The molecular defect underlying canine fucosidosis

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Abstract
Fucosidosis is a lysosomal storage disease which affects humans and English springer spaniel dogs. The disease is recessively inherited in both species and results from a deficiency of the enzyme α-L-fucosidase. We have recently cloned and sequenced the canine fucosidase gene (EMBL sequence admission number X92448 (cDNA) and X92671-X92678 (individual exonic data)). The gene spans 12 kb and consists of eight exons. SSCP based mutation analysis of affected animals was carried out on the coding region of this gene both with exonic primers, and intronic primer pairs for each exon. A 14 base pair deletion of the cDNA was identified at the 3' end of exon 1 in fucosidosis affected animals. Surprisingly, PCR based genomic cloning of DNA from these animals showed an identical deletion in this DNA, ending at the start of intron 1. This change causes a frameshift and, in consequence, 25 novel codons are transcribed in exon 2 before the first of two adjacent premature stop codons is encountered.

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Key words: canine fucosidosis; α-L-fucosidase; lysosomal storage disease; mutation detection.

The lysosomal storage disease fucosidosis has been described in humans and also in pedigree English springer spaniels in Australia and in the United Kingdom. Affected dogs have a profound deficiency (0-5% of the control mean) of the enzyme α-L-fucosidase which catalyses removal of fucose moieties from the oligosaccharide side chains of glycoproteins and glycolipids. Pathology and clinical signs are the result of the lysosomal accumulation of fucose containing glycoconjugates. The central nervous system displays particular marked storage abnormalities. Heterozygotes are without symptoms and possess fucosidase activities of approximately 50% of the control mean. The disease in the springer spaniel manifests as a mainly neurological syndrome with mixed motor and mental function deficits including progressive ataxia, proprioceptive difficulties, apparent blindness, change in temperament, dysphagia, dysphonia, and loss of learned behaviour. As the disease progresses there is a rapid deterioration in body condition with muscle wasting. Affected dogs die or are euthanased by 3 to 4 years of age. The canine disease has been likened to the delayed onset human form because dogs are clinically normal until 18 months of age. Thus, disease similarities have led to the use of springer spaniel dogs as models in bone marrow transplantation trials.

Both the canine and the human diseases are inherited as autosomal recessive conditions. Pedigree analysis of canine families suggests that a common British founder is present in all affected lines, including those in Australia which were derived from exported British breeding stock. Testing for carrier status in dogs has been conducted in the UK by analysis of enzyme activity. Heterozygotes should have enzyme activities that are approximately 50% of the control mean. The division between normal and heterozygous dogs is, however, often indistinct and a molecular description of the disease causing mutation would be valuable in allowing definitive designations of genotype within the springer spaniel population.

The cDNA for human α-L-fucosidase (FUCA1) has been cloned, and the genomic organisation established. In addition, most families with affected family members have had the molecular basis of the disease investigated. Early analyses described the obliteration of an EcoRI site in six patients from five fucosidosis families and the deletion of the last two exons at the 3' end of the gene. As more patients are analysed, it has become clear that there is considerable mutational variety within the patient groups studied. Most of the mutations described involve single base substitutions or small deletions or insertions. One splice site mutation has been identified. No obvious mutation “hotspots” have so far been identified in human fucosidosis.

We have recently cloned and sequenced the canine gene for fucosidase (Skelly et al, submitted for publication). The nucleotide sequence data used in this paper will appear in the EMBL, Genbank, and DDBJ Nucleotide Sequence Databases under the accession numbers X92448 (cDNA sequence) and X92671-X92678 (sequences of exons 1-8). In this study we have used SSCP and sequence analysis to investigate the mutation responsible for canine fucosidosis.

Materials and methods
The dogs investigated in this work were from pedigree English springer spaniel breeding colonies in the UK. Affected animals were identified by enzymology as previously described following referral to the Department of Clinical Veterinary Medicine, University of Cambridge, or were identified from samples submitted directly by veterinarians.
SSCP ANALYSIS

SSCP analysis was performed across the whole gene on genomic DNA isolated from blood or from fibroblast cultures by the guanidinium hydrochloride method of extraction.20 SSCP analysis of fucosidase cDNA was also performed; however, the RT-PCR products generated did not span the whole of the coding region (figure 1A). Once intronic and splice site sequence information was available, mutation analysis of the exons and flanking regions using intronic primers was deemed preferable. PCR products for SSCP analysis were in all cases smaller than 350 bp. Genomic and RT-PCR products from normal and affected dogs were amplified as described below, denatured in formamide dye, and electrophoresed at room temperature in non-denaturing gels (Hydrolink MDE gel solution, AT Biochem) at 6 to 8 W for 12 to 14 hours.21 For each exon or RT-PCR product analysed, SSCP was performed both with and without the addition of 10% glycerol.

PCR AMPLIFICATION

For amplification before SSCP analysis of each exon plus the flanking regions, primers were designed in adjacent intronic sequences and were situated approximately 50 base pairs from the consensus sequences surrounding each splice site (fig 1B, table). For exons 2-8 reactions were carried out using 0.5 U of Taq polymerase (Gibco-BRL) with the supplied buffer, in a volume of 50 µl containing 1.5 mmol/l magnesium chloride, 50 pmol of each primer, 0.2 mmol/l of each dATP, dCTP, dGTP, and dTTP (Gibco-BRL), 5 µCi 32P-dATP, and 0.1 mg/ml BSA. Genomic amplification of exon 1 with a variety of primers led to non-specific amplification of spurious products even at raised temperatures. Because of this, genomic amplification of the 5’ portion of exon 1 was performed using the enzyme Pfu polymerase (Stratagene) in the supplied buffer in a total reaction volume of 100 µl, containing 50 pmol of each primer, 0.2 mmol/l of each dATP, dCTP, dGTP, and dTTP, 5 µCi 32P-dATP, and 10% dimethyl sulphoxide. For the 3’ end of exon 1, and using a variety of primers and conditions, the correct product was present (as identified by blot hybridisation using a human probe), but constituted only a small fraction of the total reaction products.

Successful amplification and subsequent SSCP analysis of the 3’ end of exon 1 was restricted to RT-PCR alone. Poly-A+ RNA was extracted from leucocytes or cultured fibroblasts using the QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech). mRNA was reverse transcribed from gene specific cDNA synthesis primers using the enzyme Tth polymerase in the presence of manganese ions at temperatures between 65 and 70°C (fig 1A). These primers were designed to have Tm values between 66 and 72°C so that reverse transcription could be carried out at raised temperatures (table). The first strand cDNA was then used as a template for PCR. All primers were designed manually as above. PCR was performed as described.
PCR products were generated for each of the exons 2-8 using intronic primers. For each of these exons a single product was amplified which corresponded to the predicted size. SSCP analysis of these exons showed no differences between normal dogs, heterozygous dogs, and dogs known to have been homozygous for the fucosidosis allele (data not shown). Exon 1 was amplified in two parts. Difficulties were experienced in amplification of this exon, probably at least in part because of the G/C richness of both the exon and its surrounding introns. The 5' end of the exon, when amplified using Pfu DNA polymerase and analysed by SSCP, showed no differences on PCR between normal, heterozygous, and homozygous mutant dogs. The 3' end of the exon could not be amplified cleanly.

SSCP analysis was performed across the majority of the cDNA sequence using RT-PCR. This analysis of fibroblast and leucocyte mRNA from canine fucosidase normal and affected animals showed only one mobility shift, which was located in the 3' portion of exon 1 (fig 2). This PCR product was cloned and sequenced in both normal and affected dogs and a 14 base pair deletion of the mRNA was identified at the 3' terminus of the exon (fig 3A). This deletion causes a frameshift within the transcript and a premature stop codon is produced downstream in exon 2. In the mutant cDNA, base 393 (EMBL accession number X92448) is now spliced to base 408 with a resultant frameshift in exon 2 of one nucleotide 5'. The mutant protein thus contains 25 novel amino acids before the first of two adjacent premature stop codons is encountered.

In order to determine the nature of this mutation in genomic DNA, amplification of the 3' end of exon 1 was performed on genomic DNA from an affected animal using the primers described (table, fig 1B). As this PCR did not amplify with sufficient specificity, shotgun cloning of the size fractionated PCR products into pCR-II (Invitrogen) was necessary. Subsequent identification of the correct clone by sequence analysis allowed determination of the DNA sequence underlying the mutation. Surprisingly, this DNA also showed a deletion which was coextensive with that seen in the cDNA; that is, the final 14 base pairs of exon 1 were missing but the whole of intron 1 was present (fig 3B).

Discussion

In this paper we describe an alteration in the gene and transcript for the enzyme fucosidase in dogs suffering from fucosidosis. This deletion in the mRNA can be predicted to have major consequences in the subsequent translational processes because the frameshift induced by the deletion produces a premature termination codon. Hence it is predicted that the protein product would be foreshortened and consequently inactive and probably unstable. The absence of material cross reacting with antibodies raised against normal canine fucosidase in tissues of dogs suffering from fucosidosis could be because of protein in-
stability or of the absence of antigenic epitope in the mutant protein.24

The mutation underlying the deletion in the RNA is a coextensive deletion of the DNA. The splice donor site at the end of exon 1 in normal dogs takes the form "CCG/GTGAGT.". This sequence, with a C at position –2, is itself less close to a splice donor consensus than the splice site created by the deletion in the mutant gene "CAG/GTGAGT.". It is therefore unsurprising that the latter splice site is used efficiently.

The description of the disease causing mutation has important consequences for the English springer spaniel breed. Until now, heterozygotes have been identified on the basis of a reduced enzyme activity.8 This method is unreliable and far from satisfactory when breeding programmes are being established. The description of the mutation at the molecular level will allow a test to be designed which will identify the mutant allele specifically, so that heterozygous dogs can be identified easily and rapidly.

Fucosidosis in English springer spaniels provides a useful animal model with which to study the human disease. Dogs diagnosed biochemically as being homozygous for the disease-causing mutation have already been used in trials to assess the efficacy of bone marrow transplantation.7 25 26 As a consequence of this work, bone marrow transplantation has been attempted in a human patient.27 Now, with the therapeutic emphasis turning towards the possibility of gene replacement therapy, a fuller molecular understanding of the canine disease must be beneficial. Preliminary assessments of gene transfer, using retroviral vectors, have been conducted in human and canine fucosidosis fibroblasts cultures.28 The early identification of affected animals and the knowledge of the cDNA and genomic organisation of canine fucosidase could prove invaluable when it comes to designing such gene replacement protocols using the springer spaniel as a human disease model.

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