Cystatin B-deficient mice have increased expression of apoptosis and glial activation genes

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Received March 9 2001; Revised and Accepted July 5, 2001

Loss-of-function mutations in the cystatin B (Cstb) gene cause a neurological disorder known as Unverricht–Lundborg disease (EPM1) in human patients. Mice that lack Cstb provide a mammalian model for EPM1 by displaying progressive ataxia and myoclonic seizures. We analyzed RNAs from brains of Cstb-deficient mice by using modified differential display, oligonucleotide microarray hybridization and quantitative reverse transcriptase polymerase chain reaction to examine the molecular consequences of the lack of Cstb. We identified seven genes that have consistently increased transcript levels in neurological tissues from the knockout mice. These genes are cathepsin S, C1q B-chain of complement (C1qB), β2-microglobulin, glial fibrillary acidic protein (Gfap), apolipoprotein D, fibronectin 1 and metallothionein II, which are expected to be involved in increased proteolysis, apoptosis and glial activation. The molecular changes in Cstb-deficient mice are consistent with the pathology found in the mouse model and may provide clues towards the identification of therapeutic points of intervention for EPM1 patients.

INTRODUCTION

Loss-of-function mutations in the human cystatin B (Cstb) gene cause a childhood form of epilepsy known as Unverricht–Lundborg disease (EPM1) (1). EPM1 is an autosomal recessive inherited disorder, characterized by progressive neurological dysfunction, myoclonic seizures and tonic-clonic seizures (2). Cstb is a known in vitro inhibitor of cathepsins B, H, L and S (3–4); however, in vivo targets and the molecular events leading to the neurological symptoms found in EPM1 when Cstb is lacking are unknown.

To gain new insights into the molecular basis for EPM1, we previously generated mice lacking Cstb (5). These animals develop progressive ataxia and myoclonic seizures at a young age, providing a mammalian model for the human disease. Cstb-deficient mice display extensive apoptotic cell death in cerebellar granule cells, which likely lead to the rapid progression of ataxia in these mice. In addition to providing an experimental model to study apoptotic granule cells, these mice provide a valuable resource for examining other biochemical or cellular changes that occur when Cstb is lacking.

Transcript profiling experiments have identified abnormal mRNA levels in pathological states, providing insights into the molecular bases of diseases and a way to stratify disease populations for proper therapeutic intervention (6,7). We applied these approaches to the study of EPM1 in an effort to identify altered pathways or genes displaying altered mRNA levels in Cstb-deficient mice. We used modified differential display, oligonucleotide microarray hybridization and real-time quantitative reverse transcriptase polymerase chain reaction (Q-RT–PCR) to analyze RNA levels between Cstb-deficient mice and control littermates, from both pathological and non-pathological tissues. Our results revealed several differentially expressed genes, providing further insight into the underlying molecular events involved in this disease, and therefore hinting at further points where diagnostic evaluation or therapeutic intervention may be successful. These genes also allow an entry point to examine the phenotypic and gene expression differences between these mice and human patients.

RESULTS

Expression profiling strategies

To maximize the likelihood of identifying genes with abnormal transcript levels in Cstb-deficient mice, we used both modified differential display and oligonucleotide microarray chip hybridization. Changes in the gene expression, initially identified by either screening strategy, were validated by using Q-RT–PCR assays. Furthermore, an RNA panel of knockout/control pairs, independent of the original RNAs used in the screening strategies, was added to the Q-RT–PCR analysis. This additional panel was used to segregate stochastic gene

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expression changes from those that are biologically relevant to the mutant population. To eliminate potential genetic background effects, all animals studied were isogenic except for those with the introduced Cstb mutation (5).

For the differential display analysis, we isolated whole brain and kidney RNAs from an 8-month-old symptomatic Cstb-deficient mouse and a wild-type sibling. cDNAs were synthesized in duplicate from the RNAs, followed by restriction enzyme digestion, adaptor ligation, PCR amplification, gel electrophoresis and band excision of transcripts of interest (8). Identities of bands were determined by direct sequencing. By visual evaluation of the differential display gels, we found 85 genes to have decreased transcript levels and 57 genes to have increased levels in Cstb-deficient mice. Data from two representative genes, the metallothionein II gene and the kidney androgen regulator gene, are shown in Figure 1.

RNAs from whole brain (excluding cerebellum) and cerebellum from two pairs of knockout/control mice, independent of the pair used for differential display, were used in microarray hybridization. We synthesized cDNA from each RNA sample, which we converted to cRNA and used as a substrate for microarray hybridization. Twenty base pair synthesized oligonucleotide probes, specific for approximately 11,000 transcripts, were present on the microarrays. Of the transcripts represented on the chip, we found 440 genes to be present at lower RNA levels, and 560 genes to be present at higher RNA levels in knockout mouse cerebellum and brain tissues in comparison to control mouse tissues (see Materials and Methods for selection criteria). Figure 2 shows examples on the microarray of the same genes used in the differential display example above (Fig. 1).

Validation of transcript differences by Q-RT–PCR

We chose 38 genes for Q-RT–PCR validation based on suitability for Q-RT–PCR primer design, amount of change observed in either differential display or microarray hybridization, or involvement in RNA metabolism, mitochondrial respiration, or neural development. Eighty-three percent (29/35) of transcripts discerned to be altered on the microarray analysis and 100% (4/4) of transcripts altered on differential display analysis were confirmed in Q-RT–PCR analysis.

In addition to validating gene expression differences in the original knockout and control samples identified in the screening processes described above, an independent panel of at least five pairs of knockout/control mice was included in the Q-RT–PCR testing to reduce the level of stochastic expression variation from our final analysis. Seven transcripts in addition to Cstb were consistently altered in the Cstb-deficient population when the Q-RT–PCR panel was expanded to include individuals not originally analyzed by differential display or on the micro-array. Thus, 21% (8/38) were identified as consistently (qualitatively across at least seven out of eight paired knockout versus control mice) and significantly (2-fold or greater) different in transcript levels in brain tissue RNAs of knockouts versus controls (Table 1). The other 30 genes tested in the expanded Q-RT–PCR panel gave variable results in the extended mouse panel, indicating wide gene expression differences between isogenic knockout and control pairs of mice (Fig. 3). In addition to the seven genes shown in Table 1, we tested all mice in the panel by Q-RT–PCR for Cstb RNA levels, and confirmed the expected (dramatic) decrease in the amount of Cstb transcript in all tissues examined in Cstb-deficient mice compared with controls (data not shown).

Transcript differences in isogenic mice due to stochastic effects

The extended Q-RT–PCR RNA panel allowed us to distinguish genes that displayed consistent expression patterns (Table 1) from those that had variable expression (Fig. 3). The correlation between the initial microarray/differential display data and the subsequent Q-RT–PCR analysis was 84% (32/38) when the same RNA samples were examined. In contrast, only 21% (8/38)
Table 1. Q-RT–PCR results of transcripts found to be significantly altered in CSTB-deficient mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>Original microarray expression data in brain tissue</th>
<th>Expression pattern in multiple pairs of mouse neurological samples (number of pairs: total number of pairs tested)</th>
<th>Expression pattern in multiple pairs of mouse liver samples (number of pairs: total number of pairs tested)</th>
<th>Expression pattern in multiple pairs of mouse kidney samples (number of pairs: total number of pairs tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsin S</td>
<td>++</td>
<td>(+) (7:8)</td>
<td>(+++) (6:6)</td>
<td>(+) (2:3)</td>
</tr>
<tr>
<td>Metallothionein II</td>
<td>+</td>
<td>(+++++) (8:8)</td>
<td>(++++++) (6:6)</td>
<td>(+++++) (3:3)</td>
</tr>
<tr>
<td>β2-microglobulin</td>
<td>++</td>
<td>(+) (8:8)</td>
<td>(++) (5:6)</td>
<td>Variable expression (-6 to +16)</td>
</tr>
<tr>
<td>Glial fibrillary protein</td>
<td>+++</td>
<td>(+++++) (7:8)</td>
<td>(+) (3:6)</td>
<td>(--) (2:3)</td>
</tr>
<tr>
<td>Apolipoprotein D</td>
<td>+</td>
<td>(++) (7:8)</td>
<td>(+) (4:6)</td>
<td>(--) (2:3)</td>
</tr>
<tr>
<td>ClqB</td>
<td>+++</td>
<td>(++) (7:7)</td>
<td>(+) (5:6)</td>
<td>(--) (2:3)</td>
</tr>
<tr>
<td>Fibronectin I</td>
<td>++</td>
<td>(+) (8:8)</td>
<td>(++++++) (4:6)</td>
<td>(++++++) (2:3)</td>
</tr>
</tbody>
</table>

Seven genes were tested on several pairs of control/knockout mice over several tissues. (+), over-expression of a gene; (–) under-expression of a gene. The number of (+) or (–) indicate level of fold change between knockout and control pairs. One (+), 2-fold increase in transcript in the knockout mice; (++) 2–4-fold increase, (++) 2–6-fold increase; (++++), 2–12-fold increase; (+++++), 2–23-fold increase; (++++++), 2–430-fold increase; the same codes apply for the (–) symbols in the table. The ratio is the number of pairs that showed increased levels of the transcript of interest compared to the total number of pairs included in the panel (e.g. 7:8 means seven out of eight knockout/control mouse showed increased expression in the knockout mouse). β2-microglobulin showed variable expression, ranging from 6-fold decreased expression to 16-fold increased expression in knockout kidney tissue.

DISCUSSION

In this study, we sought to identify genes displaying altered transcript levels in neuronal tissue from Cstb-deficient mice, targets that may aid in explaining the molecular mechanism behind the human disease, EPM1. We identified consistent alterations in the transcript levels of seven transcripts in these mice, including the protease cathepsin S, a known target of Cstb. In addition, we found that transcripts consistently increased in brain tissue from Cstb-deficient mice encode proteins involved in responding to neuronal damage. For instance, we found that the lack of Cstb results in the accumulation of several transcripts encoding proteins that are known markers of microglia activation, which commonly occurs following neuronal loss. While these data are consistent with the previously described neuronal loss in the cerebellum in Cstb-deficient mice, they also suggest the presence of neuropathology in other regions of the brain.

Because Cstb is known to inhibit the proteases cathepsins B, H, L and S in vitro, we examined the transcript levels from these genes in detail. While the regulation of protease activity could be entirely at the level of uninhibited protein availability, it is also possible that regulatory feedback loops exist that alter the levels of the protease transcripts in response to the lack of the protease inhibitor Cstb. In Cstb-deficient mice, we found that the levels of cathepsins B, H and L were variable, although we were unable to identify any common alteration. In contrast, we found that increased levels of cathepsin S were a consistent and significant finding.

These data indicate that, while Cstb has the ability to inhibit various cathepsin family members in vitro, only cathepsin S transcripts have increased levels when Cstb is lacking in vivo. Cstb is widely expressed and has been shown to inhibit apoptosis in cerebellar tissue (5). The increased levels of cathepsin S mRNA in mice deficient for Cstb may be a key factor in initiating or propagating the apoptotic cascade, possibly as an uncontrolled positive feedback loop. Our finding of increases in cathepsin S in mice lacking Cstb suggests that the key apoptotic trigger may involve cathepsin S.

In addition to increased cathepsin S transcript levels from Cstb-deficient mice, five other genes showed increased mRNA levels in brain tissue (Table 1). These genes include those...
encoding β2-microglobulin, Gfap, apolipoprotein D, fibronectin 1 and C1qB. These transcripts may be present in higher amounts in knockout mice as a result of glial activation. β2-microglobulin and apolipoprotein D have been reported to increase in reactive astrocytes (a type of glial cell) (9–11). Fibronectin 1 has been demonstrated to be secreted by glial cells and to be involved in astroglial proliferation (12,13). C1qB is predominantly expressed in monocytic/macrophage cells such as microglia (14) and Gfap is widely used as a marker for microglia (15). In support of the expression data, immunocytochemistry analyses with antibodies that recognize GFAP indicate that there are increased levels of the protein in Cstb-deficient brains (P.Shannon, L.A.Pennacchio, B.A.Minassian and R.M.Myers, manuscript in preparation).

Reactive fibrillar astrocytes are capable of producing dense microglial scars, which create a physical barrier between damaged and healthy cells (16). This biological phenomenon seems to be a prevalent adaptation against many forms of neural damage. Glial activation has been suggested in other neurodegenerative diseases such as scrapie, Alzheimer’s disease and Creutzfeldt–Jakob’s disease (17–19). Neuropathology outside the cerebellum was not originally reported in Cstb-deficient mice (5), although EPM1 in humans results in significant loss of neuronal tissue in other regions of the brain. Our novel finding of increased microglial activation in non-cerebellar neuronal tissue strongly suggests that there is pathology in brain tissues in addition to the cerebellum. In parallel studies, we identified widespread neuropathology outside the cerebellum in this analysis (P.Shannon, L.A.Pennacchio, B.A.Minassian and R.M.Myers, manuscript in preparation). Thus, the identification of genes with aberrant transcript levels in brain tissue is consistent with the discovery of non-cerebellar neuropathology, strengthening Cstb-deficient mice as a model for EPM1.

One of the seven transcripts showing consistent expression alterations in the Cstb-deficient mice is associated with tissue damage response (20). Metallothionein II transcripts are far more abundant in Cstb-deficient mice in all CNS and non-CNS tissue included in this study (Fig. 4) which may indicate that Cstb is integral to the normal function of tissues outside of the CNS.

The lack of Cstb in humans and mice has previously been shown to have severe consequences, leading similarly in both species to progressive neurological decline, ataxia and seizures (1,5). In this paper, we report molecular changes at the transcript level in response to the loss of Cstb. Activation of microglia in brain tissues from Cstb-deficient mice supports the identification of pathological changes in regions of the brain in addition to the cerebellum that were not reported in the initial characterization of these animals. Furthermore, the increased levels of cathepsin S, a known target of Cstb, suggest that a feedback mechanism exists which results in increased cathepsin S mRNA when Cstb is lacking.

MATERIALS AND METHODS

RNA source

Tissue from whole brain, liver, kidney, heart, cerebellum and cortex was dissected from age- and sex-matched Cstb-deficient mice and their wild-type littermates. All mutant mice used in this study were symptomatic for the disease. Both control and mutant mice were strain 129SvJ(5). Total RNA was isolated according the manufacturer’s recommendations (Qiagen, Valencia, CA).

DNAse I treatment of RNA

Total RNA was DNase I-treated before differential display or real-time quantitative PCR analysis. One-tenth the volume of 10× DNase I buffer and 2 U of DNase I enzyme (Ambion, Austin, TX) was added to the total RNA and incubated at 37°C for 30 min. We purified RNA by either adding one-tenth the volume of DNase Inactivation Reagent (Ambion) and subsequent centrifugation to remove DNase I or by phenol/ chloroform extraction and subsequent ethanol precipitation.

Differential display (READSTM analysis)

Three micrograms of DNase I-treated total RNA was reverse transcribed to construct first strand cDNA (8). Second strand cDNA synthesis followed. The cDNA was then digested with one of several enzymes (PstI, BsrG I, SacI, HindIII, XbaI, Ncol). We used a total of six enzymes in combination with 12 primers independently in the brain and kidney differential display procedure, representing analysis of approximately 11 000 transcripts per tissue. Subsequently, the digested cDNA was ligated onto an adaptor and the material was amplified and labeled with 35P through PCR for 30 cycles. The PCR products were electrophoresed over a 4% polyacrylamide gel (Invitrogen/Life Technologies, Grand Island, NY). The gels were then dried and imaged on x-ray film. Bands were extracted from the gels and sequenced.

Affymetrix chip hybridization

RNA from each mouse was analyzed independently. Biotin-labeled cRNA was synthesized from 200 µg of total RNA and hybridized over the Affymetrix Mouse 11K sub A and sub B
GeneChips® with a total of 11 000 Affymetrix hybridization elements, which represent approximately 9700 distinct genes. After hybridization, the GeneChips® were washed and stained with streptavidin-phycocerythrin using the GeneChip Fluidics Station 400. DNA chips were read with a Hewlett-Packard GeneArray scanner. Chip hybridization signals were set to a target intensity of 100 and normalized by using Affymetrix’s algorithm for all probe sets. Only genes whose average difference scores were >200 and showed 2-fold or more under- or over-expression in knockout mice compared with control mice were included in the chip analysis.

Q-RT–PCR analysis

ABI 7700 real-time sequence detection machines were used for analysis. All primers were developed with the ABI Primer Express program. TaqMan® One-Step RT–PCR Master Mix Reagents kit (PE Corporation, Foster City, CA) and SYBR green were used to detect mRNAs.

The expressed sequence tag (EST) represented by GenBank accession no. W83013 was the reference gene used to normalize all test samples before fold-change analysis. This gene was determined by GeneChip® analysis via SpotFire® visualization of microarray data to be unchanging between wild-type and knockout mice in whole brain, cerebellum, liver, kidney spleen and heart tissues.

Each knockout mouse was paired with its wild-type sibling, who was of the same sex and age and raised under the same environmental conditions. Fold change in gene expression was solely determined by pair-wise comparisons. Approximately 10 ng of total RNA was used for each Q-RT–PCR assay, and each gene was measured in triplicate. All real-time Q-RT–PCR fold changes were determined by using a standard curve with a correlation coefficient of 0.9 or above.

While this paper focused on the expression differences between control and Cstb knockout mice, there were several genes that displayed remarkable similarity in expression level in neurological tissues across eight pairs of mice. These genes include Sult2b1, Pitrk, Spnb3, Ndr2, Adcy6, CplX2 and Atp9a, and may be of interest for future use as housekeeping genes in Cstb knockout mouse neurological tissues. These genes were derived from Q-RT–PCR assays, using EST GenBank accession no. W83013 as a reference gene.

ACKNOWLEDGEMENTS

We thank Mr Michael Cariaso for bioinformatic assistance on the Affymetrix Mouse GeneChip® and Dr Christopher Edwards for critical reading, discussion and review of the manuscript. L.A.P. was funded in part by an American Epilepsy Society Research Fellowship and an appointment to the Alexander Hollaender Distinguished Postdoctoral Fellowship Program sponsored by the U.S. Department of Energy, Office of Biological and Environmental Research, and administered by the Oak Ridge Institute for Science and Education.

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