Canine CD34: Cloning of the cDNA and Evaluation of an Antiserum to Recombinant Protein

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Increasingly, enriched populations of hematopoietic progenitors are used in experimental and clinical transplantation studies. The separation of progenitors is based on the expression of CD34, a marker preferentially expressed on progenitor cells. The dog model has been important for preclinical transplant studies, because it has proven predictive for outcomes in human hematopoietic stem cell transplantation. To identify and isolate canine hematopoietic progenitors, we have cloned a cDNA encoding a CD34 homologue from a canine myelomonocytic leukemia cell line, ML2. The CD34 homologue cDNA predicts an amino acid sequence that is highly conserved with human and murine CD34 in the cytoplasmic domain, transmembrane domain, and C-terminal end of the extracellular domain, but shows considerable divergence from these sequences at the amino-terminal end of the protein. In Western blotting studies, canine CD34 homologue (caCD34) appears to be a heavily and variably glycosylated protein with a molecular weight of approximately 100 kD and shows some tissue-specific differences in protein mass. To evaluate the expression of caCD34 protein, the extracellular domain of caCD34 was expressed as an Ig fusion protein and used as an immunogen to generate a rabbit polyclonal antisera. The antisera reacted against canine bone marrow cells stained brightly with antibodies to caCD34 and this population was 25- to 50-fold enriched for colony-forming units-granulocyte-macrophage as compared to unfractionated marrow mononuclear cells. These findings suggest that the canine CD34 homologue is expressed on bone marrow progenitor cells and, thus, that this molecule should be a valuable marker for identifying and isolating canine hematopoietic progenitors for experimental hematopoiesis and stem cell transplantation. © 1996 by The American Society of Hematology.

CD34 is a Type 1 transmembrane protein expressed primarily on primitive hematopoietic progenitor cells and vascular endothelium from many tissues. Cell surface expression of CD34 is developmentally regulated in hematopoiesis and is inversely related to the stage of differentiation, such that CD34 expression is lost beyond the committed progenitor stage. This pattern of expression suggests an important role of CD34 in early hematopoiesis. The genes for human and murine CD34 have been cloned and have significant homology of the genomic structure, cDNA sequence, amino acid sequence, and predicted protein structure. Predicted structural features of these proteins suggest properties of an adhesion molecule of the sialomucin class of molecules and murine studies showed that vascular progenitor cells may be a ligand for leukocyte L-selectin with a possible in vivo role in vascular adhesion. The function of CD34 is not fully understood and human and murine CD34 have no close homology to other known proteins.

The restricted expression of CD34 has allowed for antibodies to human CD34 and to murine CD34 to be used to identify and enrich hematopoietic progenitors as determined by in vitro and in vivo studies. In humans, monoclonal antibodies (MoAbs) to CD34 recognize about 1.5% of marrow mononuclear cells and CD34-enriched progenitor cells, derived from marrow or peripheral blood stem cells (PBSC), have the capability to fully restore hematopoiesis after treatment with myeloablative chemotherapy. CD34 selection technology is an active area of clinical research as a method for purging autografts of tumor or T-cell-depleting stem cell allografts to prevent graft-versus-host disease (GVHD). The dog has a proven role in experimental marrow transplantation and results of canine transplantation studies have predicted findings that have led to important advances in allogeneic transplantation. Because no marker of early canine hematopoietic progenitors has previously been reported, we undertook to determine whether the canine homologue for CD34 (caCD34) would be such a marker. We describe here the cloning of a cDNA for caCD34 and the characterization of cells expressing this protein.

MATERIALS AND METHODS

Cell lines and cell culture. The canine leukemia cell lines ML1, ML2, ML3 (all myelomonocytic), 1390 (CD8+ leukemia) (ML2, ML3, and 1390 were derived from spontaneous canine leukemias and provided by P. Moore, University of California, Davis, CA), and CLG-90 (large granular cell leukemia; M. Wellman, manuscript in preparation) were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 25 mmol/L HEPES, 0.1 mmol/L minimum essential medium (MEM) nonessential amino acids, 1 mmol/L sodium pyruvate, and 0.05 mmol/L 2-mercaptoethanol. Jugaline vein endothelial cells from normal dogs were purchased from a canine myelomonocytic leukemia cell line, that is highly conserved with human and murine CD34 in tors, we have cloned a cDNA encoding a CD34 homologue that is used in experimental and clinical transplantation studies. The separation of progenitors is based on the expression of CD34, a marker preferentially expressed on progenitor cells. The dog model has been important for preclinical transplant studies, because it has proven predictive for outcomes in human hematopoietic stem cell transplantation. To identify and isolate canine hematopoietic progenitors, we have cloned a cDNA encoding a CD34 homologue from a canine myelomonocytic leukemia cell line, ML2. The CD34 homologue cDNA predicts an amino acid sequence that is highly conserved with human and murine CD34 in the cytoplasmic domain, transmembrane domain, and C-terminal end of the extracellular domain, but shows considerable divergence from these sequences at the amino-terminal end of the protein. In Western blotting studies, canine CD34 homologue (caCD34) appears to be a heavily and variably glycosylated protein with a molecular weight of approximately 100 kD and shows some tissue-specific differences in protein mass. To evaluate the expression of caCD34 protein, the extracellular domain of caCD34 was expressed as an Ig fusion protein and used as an immunogen to generate a rabbit polyclonal antisera. The antisera reacted against canine bone marrow cells stained brightly with antibodies to caCD34 and this population was 25- to 50-fold enriched for colony-forming units-granulocyte-macrophage as compared to unfractionated marrow mononuclear cells. These findings suggest that the canine CD34 homologue is expressed on bone marrow progenitor cells and, thus, that this molecule should be a valuable marker for identifying and isolating canine hematopoietic progenitors for experimental hematopoiesis and stem cell transplantation. © 1996 by The American Society of Hematology.

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from Endotech (Indianapolis, IN) and cultured according to the manufacturer’s instructions.

Northern blotting. Total cellular RNA was prepared from cell suspensions by guanidinium isothiocyanate lysis20 using RNAzol A (Texas Biotech, Houston, TX). Fifteen or 30 μg of RNA was run on a 1% glyoxal denaturing agarose gel and transferred to nylon membranes. Membranes were probed with a human CD34 cDNA probe (kindly provided by Dr D. Tenen, Harvard Medical School, Cambridge, MA) radiolabeled with [α-32P]dCTP (NEN Dupont, Boston, MA) using the random hexanucleotide priming method.21

Reverse transcription-polymerase chain reaction (RT-PCR). PCR primers 34A and 34B were designed from sequences of highest homology when comparing human and murine CD34 cDNAs. Primers were (see Fig 1A) (a) 34A (sense) 5'-CCGAATTCGCTC-TTGGCAGCTGCTGGAGG; (b) 34B (antisense) 5'-CCGAAATTC-ACGTTGGTCTTGCTGAAATG; (c) 34C (sense) 5'-TACGAGCCTTGGACAGGATGCTGCCCAGGCA; and (d) 34D (antisense) 5'-CGAAGATATGCTTGGTTAGGTAATGAC. EcoRI (A, B, and D) and HindIII (C) restriction sites (underlined) were included. Reverse transcription and PCR were performed as previously described.22 Samples were electrophoresed through 2% agarose gels and analyzed by ethidium bromide staining and/or probing of Southern blots with caCD34-specific [γ-32P]dATP end-labeled oligonucleotides. PCR products were cloned into the EcoRI restriction site of pBluescript (Stratagene, La Jolla, CA) or directly into pT7Blue T-Vector (Novagen, Madison, WI)23 and sequenced as described below. To extend the 5' end of the cDNA, rapid amplification of cDNA ends24 (5' RACE) was performed using two commercial kits (GIBCO BRL [Gaithersburg, MD] and Clontech [Palo Alto, CA]) as described in the instructions from the manufacturers.

Screening of canine cDNA and genomic phage libraries. A random-primed cDNA library was made in Lambda ZAP (Stratagene) from poly-A RNA purified from the ML2 cell line. Inserts were cloned nondirectionally into EcoRI restriction sites. Nylon filters (Amer sham, Arlington Heights, IL) lifted from plates containing 1.2 × 106 plaques were probed in buffer containing 50% formamide at 42°C with caCD34-specific probes radiolabeled by random hexamer priming. Filters were washed at a final stringency of either 0.5× or 0.1× SSPE at 42°C and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY) at -70°C for 1 to 3 days. Phage elutes from positive clones were evaluated for insert size by PCR using 34A and 34B or gene-specific primers (GSP), combined with vector-specific RNA-polymerase site primers, T3 or T7. Clones selected for further characterization were converted to plasmids (pBluescript SK(-)) by an in vivo excision.25 A canine genomic phage library made in Lambda Fix II (Stratagene) was screened with a cDNA clone and then with genomic subclones. Clones were characterized by restriction enzyme mapping, Southern blotting with OGP oligonucleotides, and PCR using primer pairs predicted to be located in different exons of caCD34. Clones were sequenced on both strands using the diodeoxynucleotide method26 or by PCR-based cycle sequencing with the Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Inc (Foster City, CA) using an Applied Biosystems 373A DNA sequencer. Sequence analysis was performed with Genepro 4.2 software (Riverside Scientific Enterprises, Bainbridge Island, WA) and software from Genetics Computer Group (GCG) Inc (Madison, WI).

Production of recombinant caCD34. An expression construct for the extracellular domain of caCD34 was made by PCR using primers 34F (93 bp; sense, 5'AGGAAGCTTCTCGAGATGCAGTCTGGTCCGAGGCGCAGGGCCGAGGAGATGCGCGGGGCTTGAGCCCGCTTGGCTGCGTCAGTCTCG; amino acid sequence, MLVRRGARAGPRMPRGWTALCLLSLL) with 5' HindIII and Xho I restriction sites (underlined) and 34G (32 bp, antisense) 5'-CTCCAGATCTGGCTTGGGGAATAGCTCTGGT with a Bgl II restriction site (underlined). The PCR product was cloned into an expression plasmid containing a murine Ig heavy chain sequence. A caCD34-murine Ig fusion protein (CD34-Ig) and a control fusion protein (murine CTLA4-Ig containing the same murine Ig sequence) were produced by transient expression in COS cells and purified as previously described.27 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed.
with precast 4% to 20% gels (Novex, San Diego, CA) using the manufacturer’s instructions. Automated N-terminal sequencing was performed at the University of Victoria Microanalytical Centre (British Columbia, Canada) using an Applied Biosystems model 473 pulsed liquid sequencer.

Immunization of rabbits and production of affinity-purified anti-CD34 polyclonal antiserum (RPecaCD34). A female New Zealand white rabbit was used to generate a polyclonal antiserum to caCD34 as follows. Primary immunization was with combination of 2 mg CD34-lg in 250 μL phosphate-buffered saline (PBS), 250 μL septic montanide ISA 50, and RIBI adjuvant injected subcutaneously in 50-μL aliquots at 10 sites. The animal was boosted at 1 month with 1 mg CD34-lg, at 6 months with ML2 cells, at 8 months with ML2 cells, and at 10 months with a combination of ML2 cells and canine endothelial cells from primary culture. Rabbit IgG from serum was purified on immobilized recombinant protein A (1A-300; Repligen, Cambridge, MA). The subfraction of IgG specific for CD34 was then isolated in two steps. First, IgG reactive with the IgG tail of the immunogen was removed by adsorption to a column of the immobilized control fusion protein (CTLA4-lg). Second, IgG reactive to CD34-lg was affinity-isolated on a column of immobilized CD34-lg. Columns were prepared by immobilization of protein onto CNBr-activated Sepharose 4B using the manufacturer’s instructions (Pharmacia Biotech, Uppsala, Sweden).

Anti-CD34 enzyme-linked immunosorbent assay (ELISA). The anti-CD34 ELISA was performed as described previously with the following modifications. Immunlon 2 (Dynatech) flat-bottom plates were coated with 3 μg/mL CD34-lg diluted in 0.05 mol/L bicarbonate binding buffer, pH 9.6. Bound antibody was detected with a 1:8,000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG antibody (Southern Biotechnology, Birmingham, AL). Western blotting. Cells were washed in PBS and lysed with a solution of 1% NP40, 150 mmol/L NaCl in 50 mmol/L Tris, pH 8.0. Nuclei were removed by 10 minutes of centrifugation at 10,000 g and lysates were stored at −70°C. Lysates were electrophoresed in a reduced Tris/glycine SDS 8% polyacrylamide gel (Novex). Protein was transferred to a precut polyvinylidene difluoride (PVDF) membrane (Novex) using a wet Western transfer apparatus (Novex) in Tris-glycine transfer buffer. Membranes were blocked in 5% nonfat dry milk in PBS and then incubated with either RPecaCD34 or control rabbit serum followed by incubation for 1 hour with goat antirabbit alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, IN) diluted in blocking solution. Membranes were washed four times with TTBS (2.5 mmol/L Tris, 50 mmol/L NaCl, 0.5% Tween-20, pH 7.5) and proteins were visualized with Western blue AP substrate (Promega).

Flow cytometry. The following antibodies were used: RPecaCD34, S50 (murine IgG1 MoAb, anti-CD44, as a positive control), 31A9 (a murine IgG1, nonreactive with canine hematopoietic cells), and normal rabbit serum. RPecaCD34 was used at 10 μg/mL, rabbit serum at a dilution of 1:100, and S5 and 31A at 5 μg/mL. A fluorescein isothiocyanate (FITC)-conjugated polyclonal goat-antirabbit antibody (Caltag, San Francisco, CA) or a phycoerythrin (PE)-conjugated polyclonal goat-antirabbit antibody (Southern Biotechnology) and an FITC-conjugated goat-antimouse polyclonal antibody (Caltag) were used as secondary-stage antibodies. Ficol-Hypaque–separated bone marrow mononuclear cells (BMMC) and peripheral blood mononuclear cells adjusted to 5 × 107 cells/mL were stained for 20 minutes at 4°C at each stage and washed with PBS/2% horse serum. For some experiments, 100-μL aliquots of unfractionated marrow or blood were stained after the addition of 50 μL of PBS/2% horse serum and washed after each stage with PBS/2% horse serum, and then erythrocytes were lysed with hemolytic buffer. Cells were fixed in 1% paraformaldehyde before analysis. Cell lines and cultured endothelial cells were incubated with RPecaCD34 (10 μg/mL), CD34-lg (100 μg/mL), or a combination of the two. Cells were then washed with PBS, incubated with FITC-conjugated secondary antibodies, and washed with PBS. Flow cytometry was performed on a FACScan (Becton Dickinson, San Jose, CA) or FACSStar (Becton Dickinson), and the list mode data were analyzed using Reproman software (True Facts Software Inc, Seattle, WA) and Cellquest Software (Becton Dickinson).

 Colony-forming unit–granulocyte-macrophage (CFU-GM) assays. BMMC (2 × 105/mL) were stained with RPecaCD34 as described above, resuspended in PBS/2% horse serum, and sorted on a FACS Star (Becton Dickinson). Lineage depletion of canine BMMC was performed with the following MoAbs: JD3 (anti-CD8),11 1E4 (anti-CD4),12 Tul4 (antimonocyte; DAKO, Glostrup, Denmark), and DM5 (antigranulocyte),12 using the magnetic activated cells sorter (MACS; Miltenyi Biotec, Sunnyvale, CA) separation system as per the manufacturer’s instructions. Sorted and immunomagnetically separated cell fractions were washed twice and 500, 103, 5 × 103, or 103 cells per plate were assayed for granulocyte-macrophage progenitor cells (CFU-GM), as previously described.13

Immunoperoxidase staining of tissue sections. Normal dog tissues were snap frozen in liquid nitrogen and 6-μm sections were cut onto glass slides using a Tissue Tek cryostat (Miles Scientific, Naperville, IL). Air-dried slides were fixed in acetone and in formyl calcium, and staining was performed using a TechMate 1000 Immunostainer (BioTek Solutions, Inc, Santa Barbara, CA). Sections were treated with 5% goat serum/2% bovine serum albumin (BSA) fraction (Calbiochem, La Jolla, CA), Tween-20, and 0.4% sodium azide. Staining with RPecaCD34 (2.5 μg/mL) was detected by sequential applications of biotinylated goat-antirabbit (Vector, Burlingame, CA) and horseradish peroxidase-streptavidin (Zymed, San Francisco, CA) followed by diaminobenzidine (Polysciences, Warrington, PA) at 0.5 μg/mL, 0.1% NiCl2, and 0.01% H2O2. Slides were counterstained with 0.1% acridine orange/0.1% safronin O and dehydrated rapidly through graded alcohols. As negative controls, staining was performed on canine tissues without primary antibody and on human lung to assess nonspecific tissue reactivity of RPecaCD34. A rabbit polyclonal antihuman factor VIII (Dako, Carpinteria, CA) was used as a positive control to identify endothelial cells.

RESULTS

Isolation of the cDNA for caCD34. In Northern blotting studies using a human CD34 probe, the ML2 cell line yielded a transcript of approximately 2.8 kb, slightly larger than that from the KG1 myeloid leukemia cell line (human), which is approximately 2.6 kb. This transcript was not detected from ML1 or BMMC. In RT-PCR studies using primers 34A and 34B, a strong amplification signal of approximately 450 bp was detected from both KG1 and ML2, whereas weak signals were detected from ML1 and canine bone marrow, consistent with Northern blotting results. Sequence of the PCR fragment isolated from ML2 was highly homologous with 3’ coding sequence of human and murine CD34 cDNAs and this fragment was used as a canine-specific CD34 probe to screen the ML2 cDNA library. Initial screening yielded 60 positive clones and, based on PCR analyses of the insert sizes three clones, 54-1, 44-3, and 28-1 (Fig 1A), were further characterized. Clone 54-1 extended 500 bp further 5’ than any other clone and contained a 3’ 135-bp insert not found in the other two clones that suggested the presence of an alternatively spliced transcript. Clone 54-1, when used to probe the Northern blot previously described, hybridized to transcripts from ML-2 and KG-1 of identical size to those
that hybridized to the human CD34 probe (data not shown). Attempts to extend the 5’ sequence by rescreening the cDNA library with a 500-bp 5’ cDNA probe from clone 54-1 and with 5’ RACE were unsuccessful, suggesting the presence of extensive 5’ secondary structure in the RNA molecule.

Additional cDNA sequence for the signal peptide and 5’ untranslated region was obtained after screening a canine genomic library with clone 54-1. Two unique clones, CD34-27 and CD34-14, together extended from 5’ of predicted exon 2 to 3’ of predicted exon 8 (Fig 1B), but neither hybridized to the exon 1 oligonucleotide 34E (antisense), 5’-CAGCAGACTGACCAGGCAGAG, designed from clone 54-1. A 3.8-kb EcoRI subclone from CD34-14 was used to sequence the 3’ end of the cDNA, including the poly A signal. After rescreening the library with a 5’ 1.1-kb BamHI fragment from CD34-27, two clones, CD34-2 and CD34-5, hybridized to the oligonucleotide 34E (Fig 1B). Because of the very high GC content in the presumed first exon, deletion constructs of a 5.0-kb Sal I/BamHI subclone from CD34-5 were made to facilitate sequencing (Fig 1B). Also, a 131-bp PCR cDNA fragment amplified from ML2 cDNA with primers 34C and 34D (Fig 1A) was sequenced to determine the signal peptide nucleotide sequence. Based on restriction mapping and PCR studies of phage clones, the likely genomic structure of caCD34 was determined (Fig 1B).

The nucleotide sequence of the caCD34 cDNA, derived from both cDNA and genomic clones, is shown in Fig 2. The canine cDNA is 135 bp longer than the human CD34 cDNA, consistent with the transcript sizes found by Northern blotting. The transcription start site has not been formally mapped, but is predicted from computer alignments of canine sequence with human cDNA and promoter sequences. Additional sequence 5’ of the proposed cDNA sequence (data not shown) showed homology to the human CD34 promoter sequence,34 and also showed a TATA box motif 34 bp upstream of the proposed start of transcription. Exon 1 (nucleotides 1 through 338) was extremely GC rich (82.5%) and may be able to form stem-loop-stem structures that have been implicated in translational regulation. A variant polyadenylation signal, AAUUA,35 started at nucleotide 2730 and is identical to the poly A signal seen in the human gene. Several AU motifs (AUUUUA, AUUUUUUA) that may have a role in mRNA stability were found in the 3’ untranslated region.

Analysis of the predicted amino acid sequence. The main open reading frame of the cDNA is 1167 bp and began with the start codon AUG at nucleotide 260 and ended with the stop codon UGA (Fig 2A). This encodes a protein of 389 amino acids with a predicted molecular weight of 41 kD that has the features of a type one transmembrane protein. The mature protein was predicted using SIGCLEAVE (GCC) to begin with either the asparagine at residue 32 or the glutamic acid at residue 34. An extracellular domain of 260 to 262 amino acids is characterized by an amino-terminal region of approximately 150 amino acids with a high serine and threonine content and with the potential for extensive O-linked glycosylation. There are 7 potential N-linked glycosylation sites in the extracellular domain, of which 5 are found within this N-terminal region. The C-terminal end of the extracellular domain contains 6 cysteine residues between amino acids 182 and 247, which suggests that disulfide bonding may be important for structure of the protein. A 23 amino acid transmembrane domain extending between residues 294 and 316 is predicted based on the presence of a hydrophobic domain and homology to the human and murine sequences. The cytoplasmic domain is predicted to be 73 amino acids and has two protein kinase C (PKC) phosphorylation consensus sequences32 and a potential tyrosine phosphorylation site34 at amino acid 334 (Fig 2A).

Translation of the sequence from clone 54-1 (Fig 2B) encoded an in-frame early stop codon for the main open reading frame that results in a short cytoplasmic domain of 16 amino acids, rather than 73 amino acids predicted by sequence from clones 44-3 and 28-1. Competitive RT-PCR studies were performed to evaluate the presence and relative quantity of the two caCD34 transcripts using primers that flanked sequence encoding both transcripts (Fig 1A). Analysis of the ML2 line, several canine tissues, cultured endothelial cells, and bone marrow (data not shown) showed that in each cell type the alternatively spliced transcript represented no more than 10% of the mRNA for caCD34.

A search of the Genbank and Protein Identification Resource (PIR) databases did not identify any proteins with significant homology to caCD34 apart from the two caCD34 sequences already reported. A comparison of the amino acid sequence from dog with those of the human and mouse (Fig 3) showed an overall amino acid homology between dog and human of 69% and between dog and mouse of 62%. Homologies of the cytoplasmic and transmembrane domains between the three species were high (>86%), whereas homologies in the extracellular domain were much lower: dog:human, 60%; dog:mouse, 55%; and human:mouse, 56%. The overall structure of mature CD34 proteins appears very similar among these species, with the N-terminal portion of the extracellular domain being rich in serine and threonine residues. However, sequence homology is lower in this N-terminal region than further towards the C-terminal end of the molecule. The C-terminal 100 amino acids of the extracellular domain are cysteine rich, with the number and location of the cysteine residues being highly conserved; this suggests that the structure of these proteins may be very similar. The transmembrane and cytoplasmic domains are highly conserved, including cytoplasmic PKC and tyrosine phosphorylation motifs, which suggest conservation of intracellular signalling pathways. The three species all produce an alternatively spliced variant with an additional exon which, although different in size and nucleotide sequence, in each case encodes an identical 4 amino acids before a stop codon that truncates the intracellular domain.

Expression and analysis of recombinant caCD34. To prepare antisera to examine the cellular expression of caCD34 protein, a fusion protein (CD34-Ig) was produced. Using primers 34F and 34G and clone 54-1 as PCR template, a 906-bp fragment was amplified encoding a 99-bp signal peptide sequence and the 780-bp extracellular domain of caCD34 (Fig 4A). This fragment was digested with HindIII and Bgl II and subcloned into HindIII and BamHI sites of a pLN vector that contained cDNA sequence encoding the
A.

cccctgctccgacctgagggagaaaccggcggcgccacccggccgccgctgctccgagcgccggctggagccgagcgctcgcg

gtgcggcggccccagcggagggcgacggcgcggcggcggcggcggcggcggcggcggcggcggcggcggcggcgcgtcccggggccgagccgaac

gccctccggcccccgctcccgtcccccgcctgcggggctgagccgagcgctcgcg

1996

B.

tggagccctacaggagaaaggctggAGCTGGAACCC~ATCGCTCTTCAGGAAG~GGAGTCT~CACATGCAGCTACAA

starting at nucleotide 2729. Features include transmembrane domain (italics/underlined), highly conserved cysteine residues (bold), and potential N-linked glycosylation sites INXTIS; sequence shown in lowercase at each end of the cDNA was determined from genomic phage clones. The polyadenylation signal is underlined. Potential PKC (double underlined) and tyrosine phosphorylation sites (bold) present in the cytoplasmic domain (B) Sequence from clone 54-1 that encodes a stop codon (double underlined) that would truncate the cytoplasmic tail after 16 amino acids (GenBank accession no. U49457). The amino acid leader peptide (underlined). amino acids of the amino acid sequence are highlighted following. (A)

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hinge, CH2, and CH3 domains of a murine Ig heavy chain. DNA sequencing verified a correct construct that encoded amino acids 1 through 26 of the human CD34 signal peptide and residues 14 to 293 of the predicted caCD34 sequence fused in frame to the hinge region of murine heavy chain Cy2a. CD34-Ig was produced by transient expression in COS cells and purified using immobilized protein A. The molecular weight of CD34-Ig determined by SDS-PAGE under nonreducing conditions was ~220 kD, with three distinct species being discernable (Fig 4B). Upon reducing conditions, 2 diffuse bands were visible at ~90 and ~115 kD, consistent with the protein being a homodimer. Because the predicted molecular weight of CD34-Ig was 50 kD, this suggested that the protein was glycosylated. Treatment of CD34-Ig with N-glycosidase caused a reduction in molecular weight (data not shown), confirming the presence of N-linked carbohydrate. Automated N-terminal sequencing was performed to confirm correct processing of the human signal peptide sequence. This produced a sequence ETVTIPXTVE-P350****= gap in sequence.

**Fig 3. Comparison of the amino acid sequences from the species for which a full-length CD34 sequence has been reported. There is high homology in the cysteine-rich region of the extracellular domain, the transmembrane domain, and the cytoplasmic domain, but there is marked divergence of sequence in the amino-terminal end of the molecule. Domains of caCD34 were predicted by computer analysis of the protein structure and the homology to human and murine proteins. Signal peptide sequences are shaded. Alignments were performed using PILEUP (GCG).**

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preincubation of antibody with CD34-Ig (Fig 6), but not with a control fusion protein (data not shown), indicating that binding of RPacaCD34 to cell surface epitopes also expressed by CD34-Ig. Endothelial cells, ML3, and 1390 all expressed high-level caCD34, approximately 1 log of fluorescence greater than ML2. ML1 and CLGL did not express detectable surface CD34. These results were consistent with PCR-based assays of CD34 mRNA in which strong amplification signals were detected from ML2, ML3, endothelial cells, and 1390 cells, whereas CD34 expression was weak or absent from ML1 and CLGL 90 (data not shown). Analysis of bone marrow reproducibly showed that RPacaCD34 stained brightly approximately 1% of cells. These were primarily small mononuclear cells with low side scatter that were not detected in canine peripheral blood (Fig 7). Cells were only considered CD34" if bright staining was found as compared with other cells. Some dim staining of larger cells with intermediate side scatter was also observed, and these cells had the light scattering features of monocytes.

In preliminary experiments, immunohistochemistry of dog tissues was performed. Positive staining of canine vascular endothelium with little nonspecific background reactivity was found (Fig 8). To further assess nonspecific staining and whether RPacaCD34 would recognize human endothelial CD34, the human lung was also tested. There was no staining of human lung tissue, indicating that RPacaCD34 did not contain antibodies that were crossreactive with human vascular CD34. Similarly, RPacaCD34 did not stain the human CD34+ leukemia cell line KG1 (data not shown). These results were somewhat surprising in view of the shared amino acid homology seen in the C-terminal end of the extracellular domains of CD34. The staining pattern in canine lung was very similar to that seen with antihuman factor VIII staining of canine lung and human lung (data not

Fig 4. (A) Chimeric plasmid construct for the expression of the extracellular domain of caCD34 used to transfect Cos cells for production of CD34-Ig. (B) Analysis of CD34-Ig by SDS-PAGE and coomassie staining. CD34-Ig is expressed as a homodimer and has a molecular weight of 95 to 100 kD under reducing conditions, whereas the predicted molecular weight is 50 kD. There appears to be more than one isoform being expressed, most likely due to variable glycosylation. A murine IgG monoclonal is run as a control.

Fig 5. Western blotting analysis of cell lines and cultured vascular endothelium using RPacaCD34 and control rabbit serum. Whole cell lysates were prepared as described in the Materials and Methods and 15 μL of each was loaded on to the gel. Lanes are as follows: 1, CD34-Ig (0.5 μg); 2, CLGL; 3, 1390; 4, ML3; 5, cultured canine endothelial cells. Size markers indicate molecular weight in kilodaltons.
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A cDNA library made from the canine leukemia cell line ML2 was used to clone a cDNA for the canine homologue for CD34. The isolated cDNA sequence encoded the mature protein but not the entire signal peptide sequence, and additional 5' and 3' cDNA sequence was determined from genomic clones. Attempts to isolate cDNA clones that extended 5' of the signal peptide sequence were unsuccessful, suggesting that the RNA molecule had considerable secondary structure. Exon 1 of caCD34 is extremely GC rich (82.5%), significantly more so than that of exon 1 of the human gene (62%), and this accounted for failures of reverse transcription and difficulty in DNA sequencing of this region. The GC-rich sequence including and immediately 5' of the signal peptide sequence can possibly form stem-loop-stem structures that may be important in posttranscriptional regulation of protein expression. CpG islands found in the first exon may be important in regulating tissue-specific gene expression by changes in methylation status. The genomic structure of caCD34, including the size of the gene (25 kb between exon 1 and the polyadenylation signal), and the proposed intron/exon structure are very similar to that of the human and murine CD34 genes. The promoter region is currently being sequenced to identify possible control elements that regulate CD34 expression in dogs, which may be important for subsequent attempts to control gene expression in canine hematopoietic stem cells.

This is the third CD34 cDNA cloned, and Genbank and PIR database searches showed no sequences with significant homology to caCD34 with the exception of human CD34 and murine CD34. Because the function of CD34 remains undetermined, it was expected that comparisons of the molecule from the three species would be informative as to the important functional domains of CD34. The predicted protein structure from all three species shows marked homology, with canine and human CD34 showing greater amino acid homology than either does to the murine protein. At the N-terminal end of CD34 there appears to have been only limited evolutionary pressure to conserve specific amino-acid sequence. Extensive O-linked glycosylation takes place largely on the N-terminal serine and threonine residues of the human CD34 molecule. Tissue-specific glycosylation differences may affect the tissue-specific functions of CD34, as suggested by the finding that L-selectin binds to CD34 expressed on vascular endothelium but not to CD34 expressed on hematopoietic cells. The conservation of the cysteine-rich area in the C-terminal end of the extracellular domain suggests the presence of critical regions for the extracellular functions.

DISCUSSION

A flow cytometry analysis of CD34 expression on cultured endothelial cells and canine leukemia cell lines using RPcaCD34. Cell lines were stained with RPcaCD34 and a second-stage FITC-conjugated goat antirabbit polyclonal antibody as described in the Materials and Methods. In each case, staining with RPcaCD34 is the shaded plot, the broken line shows staining with antibody that was preincubated with CD34-lg, and the solid line shows staining with only the second-stage antibody.

shown), except that staining was more intense with RPcaCD34. Intense staining was noted in high endothelial venules from lymph node and, strikingly, in the capillaries of the myocardium.

Progenitor assays. To evaluate whether RPcaCD34 recognized canine hematopoietic progenitors, the brightest 1% of BMMC that stained with RPcaCD34 were isolated by cell sorting and assayed for content of CFU-GM. In two separate experiments, CD34⁺ cells were 25- to 50-fold enriched for CFU-GM as compared with CD34⁻ cells, unfractionated PBMC, or BMMC that had undergone a partial lineage depletion (Table 1). In experiment 2, the result was qualitatively the same, but there was substantially better colony growth, probably due primarily to greater experience using the polyclonal antiserum in flow cytometry experiments and to sample to sample variation in progenitor content.

Log Fluorescence Intensity

Relative Cell Number

Endothelial Cells

1390

ML-2

ML-3
Fig 7. Flow cytometry of canine bone marrow and peripheral blood showing side scatter versus CD34 (log PE) to illustrate patterns of staining with R+caCD34. Unfractionated bone marrow (C) and peripheral blood (D) were stained with R+caCD34 followed by a second-stage PE-conjugated polyclonal goat-antirabbit antibody. Red blood cells were lysed with hemolytic buffer. Controls shown for marrow (A) and peripheral blood (B) were the second-stage PE-conjugated goat-antirabbit antibody. Debris was gated out before analysis based on forward light scatter and side scatter properties of the cells. The percentage of CD34+ cells (in boxes) is an arbitrary estimate determined by setting a rectangular gate on cells with low side scatter expressing a high level of reactivity to R+caCD34. Some nonspecific staining of cells with low-intermediate side scatter is seen, and these cells have the forward versus side scatter characteristics of monocytes (data not shown).

of the molecule and/or for maintaining tertiary structure. Cytoplasmic PKC and tyrosine phosphorylation domains may be potentially critical for intracellular signalling functions of CD34.

An alternatively spliced transcript of CD34 encodes a premature stop codon that truncates the cytoplasmic domain. Although the size of the alternatively spliced exon differs in dog, human, and mouse, in each case an identical truncation of the intracellular domain of CD34 is found. The function of the truncated protein is still unknown, although a role in progenitor differentiation associated with loss of signalling functions has been proposed. In mice, the variant transcript was detected in both hematopoietic and nonhematopoietic tissues and may contribute up to two thirds of the CD34 mRNA, depending on which tissue is examined. In human CD34+ cells, the variant transcript was expressed in relatively higher proportion in the more differentiated CD38+ subpopulation as compared with the CD38− subpopulation. In several canine tissues, the variant transcript comprised less than 10% of the CD34 mRNA, suggesting possible differences to those observed in murine and human tissues.

A recombinant fusion protein encoding the extracellular domain of caCD34 was produced once the sequence and reading frame of the cDNA was determined. To generate a disulfide-linked glycosylated molecule, CD34-Ig was pro-
The expression of caCD34 as an Ig fusion molecule facilitated ease of purification using protein A columns. Antibodies specific to the caCD34, isolated by a two-step affinity-binding process, showed relatively little nonspecific staining when used for flow cytometry and were useful for initial characterizations of canine hematopoietic cells. However, MoAbs will be required for large-scale cell separation for experimental transplantation studies as well as for more accurate flow cytometry applications.

The positive staining of canine vascular endothelium by RpacaCD34 was consistent with patterns of CD34 expression that have previously been reported for human and murine tissues. Northern blotting studies in mice and humans showed that CD34 is expressed in many tissues. The exact cellular origin of these transcripts has not been determined in humans, although murine studies suggest a likely vascular origin in most instances. In humans, patterns of CD34 expression have not been determined using a polyclonal antiserum. Because CD34 epitope differences may...
Table 1. Analysis of CFU-GM From Sorted CD34+ Cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cell No. *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^3</td>
</tr>
<tr>
<td>CD34+ 1</td>
<td>3.5</td>
</tr>
<tr>
<td>CD34+ 1</td>
<td>3.5</td>
</tr>
<tr>
<td>CD34+ 2</td>
<td>63</td>
</tr>
<tr>
<td>CD34+ 2</td>
<td>1</td>
</tr>
<tr>
<td>BMMC 2</td>
<td>6</td>
</tr>
<tr>
<td>LIN- 3</td>
<td>0</td>
</tr>
<tr>
<td>LIN- 3</td>
<td>0</td>
</tr>
<tr>
<td>LIN- 4</td>
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</tr>
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</tr>
<tr>
<td>BMMC 3</td>
<td>ND</td>
</tr>
<tr>
<td>BMMC 5</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values represent average from duplicate plates. Numbers 1 through 5 refer to dogs used in the experiments.

Abbreviations: ND, not done; CD34+, sorted caCD34+ cells; CD34-, sorted CD34™ cells; LIN-, lineage-depleted BMMC; LIN+, lineage-positive BMMC.

* Number of cells plated in experiment.

† Results were calculated on basis of plating 5 x 10^6 cells.

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Canine CD34: cloning of the cDNA and evaluation of an antiserum to recombinant protein

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