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Research paper

Identification of a candidate therapeutic antibody for treatment of canine B-cell lymphoma

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ABSTRACT

B-cell lymphoma is one of the most frequently observed non-cutaneous neoplasms in dogs. For both human and canine BCL, the standard of care treatment typically involves a combination chemotherapy, *e.g.* "CHOP" therapy. Treatment for human lymphoma greatly benefited from the addition of anti-CD20 targeted biological therapeutics to these chemotherapy protocols; this type of therapeutic has not been available to the veterinary oncologist. Here, we describe the generation and characterization of a rituximab-like anti-CD20 antibody intended as a candidate treatment for canine B-cell lymphoma. A panel of anti-canine CD20 monoclonal antibodies was generated using a mouse hybridoma approach. Mouse monoclonal antibody 1E4 was selected for construction of a canine chimeric molecule based on its rank ordering in a flow cytometry-based affinity assay. 1E4 binds to approximately the same location in the extracellular domain of CD20 as rituximab, and 1E4-based chimeric antibodies co-stain canine B cells in flow cytometric analysis of canine leukocytes using an anti-canine CD21 antibody. We show that two of the four reported canine IgG subclasses (cIgGB and cIgGC) can bind to canine CD16a, a receptor involved in antibody-dependent cellular cytotoxicity (ADCC). Chimeric monoclonal antibodies were assembled using canine heavy chain constant regions that incorporated the appropriate effector function along with the mouse monoclonal 1E4 anti-canine CD20 variable regions, and expressed in CHO cells. We observed that 1E4-cIgGB and 1E4-cIgGC significantly deplete B-cell levels in healthy beagle dogs. The *in vivo* half-life of 1E4-cIgGB in a healthy dog was ~14 days. The antibody 1E4-cIgGB has been selected for further testing and development as an agent for the treatment of canine B-cell lymphoma.

1. Introduction

Canine lymphoma is a significant veterinary problem, comprising up to a quarter of all canine cancers. The majority of canine lymphomas (≥60%) are of B-cell origin (B-cell lymphoma; BCL), while the remainder are T-cell derived or of mixed B-and T-cell immunophenotype (Modiano et al., 2005). The current standard of care for canine BCL is a protocol employing several chemotherapeutic drugs, commonly cyclophosphamide,

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hydroxydaunorubicin/doxorubicin, oncovin/vincristine and prednisone (CHOP therapy; Garrett et al., 2008). Left untreated, dogs with lymphoma typically only survive about 6 weeks from time of diagnosis. CHOP therapy extends the survival time of dogs with BCL to 10–14 months (Richards and Suter, 2015). Most dogs experience complete remission followed by one or more relapses. CHOP therapy is rarely curative in dogs, possibly because veterinary protocols tend to be less dose-intensive as compared to human CHOP protocols; in fact, only ~20% of treated dogs will survive beyond 2 years (Richards and Suter, 2015).

The most common form of BCL in humans is non-Hodgkins lymphoma (NHL). The advent of the monoclonal antibody rituximab revolutionized human BCL treatment by significantly increasing response and survival rates in NHL patients over chemotherapy alone. The standard of care for human NHL is now rituximab in combination with CHOP (R-CHOP) (Dotan et al., 2010; Molina, 2008). The target of rituximab is CD20, a tetra-membrane spanning protein expressed on B-cell lymphomas and normal B-cells from the pre-B-cell through memory B-cell stages. The fact that CD20 is not expressed on plasma cells makes it a particularly attractive therapeutic target, as depletion of CD20-positive B-cells does not eliminate the antibody response to foreign pathogens (van Meerten and Hagenbeek, 2010). The actual function of CD20 is not well understood, but it may play a role in Ca^{2+} transport across the plasma membrane (Bubien et al., 1993; Li et al., 2003).

Rituximab is a chimeric antibody, composed of mouse variable regions that bind to CD20 and a human IgG1 constant/Fc region that mediates effector functions of the antibody. Rituximab works by depleting CD20-positive B-cells, largely *via* antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (Beers et al., 2010). It has been shown that, as is the case in humans, canine B-cell lymphomas express CD20 but canine T cell lymphomas do not (Jubala et al., 2005; Kano et al., 2005). For this reason, a rituximab-like therapeutic specific to canine CD20 could potentially be effective in treating BCL in dogs. Rituximab does not bind to canine CD20, likely due to a lack of conservation of the rituximab epitope in the canine protein (Impellizeri et al., 2006; Jubala et al., 2005), making it impossible to use rituximab for treatment of canine BCL. Here, we describe the generation and characterization of a chimeric anti-CD20 antibody specifically designed for therapeutic B-cell depletion in dogs. This reagent should enable the evaluation of the effectiveness of anti-CD20 therapy for BCL in dogs.

2. Materials and methods

2.1. Cloning and expression of canine and human CD20 ECD2 fusion proteins and hybrids

The second extracellular domain (ECD2) of canine and human CD20 was cloned by RT-PCR. RNA was isolated from canine or human PBMCs (Bioreclamation, Westbury, NY) using Qiagen RNeasy and QIAshredder kits (Qiagen, Valencia, CA). The ThermoScript RT-PCR system (Life Technologies, Carlsbad, CA) was used to generate cDNA using random hexamers. Canine CD20 was amplified from cDNA using primers BE-001 and BE-003, and human CD20 was amplified from human cDNA using primers BE-142 and BE-147. PCR products were then cloned into the pFUSE-mFc2 (IL2ss) and pFUSE-hIgG2-Fc2 vectors (InvivoGen; San Diego, CA) at *EcoR*I and *Bgl*II sites.

Hybrid versions of canine CD20 containing human CD20 sequence were also generated for epitope mapping studies. Versions 1 and 2 were made by amplifying canine ECD2 with primers BE-001 and BE-146 and human ECD2 with primers BE-145 and BE-147, then fusing these cassettes by overlapping PCR using primers BE-001 and BE-147 (Version 1 was an unexpected product of this cloning reaction). Version 3 was made by amplifying cloned WT canine ECD2 with primers BE-001 and BE-148. Version 4 was made by amplifying cloned Version 2 with primers BE-001 and BE-149. Final PCR products were cloned into pFUSE-mFc2 (IL2ss) as above.

Plasmid DNA for each construct was transfected into 293FS cells using 293Fectin reagent (Life Technologies). Protein was purified from cell supernatants using an AKTA FPLC liquid chromatography system and HiTrap Protein A or Protein G HP columns (GE Healthcare, Piscataway, NJ). Proteins were eluted using Ig Elution Buffer (Thermo Fisher Scientific/Pierce, Rockford, IL) and buffer exchanged into PBS by dialysis.

2.2. Hybridoma generation and screening

Mice were immunized with canine CD20-ECD2-mFc protein using a RIMMS-based protocol (Kilpatrick et al., 1997), and hybridomas were generated and subcloned essentially as previously described (Cohen et al., 2005), with the exception that F0 cells were used as the fusion partner instead of SP2/0 cells. Hybridoma supernatants were screened by ELISA as described (Cohen et al., 2005) using canine CD20 ECD2-hFc as bait. Supernatants from ELISA-positive hybridomas were then tested by flow cytometry for binding to native canine CD20 on the surface of canine B-cell lymphoma cells.

2.3. Preparation of canine tumor cells

Canine B-cell tumor samples were obtained from Veterinary Specialty Hospital (San Diego, CA). Tumors were dissociated into single cells for flow cytometry analysis as follows: excised tumors were placed in DMEM:F12 (Thermo Fisher Scientific/Hyclone) supplemented with 10% FBS (Life Technologies) and 1% antibiotic/antimycotic (Sigma, St. Louis, MO). Following media aspiration, tumors were chopped with a straight-edge razor in a 10 cm tissue culture dish until a fine minced paste consistency was achieved. Tissues were then digested in media with collagenase 3 (Worthington, Lakewood, NJ) for 2–3 h. Occasional pipette trituration of the mixture was used to aid in digestion. The resulting cell suspension was then passed successively through 100 μM and 40 μM strainers (BD Pharmingen, San Jose, CA). Cells were spun at 1000 rpm for 5 min and washed with media prior to flow cytometry use