RESEARCH COMMUNICATION

Expression of the Wilson disease gene is deficient in the Long-Evans Cinnamon rat

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Long-Evans Cinnamon rats develop a necrotizing hepatitis characterized by excessive hepatic copper accumulation, defective holoceruloplasmin biosynthesis and impaired biliary copper excretion. To elucidate the molecular basis of this defect, a cDNA clone encoding the rat Wilson disease gene was isolated and used to examine gene expression in selected tissues from normal and Long-Evans Cinnamon rats. Although this cDNA

readily detects Wilson transcripts in liver and other tissues from normal rats, such transcripts are entirely absent from tissues derived from the Long-Evans Cinnamon rat strain. These data therefore identify the Long-Evans Cinnamon rat as the first *bona fide* animal model of Wilson disease and suggest that this rat strain may be a valuable resource in the study of this genetic disorder.

INTRODUCTION

Wilson disease is an autosomal recessive disorder of copper metabolism characterized by hepatic cirrhosis and neuronal degeneration [1]. This disorder is due to the absence or dysfunction of a P-type ATPase encoded on chromosome 13 and expressed in the livers of normal individuals [2–4]. Loss of function of this protein results in impaired intracellular hepatocyte copper transport, with copper accumulation in the cytosol and copper depletion from compartments essential for holoceruloplasmin biosynthesis and biliary copper excretion [5]. Prolonged hepatic copper accretion results in cirrhosis and liver failure with subsequent copper deposition and injury in selected extrahepatic tissues. A homologous P-type ATPase is involved in the pathogenesis of Menkes disease, which is an X-linked disorder of copper metabolism resulting in depigmented hair and neuronal degeneration [6–8].

Despite elucidation of the aetiology of Wilson disease, many important questions remain. The precise role of the Wilson P-type ATPase in intracellular copper transport is unknown, as are the effects of specific inherited mutations on any putative transport function of this protein. The mechanisms of tissue injury from copper accumulation are also poorly understood, although such mechanisms are likely to be of broad significance in defining the role of free radicals in cellular necrosis, particularly in degenerative neurological disease [9]. Clinically there is a marked discordance between phenotype and genotype in patients with Wilson disease, such that siblings with identical mutations demonstrate widely varying clinical presentations [10]. This discordance reveals that additional genetic or environmental factors are involved in the pathogenesis of this disease, but the aetiology of such factors is currently unknown.

Many of the issues cited above have been difficult to address directly in patients with Wilson disease, because affected tissues such as liver and brain are not readily available for study. For these reasons, investigators have long sought to define an animal model of Wilson disease. Recently, an autosomal recessive form of hepatitis in the inbred Long-Evans Cinnamon (LEC) rat has

been shown to be associated with excess hepatic copper accumulation, defective biliary copper excretion and impaired holoceruloplasmin biosynthesis [11,12]. The abnormalities of copper metabolism in the LEC rat suggest that the mutation in these animals may represent the rodent equivalent of Wilson disease. We directly tested this hypothesis by examining expression of the rat homologue of the Wilson disease gene in the LEC rat.

EXPERIMENTAL

Oligonucleotides corresponding to nt 122–146 (5'-GCAGAGAT-CAAGTATGACCCAGAGG-3') and 917–942 (5'-TGGCTTG-GAGAGACATGAGTTTAGCC-3') of a human Wilson disease cDNA [2] were synthesized and used to amplify a partial rat Wilson disease cDNA from a rat liver cDNA library (Clontech) as previously described [13]. A partial rat Menkes cDNA was similarly generated from a rat lung cDNA library (Clontech) using oligonucleotides corresponding to nt 1601–1627 (5'-AATTCATCTAAGTGTTACATACAGGTC-3') and nt 1980–2007 (5'-GTATGGATAATATCTCTAGGACCAATA-3') of the human Menkes cDNA [6]. The amplified rat Wilson (accession no. L28173) and Menkes (accession no. L28172) cDNAs were sequenced by the dideoxy chain termination reaction [14] and subcloned for the generation of cRNA probes [15]. The nucleotide sequences were translated and the derived amino acid sequences were aligned with the corresponding regions of the human proteins as described [16].

In these studies the inbred Long-Evans Agouti (LEA) rat strain was used as a control, since this is the normal parental strain in which the LEC mutation arose. Breeding pairs of LEC and LEA rat strains were generously provided by Professor Noritoshi Takeichi (Hokkaido University School of Medicine, Hokkaido, Japan). Adult male and female LEC and LEA rats were killed and the organs isolated and snap frozen in liquid nitrogen. RNA was isolated by dissolution of tissue in guanidinium isothiocyanate followed by CsCl gradient centrifugation [17]. RNA samples were electrophoresed in 0.7%

Figure 1 Amino acid sequence of the rat Wilson disease gene

Portions of the derived amino acid sequences of the rat Menkes (rM) and Wilson (rW) partial cDNAs are shown, and compared with the homologous regions in the corresponding human Menkes (hM) and Wilson (hW) proteins. The numbers correspond to the published amino acid sequence of the human Menkes protein [6]. Identical amino acids are shown in bold and the putative sixth copper-binding domain is boxed, with the conserved amino acids in the metal-binding motif identified (Cu).

formaldehyde-agarose denaturing gels, transferred to nylon membranes and hybridized as described [15]. Radiolabelled antisense cRNA probes were prepared by *in vitro* transcription using [³²P]CTP [18]. Additional plasmids used in this study included cDNA inserts encoding rat ceruloplasmin, albumin, transferrin and β -actin [15]. In all cases RNA blots were reprobed with actin to ensure equivalent RNA loading and transfer. All blots were hybridized at 58.5 °C and following washing were exposed to X-Omat XAR film (Kodak) at -70 °C. Genomic DNA was isolated from the spleens of LEA and LEC rats by phenol extraction, and Southern blots of restriction endonuclease-digested genomic DNA were hybridized to antisense [³²P]cRNA probes as previously described [13].

RESULTS

Partial cDNAs encoding the rat homologues of the human Wilson and Menkes disease genes were isolated by PCR using oligonucleotides derived from the corresponding human sequences. The derived amino acid sequence from a portion of the isolated cDNA clones encoding the sixth copper-binding domain revealed 81 % and 89 % identity with the human Wilson and Menkes proteins respectively (Figure 1). An absolute conservation of the metal-binding motif (Cu) was observed across species and between proteins (Figure 1). As predicted from the human data, sequences upstream of the rat Wilson and Menkes copper-binding domain were divergent, suggesting that probes from these regions would discriminate between these two genes. The nucleotide sequence of the entire 820 bp rat Wilson cDNA was 91 % identical to the corresponding human cDNA, confirming that this clone does represent the rat homologue of the human disease gene (results not shown).

To directly examine expression of the Wilson disease gene in the LEC rat, RNA was isolated from the liver of adult LEC rats and probed by RNA blot analysis using the rat Wilson disease cRNA probe (Figure 2). Using these methods, a single 7.5 kb transcript was readily detected in LEA liver (Figure 2, lane 1) which was absent in an equivalent sample from LEC liver (Figure 2, lane 2). Similar results were obtained using multiple samples from adult LEA and LEC livers, and these findings were further confirmed by identification of a transcript of identical size in liver tissue from healthy non-inbred Sprague-Dawley rats (results not shown).

The specificity of these findings was further investigated by examining expression of the Wilson disease gene in multiple tissues from the LEC rat at an age prior to the onset of hepatic cirrhosis. As observed previously, a single 7.5 kb Wilson-specific transcript was readily detected in the LEA liver under these conditions (Figure 3, lane 6). A less abundant, similar-sized transcript was also detected in LEA kidney (Figure 3, lane 3) and a 7.8 kb transcript was found in LEA spleen and stomach

Figure 2 Expression of the rat Wilson disease gene in LEC rat liver

An RNA blot analysis is shown of 10 μ g of liver RNA from LEA (lane 1) and LEC (lane 2) rats (aged 3 months) hybridized with an antisense cRNA probe specific to the rat Wilson disease gene. The arrowhead denotes the single 7.5 kb Wilson-specific transcript detectable in the LEA liver. The positions of 28 S and 18 S RNAs are also shown. The blot was hybridized and processed as described in the text and exposed to XAR film at -70 °C for 14 h.

Figure 3 Expression of the rat Wilson disease gene in organs of the LEC rat

RNA was prepared as described in the text from the various indicated tissues of LEA (lanes 1-7) and LEC (lanes 8-14) rats (aged 10 weeks). An RNA blot of 10 μ g of RNA from each organ is shown which was sequentially hybridized with antisense cRNA probes specific to the rat Wilson disease gene (WD), ceruloplasmin (Cp) and β -actin (Act). Arrowheads indicate 7.5 and 7.8 kb Wilson transcripts as well as specific ceruloplasmin and actin transcripts. The blot was hybridized and processed as described in the text and exposed to XAR film at -70 °C for 14 (WD), 6 (Cp) and 2 (act) h. Abbreviation: Mam. gl., mammary gland.

Figure 4 Expression of rat Wilson and Menkes genes in organs of the LEC rat

RNA blot analysis of 10 μ g of RNA from LEA rat (lanes 1–4 and 9–12) and LEC rat (lanes 5–8 and 13–16) organs. The blot was sequentially hybridized with antisense cRNA probes specific to the rat Wilson (lanes 1–8) and then Menkes (lanes 9–16) disease genes. Blots were processed as described in the text. Arrowheads indicate 7.5 and 7.8 kb Wilson-specific (W) and 8.0 kb Menkes-specific (Mc) transcripts. Blots were exposed to XAR film at -70°C for 14 (W) and 22 (Mc) h.

Figure 5 Southern blot of the rat Wilson disease gene

Genomic DNA was isolated from Long-Evans rat spleens as described in the text and subjected to Southern blot analysis. *EcoRI*, *BamHI* and *HindIII* restriction endonucleases were used to digest LEA (lanes 1, 3 and 5) and LEC (lanes 2, 4 and 6) genomic DNA and, following electrophoresis and capillary transfer, the blot was hybridized with an antisense cRNA probe specific for the rat Wilson disease gene. The positions (kb) of restriction fragments of *HindIII*-digested λ phage DNA are shown.

(Figure 3, lanes 2 and 7). Examination of these same organs in the LEC rat failed to detect any expression of the Wilson disease gene, despite prolonged exposure of the blot (Figure 3, lanes 8–14). When this same blot was reprobbed with a cRNA probe for rat ceruloplasmin, an equivalent abundance of ceruloplasmin-specific transcript was observed in livers from LEA and LEC rats (Figure 3, lanes 6 and 13). No quantitative differences in gene expression between the same organs in the LEA and LEC rats were observed when these same samples were reprobbed with cRNA probes specific for β -actin (Figure 3, lanes 1–7 versus 8–14) and additional liver-specific genes including albumin and transferrin (results not shown).

To determine if the deficiency of gene expression observed in the LEC rat was specific to the Wilson copper-transporting P-type ATPase, additional studies were carried out using a cRNA probe derived from the rat Menkes disease gene. As can be seen

in Figure 4, RNA blot analysis using a cRNA probe for the Wilson disease gene revealed expression of Wilson-specific transcripts in the liver, stomach and spleen of LEA but not LEC rats (lanes 1–4 versus 5–8). However, when this same blot was reprobbed with a cRNA probe encoding the rat Menkes disease gene, equivalent expression of an 8.0 kb Menkes-specific transcript was observed in stomach and lung tissue from both the LEA and LEC animals (Figure 4, lanes 9–16). Because the transcripts for these two genes are of similar size, these blots were reprobbed without stripping of the Wilson gene signal, thus ensuring that the second signal observed is indeed the rat Menkes gene.

The structure of the rat Wilson disease gene was examined in LEA and LEC rats by Southern blot analysis. Restriction enzyme digests of genomic DNA from LEA and LEC rats revealed identically sized fragments when analysed using the rat Wilson cRNA probe (Figure 5). Further analysis using cDNA probes derived from the 5' and 3' regions of the human Wilson disease gene failed to detect any differences in size or band intensity between LEA and LEC genomic DNA digested with multiple restriction enzymes and analysed in a similar fashion (results not shown).

DISCUSSION

Taken together, these data demonstrate that the Wilson disease gene is expressed as a single 7.5 kb transcript in the normal rat liver and that the LEC rat is deficient in this expression. This lack of expression is not secondary to hepatocyte injury, since it is observed prior to the onset of cirrhosis and in the face of normal expression of other hepatocyte-specific genes. Furthermore, tissue-specific expression of the Menkes disease gene is normal in these animals, suggesting that the defect in copper metabolism is specific for the Wilson P-type ATPase. In addition to hepatic expression of the Wilson disease gene, single transcripts of variable size were also observed in these studies in kidney, stomach and spleen. These extrahepatic transcripts were missing in the LEC rat, indicating that each is derived from the Wilson disease gene and again supporting the concept that deficient expression of this gene is the primary defect in these animals. The basis for the larger-sized transcript in stomach and spleen is currently unknown, but a variation in transcript size has been observed in extrahepatic expression of the Wilson disease gene in humans [2,3].

The LEC rat was originally identified as a mutant strain which arose in a closed colony of Long-Evans rats. This mutation is inherited in an autosomal recessive manner and is universally associated with the development of hepatitis by 4 months of age [19]. The nature of the hepatitis and the inherited features prompted further metabolic studies which revealed elevated hepatic copper concentrations associated with impaired copper incorporation into ceruloplasmin and defective biliary copper excretion [11,12]. The data in the present paper suggest that these abnormalities are the result of defective expression of the Wilson disease gene. While it is possible that the deficiency in expression of the Wilson disease gene observed here is secondary to other factors which regulate expression of this gene, the lack of detectable message is similar to that observed in liver tissue from some patients with Wilson disease [2]. In some cases the mutations observed in the human Wilson disease gene would not be expected to result in the lack of transcript [3,4], so further studies will need to be done to determine the relationship between these mutations and gene expression in both the human and the LEC rat. Southern blot analysis did not reveal differences in Wilson disease gene structure in the LEC rat, and elucidation of the

mechanism of this defect must await cloning of the LEC rat Wilson gene.

To our knowledge, this paper is the first description of a *bona fide* animal model of Wilson disease. Previous studies have revealed copper abnormalities in the Toxic Milk mouse and the Bedlington terrier, but in neither case is the metabolic abnormality similar to that observed in Wilson disease [20,21]. The LEC rat offers the opportunity to experimentally address questions regarding the function of the Wilson P-type ATPase in hepatocyte copper metabolism and to assess the mechanisms of hepatic injury caused by copper accumulation. Further studies will be needed to determine whether the LEC rat can also provide insight into the neurological lesions of Wilson disease; however, preliminary evidence reveals that copper does accumulate in specific regions of the LEC rat brain [22]. Environmental and genetic influences on the rate of hepatic copper accumulation and liver injury will also be amenable to study by breeding this mutation into other rat strains and manipulating exposure to exogenous hepatotoxins. Furthermore, because copper accumulation in the LEC rat liver and brain is dramatically reduced by chelation therapy, these animals hold promise for defining new modes of treatment for copper toxicity [23]. Finally, since transplantation has revealed that the primary defect in Wilson disease is confined to the liver [24], the LEC rat is a potentially valuable model for the development of useful methods for somatic gene therapy [25].

Lastly, note should be made of the remarkable incidence (> 90%) of hepatocellular carcinoma in the LEC rat [26]. This malignancy is most likely the result of hepatic copper accumulation rather than that of a second unrelated mutation, since hepatocellular carcinoma is not observed in the inbred LEA strain and can be prevented in the LEC rat by early chelation therapy [27]. While the incidence of liver cancer is not increased in patients with Wilson disease, this is probably due to the fact that chelation therapy alters the natural history of this liver disease [28]. The data in the present study indicate that an inherited deficiency in expression of the Wilson disease gene should be added to the list of genetic mutations which contribute to carcinogenesis. These data also suggest that further analysis of this process in the LEC rat may provide insight into the mechanisms underlying liver injury and cancer development in humans, as well as the unique roles of environment and genotype in cancer susceptibility [29].

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