete medium every 2–3 days, at a ratio of 1:3 (Nody-1), 1:4 (Ema, CLK and UL-1) or 1:9 (CLC). Limiting dilution cloning was performed for the UL-1 cell line only. Each cell line grew stably without the addition of growth factors for more than 100 passages.

Flow cytometry: Primary lymphoma cells or established cell lines were collected and resuspended in FACS buffer (PBS containing 2% FBS and 0.1% Na2EDTA). A total of 2 × 10^6 cells were stained with primary antibody for 30 min on ice. After incubation, cells were washed and incubated with secondary antibody for 30 min on ice. After washing, cells were fixed in 1% paraformaldehyde and stored until analysis. Primary and secondary antibodies are shown in Table 1. For every specific antibody, a corresponding isotype control was used.

Polymerase chain reaction for antigen receptor rearrangement (PARR): DNA was extracted from each cell line using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, U.S.A.) according to the manufacturer’s instructions. PARR was performed as described previously [1, 3]. Briefly, each sample was amplified by two sets of primers (major and minor) for IgH and one set of primers for TCRγ. As a positive control for the DNA extraction process, the constant region of IgM (Cμ) was ampliﬁed for each sample. As positive and negative controls for the PCR amplification, genomic DNA previously shown to have IgH or TCRγ rearrangements by sequence analysis (or not), respectively, were used [3]. PCR products were electrophoresed on 12% polyacrylamide gels, stained with ethidium bromide and visualized with a UV illuminator.

Cell proliferation assay: All cell lines described above were suspended in cell culture medium and seeded in 12-well plates. Then, CLC (1 × 10^4 cells) or other cell lines (3 × 10^4 cells) were cultured in triplicates. Cells were collected after 24, 48 and 72 hr of incubation and counted by trypan blue dye exclusion using a hemocytometer.

Gene expression analysis by real-time PCR: Lymph node specimens were obtained and frozen from two healthy Beagle dogs, which were housed indoors and maintained according to the Yamaguchi University Animal Care and Use Committee regulations. For real-time PCR analysis, total RNA was isolated from cell pellets or frozen tissues with ISOGEN II reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. Total RNA (1 µg) was treated using the Turbo DNA-free kit (Ambion/Applied Biosystems, Tokyo, Japan), and cDNA was transcribed using the Superscript III kit (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) according to the manufacturer’s instructions. Oligo dT primers were used to prime the first-strand synthesis for each reaction. cDNA was subject to real-time PCR amplification using the QuantiTect SYBR Green PCR kit (QIAGEN) according to the manufacturer’s protocol. The primers used for assaying canine KIT, PTEN, MYC, FLT3 and RPL32 were as follows: forward 239 5'-CGAAGATGTGTGAAGCACAGGA-3', and reverse 240, 5'- GTGTCGCTACCTTCTGAAT-3'; forward 235, 5'- ACCAGGACACAGGGAAACCT-3', and reverse 236, 5'-CTGGCCGTACAGAAGTGAA-3'; forward 233, 5'-CGCTGCTGCTCTAAGAGATC-3', and reverse 234, 5'-CGCTCCTGCTGCACTCCTCC-3'; forward 1003, 5'-CAGGGGATTTGTGAGGACA-3', and reverse 1004, 5'-GGCAATTCAGGGAATGTTG-3'; forward 370, 5'-TGGTATTGAGGACCAAGAAA-3', and reverse 371, 5'-GCACATCAGCACACTTCCA-3', respectively. Each assay was performed in duplicate. PCR cycling conditions were as follows: 95°C for 15 min, followed by 45 cycles of 94°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. PCR and fluorescence intensity detection were performed with the StepOne PCR system (PerkinElmer, Inc., Waltham, MA, U.S.A.). The data were analyzed using StepOne software v.2.2.2. Briefly, the PCR cycle number at the threshold was represented as C(T), and the difference between C(T) for the target and internal control (∆CT) was calculated. The value of 2^(-∆CT) was considered to represent the amount of target mRNA relative to the amount of internal control.

Engraftment of canine lymphoma cell lines into NOD/SCID mice: All experiments involving NOD/SCID mice were approved by the Yamaguchi University Animal Care and Use Committee (approval number 164). Immune-deficient NOD/SCID mice at 6–8 weeks of age were obtained from Kyudo Co., Ltd. (Saga, Japan). All mice were kept in accordance with institutional guidelines and maintained in autoclaved conditions.
isolator cages in a specific pathogen-free area.

Viability of each cell line was assessed by trypan blue dye exclusion (viability exceeded 98% in all experiments), and cells were resuspended at 20 × 10^6 cells/ml in RPMI1640 medium alone. For each cell line, 10 × 10^6 cells were injected intraperitoneally into recipient mice (four mice per group). After inoculation, mice were observed daily for any abnormal clinical signs such as weakness, ascites accumulation or dyspnea. At 21 days post-inoculation, mice were sacrificed using diethyl ether anesthesia. Ascites was collected from each mouse, and liver, spleen, kidney, pancreas, stomach, small intestine, large intestine, heart, lung, brain, spinal cord and lymph nodes were removed. Tissues were fixed in 10% neutral-buffered formalin for 24 hr, transferred to 70% ethanol and embedded in paraffin. Glass slides with 3-µm sections were prepared and stained with hematoxylin and eosin.

### RESULTS

The results of the primary lymphoma cells: Pleural fluid was collected from cases 1 and 2, and ascites were collected from cases 3, 4 and 5. All fluid samples contained malignant lymphoblastoid cells. PARR analysis of clinical samples showed a monoclonal TCRγ gene rearrangement in cases 1, 3 and 5, but not in case 2. Only case 5 displayed a biallelic rearrangement of IgH (Table 2). Flow cytometric analysis of lymphoblastoid cells from cases 2, revealed positive expression of CD3 and CD45RA, while cells were negative for CD4, CD8α, CD11b, CD21, MHCII, IgM and IgG (Table 2). Lymphoblastoid cells from case 3 were positive for CD8α, MHCII and CD90, and negative for CD4, CD21, IgM and IgG, while certain populations were positive for CD3 (Table 2). Lymphoblastoid cells from case 5 were positive for CD8α and CD45RA and negative for CD3, CD4, CD90.
TCRαβ, TCRγδ, CD21, MHCII, IgM and IgG (Table 2). Owing to limited sample availability, immunophenotyping was not performed for case 1, and PARR and immunophenotyping were not performed for case 4.

Cases 1 and 3, from which the Ema and CLK cell lines were later derived, were diagnosed as T-cell lymphoma using PARR. Case 2, from which the CLC cell line was derived, was diagnosed as T-cell lymphoma by flow cytometric analysis of CD3 expression. The cellular phenotype of cases 4 and 5, from which the Nody-1 and UL-1 cell lines were derived, could not be identified, since immunophenotyping and PARR analysis were not carried out in case 4, and PARR analysis revealed rearrangement of both TCRγ and IgH gene in case 5.

**Characteristic analysis of established cell lines**: Ema, CLC, CLK, Nody-1 and UL-1 cell lines were established from cases 1, 2, 3, 4 and 5, respectively. Ema and Nody-1 cell lines were characterized by growth in small clusters of cells, whereas CLC, CLK and UL-1 cell lines grew as single cells (Fig. 1a). Ema, CLC, Nody-1 and UL-1 cells showed mostly round morphology, and CLK cells exhibited a characteristic spindle-like shape. Analysis of intracellular morphology by Giemsa staining, revealed that all cell lines appeared to be immature, large lymphocytes (Fig. 1a). The cytoplasm of all cell lines was moderately basophilic, and the cytoplasm of CLC, Nody-1 and UL-1 had minute vacuoles. Ema, CLK, Nody-1 and UL-1 cell lines had slightly unclear nucleoli, whereas the nucleus of CLC cells contained apparent nucleoli.

**Immunophenotypic analysis of established cell lines**: Flow cytometric analysis of surface antigens revealed characteristic expression patterns for each cell line (Table 3). All cell lines expressed CD45 and CD45RA, indicating these cells were derived from lymphocytes. All cell lines were negative for CD4, CD11b, CD14, CD21, CD34, TCRαβ, IgM and IgG. In addition, the Ema cell line expressed CD3, CD90 and TCRγδ, and was negative for CD8α, CD11a, CD11c, CD18 and MHCII expression. The CLC cell line expressed CD18 and MHCII, and was negative for CD3, CD8α, CD11a, CD11c, CD90 and TCRγδ expression. The CLK cell line expressed CD8α, CD11a, CD11c, CD90 and TCRγδ expression. The Nody-1 cell line expressed CD8α, CD11a, CD11c, CD90 and TCRγδ expression. The UL-1 cell line expressed CD8α and CD18, and was negative for CD3, CD8α, CD11a, CD11c, CD90 and TCRγδ expression. Ema, CLK, Nody-1 and UL-1 cells were considered to represent T-cell phenotypes, since they expressed at least one T-cell marker (CD3 or CD8α) and were negative for all B-cell markers (CD21, IgM and IgG).
Table 3. Phenotypic characteristics of canine lymphoma cell lines

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*Antigen receptor rearrangement of the established cell lines:* No cell lines contained immunoglobulin rearrangements by PARR analysis using B major and B minor primer pairs (Fig. 1b). All cell lines, except CLC, showed single or multiple TCRγ rearrangements (Fig. 1b). Taken together, all cell lines, except CLC, displayed T-cell phenotypes, however, in the case of CLC, immunophenotype could not be inferred from flow cytometric and PARR analyses.

*In vitro cell growth analysis:* Analysis of cell growth revealed a doubling time of 14.5, 20.9, 21.5, 26.6 and 36.2 hr, for CLC, UL-1, Nody-1, Ema and CLK cells, respectively (Fig. 2). The CLC cell line displayed the fastest cell growth rate. In contrast, CLK showed the slowest cell growth rate. UL-1 and Nody-1 cells grew at comparable rates, which were faster than that of the Ema cell line.

*Gene expression analysis of cell lines:* Real-time PCR was used to investigate the expression of MYC, PTEN, KIT and FLT3 transcripts. MYC expression was clearly upregulated in all cell lines compared with normal lymph nodes, except for Ema, in which a similar expression level was identified (Fig. 3a). We identified a significant upregulation of KIT in Nody-1 cells (Fig. 3c). All cell lines displayed upregulation of PTEN (Fig. 3b) and downregulation of FLT3 (Fig. 3d) compared with normal lymph nodes.

*Examination of inoculated NOD/SCID mice post mortem:* The in vivo characteristics of these five canine lymphoma cell lines were investigated by xenotransplantation into NOD/SCID mice. A total of 75% (3/4 mice) injected with the CLC cell line died 12–13 days post-transplantation, while 25% (1/4 mice) injected with the Nody-1 cell line died 19 days post-transplantation (Table 4). Mice transplanted with other cell lines were dissected 21 days post-transplantation. Ascsites were observed in all xenotransplanted mice, except for CLK-transplanted mice (Table 4, Fig. 4a). Large amounts of ascitic fluid were present in CLC-transplanted mice compared to mice transplanted with the other three cell lines. Lymphoma cells were observed by Diff-Quick staining of smears of ascitic fluid obtained from all mice (data not shown).

Mice transplanted with the CLK cell line did not form tumors in any organs, except for one mouse that exhibited an enlarged mediastinal lymph node. All canine lymphoma cell lines, except CLK, formed tumors in some peritoneal organs in at least three out of four xenotransplanted mice (Table 4). It was difficult to accurately measure the size of each tumor, because they strongly adhered to the gastrointestinal tract serosal surface and poorly marginated. The extent of each tumor varied among each group of mice.

**DISCUSSION**

In the present study, we established five canine lymphoma cell lines and compared their in vitro and in vivo properties. Of these, Ema, CLK, Nody-1 and UL-1, were identified as
T-cell lymphoma cell lines by PARR analysis, however, the immunophenotype of CLC was not identified in this study. Although the original patient sample from which CLC line was derived was positive for CD3 expression by flow cytometry, a cellular phenotype could not be identified by PARR or flow cytometry analyses. In some cases, the cell surface markers on established cell lines are lost during long-term culture compared to the primary isolated tumor cells [11]. Flow cytometric analysis of the CLC and CLK cell lines revealed that the expression of many antigens was lost compared to the original patient samples (Table 3).

Previous studies have shown that the expression levels of various genes associated with tumorigenesis (MYC, KIT, FLT3, and PTEN) are altered in canine lymphoma cell lines owing to genomic amplification or deletion, as elucidated by CGH analysis [4]. The expression of MYC is often upregulated in canine lymphoma cell lines, partly due to a gain in copy numbers of dog chromosome 13 (CFA13) [9]. Consistent with this study, we observed upregulation of MYC in four of five cell lines. While KIT was also reported to be upregulated in several canine lymphoma cell lines, we observed overexpression of KIT in only one of five cell lines (Nody-1). Mutation of FLT3 also contributes to tumorigenesis in certain canine acute lymphoblastic leukemia cases [10]. In this study, however, we observed significant downregulation of FLT3 expression compared to normal lymph nodes in all five cell lines. This suggests that FLT3 may not be relevant to tumorigenesis in the cell lines used in this study, although we did not examine the expression of FLT3 in primary samples. Unexpectedly, PTEN was overexpressed in all five cell lines derived in this study. Previous studies demonstrated decreased PTEN expression due to homozygous deletion on CFA26 in 2 out of 5 cell lines [9], and thus the significance of PTEN overexpression in our cell lines remains to be explored.

Xenotransplantation of cell lines in this study revealed several differences in the sites and degree of tumor infiltration among the five canine lymphoma cell lines. The majority of mice injected with Ema, CLC, Nody-1 and UL-1 cells formed tumors, whereas CLK-transplanted mice did not. In keeping with this observation, the growth rate of CLK cells was significantly lower compared with the other cell lines, which may have led to early elimination of tumor cells affecting tumor formation.

In these xenotransplantation studies, four mice died before being euthanized on day 21 (one CLC, day 12; two CLC, day 13; one Nody-1, day 19; Table 4). Microscopic analysis of organs in these mice, revealed tumor cell infiltration in many organs in CLC- and Nody-1-injected mice, compared to mice injected with the other cell lines. Since mice with greater volumes of ascitic fluid tend to die earlier, the cause of death may have been because of high pressure on the pleural cavity and abdominal organs due to the ascitic fluid.

In xenotransplanted mice, the pancreas and mesentery were the most tumor-infiltrated organs for all cell lines, except CLK. These results are consistent with a previous study where canine T-cell lymphoma cell lines were transplanted intraperitoneally into NOD/SCID mice [7]. Since tumor cells were injected intraperitoneally, it is reasonable that the mesentery was often affected, however, why the pancreas was also a primary target in most mice remains unclear. Tumor infiltration of the kidney and spleen was detected only in Nody-1-transplanted mice. Tumor infiltration of other organs, including the liver and gastrointestinal tract was also severe in Nody-1-transplanted mice, compared with the other cell lines. Infiltration by lymphoma cells was detected in the visceral surfaces of most organs (Table 4 and Fig. 4). However, lymphoma cells infiltrated around the hepatic triad of the liver in Nody-1-transplanted mice, suggesting hematogenous dissemination. No infiltration was observed in the gastrointestinal tracts of mice transplanted with UL-1 cells or in the livers of mice transplanted with Ema cells. These results demonstrate clear differences in the sites of tumor infiltration between the five canine lymphoma cell lines. Thus, in addition to differences in growth rates, each cell line may preferentially disseminate to different organs, and these characteristics may be related to lymphoma pathogenesis.

In this study, lymphoma cell lines were injected intraperitoneally into mice, since most cell lines were established from ascitic samples. However, this method of delivery may
Future studies employing other routes of injection, such as intravenous or subcutaneous xenotransplantation, are also required to completely characterize these lymphomas.

In the present study, we established and characterized five canine lymphoma cell lines, Ema, CLC, CLK, Nody-1 and UL-1, using both in vitro and in vivo methods. To date, our knowledge of the mechanisms of pathophysiology underly-
ing canine lymphoma, is limited by an insufficient number of canine lymphoma cell lines. The development of several cell lines with different characteristics will significantly enhance our understanding of canine lymphoma pathophysiology and the development of new therapies. Recently, five canine B-cell lymphoma cell lines were described [11]. Comparison of the cell lines established in this study with these published cell lines will aid our understanding of canine lymphoma and the differences in prognosis or resistance to chemotherapy between T-cell and B-cell lymphoma in dogs.

ACKNOWLEDGMENTS. We would like to acknowledge the technical expertise of the DNA Core Facility of the Center for Gene Research, Yamaguchi University. This study was supported by a Grant-in-Aid for Young Scientists (A) (21688022) from the Ministry of Education, Culture, Sports, Science and Technology (Japan).

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