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The Increment of Purine Specific Sodium Nucleoside Cotransporter mRNA in Experimental Fibrotic Liver Induced by Bile Duct Ligation and Scission

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We investigated the expression profiles of rat fibrotic liver induced by bile duct ligation and scission (BDL/S) using the 3'-directed cDNA libraries. The possibility that the 3'-directed cDNA library represents the mRNA population faithfully was examined by northern blots. During the northern analysis based on fibrotic liver expression profile, we found for the first time that purine specific sodium nucleoside cotransporter (SPNT) was upregulated in BDL/S-induced fibrotic liver. To determine whether the accumulation of bile juice could affect the expression of SPNT mRNA or not, we examined the change of SPNT mRNA expression at 3, 14, 28 days after BDL/S operation. No change in SPNT expression was observed in rat liver at 3 days after surgery. In contrast, there were significant increases in SPNT expression at 14 and 28 days after surgery. We also examined whether chronic liver damage affected SPNT mRNA expression. SPNT mRNA level was significantly increased in BDL/S-induced fibrotic rat liver, whereas no significant change was obserbed in fibrotic livers chronically exposed to carbon tetrachloride or dimethylnitrosamine. From the above results, although further study might be needed, it was considered that the increment of SPNT mRNA in BDL/S liver morphological compatibility to human was remarkable.

Key words: Biliary obstruction, 3'-Directed cDNA library, Expression profile, Gene signature

INTRODUCTION

The liver is an organ with a remarkable capacity for regeneration (Hashimoto and Watanabe, 1999); cell death by apoptosis and/or necrosis in viral hepatitis, alcohol abuse or biliary obstruction induces regeneration. But insults occurring repeatedly set into a cascade of events leading to loss of acinar structure, increased connective tissue content and finally results in cirrhosis. In the end-stage of cirrhosis, the regenerative potential of the liver is impaired or even lost (Krähenbühl *et al.*, 1992; Zimmermann *et al.*, 1992), leading to hepatic decompensation and death (Gross *et al.*, 1987; Park *et al.*, 1978). New therapeutic approaches should be evaluated with the aim of improving survival and diminishing liver injury.

The gene expression patterns in cirrhogenesis are largely unknown; it stands to reason that at least some of these signals will be at the level of gene expression. The knowledge of signals that trigger necrosis and result in hepatic cirrhosis will be extremely valuable in understanding the cause of liver cirrhosis and in working towards its treatment.

The present study is to determine and characterize the specific genes, which are specifically and abundantly expressed in rat cirrhotic liver induced by bile duct ligation and scission. We have initiated a systematic survey of active genes, as well as the relative abundance of mRNA expression, in rat normal and cirrhotic liver using an expression profiling method that is based on quantitative analysis of mRNA populations. This was performed by using 3-directed cDNA libraries (Matsubara and Okubo, 1993; Itoh *et al.*, 1998), which clone has short nucleotide sequence just upstream of poly(A).

During the northern blot analysis to confirm the cirrhotic liver expression profile, we could find that mRNA of purine specific sodium nucleoside cotransporter(SPNT) was increased significantly in rat cirrhotic liver induced by biliary obstruction.

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MATERIALS AND METHODS

Surgical procedures

Male Sprague-Dawley rats weighing 200 to 250 g at the start of the study were used. Biliary cirrhosis was induced by ligation and excision of the bile duct as described (Kountouras *et al.*, 1984; Nam *et al.*, 1997). Double ligatures were placed on the common bile duct and the scission was made into the bile duct between the two ligatures. In sham operated rat, only an incision was made in the abdomen and it was closed without any damage to bile duct. The animals were sacrificed at desired time after obstruction. The CCl_4 group was treated with a oral administration of CCl_4 (Sigma, St. Louis, MO) diluted 50% (vol/vol) with mineral oil (Sigma, St. Louis, MO) at a dose of 1 mL/kg body weight for each rat. CCl_4 was administrated 2 times per week for 6 weeks. The DMN group was treated with a single intraperitoneal injection of DMN (Sigma, St. Louis, MO) diluted with saline at a dose of 10 mg/kg body weight for each rat for 4 weeks.

Characterization of hepatic cirrhosis

Serum and whole liver tissue was obtained from BDL/S and sharm rats. Hydroxyproline concentration in liver was determined by spectrophotometry (Jamall *et al.*, 1981). Serum biochemical parameters (ALT, AST, ALP, total-bilirubin, albumin) were measured. Serum biochemical parameters were measured by Autodry chemistry analyzer (SPOTCHEM™ SP4410, Arkray, Japan). Data are expressed as mean ± SD. Data were analyzed by the student's *t*-test and the *p* value less than 0.05 was considered to be signi-ficant.

Library construction and sequencing

Total RNA from each liver samples were isolated by ultracentrifugation on a CsCl gradient (Sambrook *et al.*, 1989). Poly(A) RNA was separated by using an oligo(dT)-cellulose column. Construction of the 3'-directed cDNA library and transformation into *E. coli* were as described previously (Okubo *et al.*, 1992). Briefly, cDNA was synthesized using a pUC19-based vector primer, digested by the dam-sensitive four-base cutter *Mbo*I, followed by circularization and transformation into *E.coli*. The transformant colonies were randomly selected and cultured in 96-well plates, and the inserted cDNAs amplified with flanking primers and subjected to cycle sequencing.

Comparision of DNA sequences

Sequences of 3-directed cDNA clones were compared with each other by using a computer program, DNASIS™ (Hitachi, Japan). Sequence similarities between cDNA clones, and between cDNA clones and GenBank entries, were analyzed using the BLAST program in the National Center for Biotechnology Information (NCBI, *http://www.ncbi.nlm.nih.gov*).

Northern blots analysis

Total RNA from each liver samples were isolated by ultracentrifugation on a CsCl gradient as described (Sambrook *et al.*, 1989). The *Hind* III/*Kpn* I fragment of clone pUC19 coding for each GSs cDNA clone were used as a probe. Prehybridized and hybridized were performed, as previously described (Virca *et al.*, 1990). For blotting, 15 μg of total RNA was electrophoresed on a 1% agarose gel containing 5.4% formaldehyde, transferred to nylon membranes (Hybond, Amersham, Madrid, Spain) by electroblotting, fixed by UV irradiation, and hybridized with a [$^{\alpha-32}$P]dCTP-labelled cDNA probe using a random priming kit (Amersham, Madrid, Spain).

RESULTS AND DISCUSSION

Characterization of the BDL/S rat

The general characteristics of the rats used in the current study are shown in Table I. Bile duct obstruction for 4 weeks after surgery resulted in significant increases ($p<0.01$) in liver weight and serum activity of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, total bilirubin, and albumin. Also, hydroxyproline content per gram of liver from BDL/S rat (1077 ± 195 μg/g liver) has increased to 282% compared with sham rats (382 ± 57 μg/g liver). The elevation of these parameters is the major characteristics of biliary liver fibrosis.

An expression profiles of active gene in rat normal and BDL/S cirrhotic liver

We measured expression profiles of genes in rat normal and BDL/S cirrhotic liver, which allowed us to perform

Table I. Serum biochemical values in sham operated and bile duct ligation and scission(BDL/S) operated rats

Group	n	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	t-Bilirubin (mg/dL)	Albumin (g/dL)	Hydroxyproline (mg/g liver)
Sham	8	49 ± 6	86 ± 18	298 ± 120	0.2 ± 0.1	4.86 ± 0.92	382 ± 57
BDL/S	11	153 ± 11**	605 ± 72**	1326 ± 279**	8.2 ± 0.2**	2.9 ± 0.3**	1077 ± 195**

Data are expressed as mean ± S.D.
The significance of differences as compared with the sham group **$p<0.01$.
n : number of rats

functional analyses of the genome in rat normal and BDL/S cirrhotic liver. This was achieved through sequencing randomly selected clones from a non-biased cDNA library (Matsubara and Okubo, 1993; Itoh *et al.*, 1998). This library represents the abundance of gene transcripts in the original mRNA population (Ohnishi *et al.*, 1998).

Although serial analysis of gene expression (SAGE) has been reported (Velcuescu *et al.*, 1995) to provide the most reliable means for the quantitative cataloging and simultaneous analysis of expressed transcripts in normal and disease conditions, the possibility of misrecognizing the transcripts might not be excluded because SAGE tags obtained are no more than 9-bp and can be obtained from the conserved region. In contrast, we might eliminate any possibility of misrecognition because 3'-directed cDNA library from sequences just upstream of poly(A) is originated from 3-untranslated region.

A directed cDNA library containing the 3'regions of cDNA was constructed from normal and BDL/S cirrhotic liver, 430 and 814 independent clones randomly selected

Table IIa. Expression profile of BDL/S cirrhotic rat liver

Gene signature	Insert (bp)	Frequency[a]	Accession No.[b]	Definition
L10A12	709	46	X77158	transferrin
L03H07	585	38	K01933	haptoglobin mRNA
L01A06	434	25	X68282	ribosomal protein
L10E12	321	18	S79304	Cytochrome oxidase subunit I
L03B01	432	13	J00734	fibrinogen gamma chain
L10H12	380	11	X52477	pre-pro-complementC3
L12H03	627	9	M62642	Hemopexin
L06A06	548	9	X63446	Fetuin
L09A04	487	9	U25055	Purine specific Na^+ nucleoside cotransporter
L11E04	675	7	D25221	Selenoprotein P
L08F09	481	6	M77183	Alpha-1-macroglobulin
L07C12	131	6	M20246	transthyretin
L04B11	326	6	X91992	alkB protein homolog
L05C06	436	6	M63482	Cytokeratin 8 polypeptide
L02B04	275	5	M27220	Fibrinogen B beta chain
L06E05	621	5	L40363	MHC class I RT1 AW3 protein
L09C03	616	5	V01222	preproalbumin
L06B01	65	4	D00675	Alpha-1-protease inhibitor
L06F07	634	4	M81088	EF-1-alpha mRNA
L02E01	134	4	M10614	Alpha-1-acid glycoprotein gene
L09H03	194	4	X53377	Ribosomal protein S7
L01A10	494	3	X82180	Ribosomal protein L4
L06B03	595	3	M28297	Acute-phase protein alpha-1-inhibitor 3 mRNA
L01E03	272	3	X05348	Growth hormone-regulated protein
L11G07	193	3	D11388	Rig/ribosomal protein S15
L08A02	108	3	M23572	33 DNA, exon 4 and 3 end
L07G09	116	3	D17309	Delta-4-3-ketosteroid 5-beta-reductase
L06E06	574	3	X13231	Sulfate glycoprotein 2
L06H01	459	3	M55663	MHC class II E-beta gene
L12B04	615	3	M33994	Male specific liver CYP450 g mRNA
L06G08	527	3	K03248	Phosphoenolpyruvate carboxykinase (GTP)gene
L07G01	701	3	M12337	Phenylalanine hydroxylase mRNA
L07E11	701	3	M27315	Mitochondrial cytochrome oxidase subunits I,II,and III, and ATPase subunit 6 genes

Table IIb. Expression profile of normal rat liver (Cont'd)

Gene signature	Insert (bp)	Frequency[c]	Accession No.[b]	Definition
N21G01	136	36	M24108	Alpha2u globulin
N31A04	293	19	X77158	Transferrin
N21D11	283	12	X52477	Pre-Pro-complement C3
N23H05	263	8	M13646	Alpha-carbonitrile-inducible CYP450
N19F04	479	6	L07806	Mitochondrial IF1 protein mRNA
N15H03	118	5	M20246	transthyretin
N25C11	119	4	X68282	Ribosomal protein L13a
N30F11	64	4	D00675	Alpha-1-protease inhibitor
N15E07	257	3	J02720	Arginase mRNA
N16B09	90	3	X91992	AlKB protein homolog
N16G06	104	3	M23572	33DNA, exon 4 and 3 end
N19E10	345	3	M86870	Calcium-binding protein mRNA
N19G04	86	3	X05861	Fibrinogen gamma chain

The 3'-directed cDNA clones identified in GenBank were determined by the BLAST program of the NCBI in 'nr' database.
[a]The frequency is the number of cDNA clones appearing among 814 clones.
[b] Accession No. indicates GenBank entries already deposited.
[c]The frequency is the number of cDNA clones appearing among 430 clones.

from library were sequenced. Among the resulting short sequences called gene signatures(GSs), representing just upstream of the poly(A), sequences that were considered essentially identical were lumped together to represent the same gene species. After this treatment, 311 and 512 independent GS species were obtained rat normal and BDL/S liver, respectively.

Table IIa and Table IIb show an expression profile of active genes as represented by their GSs and their activities with their relative abundance in rat cirrhotic liver and normal liver, respectively. We listed here 33 GSs in BDL/S liver and 13 GSs in normal liver that appeared 3 times or more in descending order of appearance, respectively. The profile might reflect unique features of the rat cirrhotic liver physiology. The clones of acute phase proteins, including transferrin, haptoglobin, fibrinogen, α_1-macroglobulin (Gabay and Kushner, 1999; Bistrian, 1999; Neubauer *et al.*, 1995; Hu *et al.*, 1996), were abundant in the cDNA library of livers of cirrhotic rats.

Identification of BDL/S cirrhotic liver-specific genes

In order to confirm and select the unique expression of specific genes among GSs appeared 5 times or more in cirrhotic liver, northern blot analysis was performed with RNAs derived from control normal and BDL/S liver (Fig. 1). The insert such as L01A06, L03B01, L12H03, L09A04, L11E04, L08F09, L06E05 and L09C03 were detected among abundantly appearing GSs in BDL/S liver (Table IIa). The RNA levels for L01A06, L03B01, L12H03, L09A04, and L06E05 were almost higher in the BDL/S liver than normal liver, while the RNA levels for L11E04 and L09C03 were detected considerable amounts in normal liver than BDL/S liver in spite of zero frequency in normal liver. These two exceptions might be due to below the limit of detection by randomly collected 430 cDNA sequences from normal liver.

As presented in Fig. 1, among GSs expressed highly in the cirrhotic liver, L09A04 mRNA expression level, coding for purine specific sodium nucleoside cotransporter (SPNT), was increased in BDL/S cirrhotic liver as compared to normal liver. Interestingly, the altered expression of SPNT has not been reported yet in association with liver injury such as cholestasis or cirrhotic state.

A SPNT system has been localized to the bile canalicular membrane (Kitamura *et al.*, 1991). The importance of bile canalicular sodium-dependent nucleoside transporter system is related to the following unique functions of liver (Che *et al.*, 1995): first, a concentrative nucleoside transport system is physiologically desirable for maintaining the nucleoside concentration in hepatocytes and preventing nucleoside loss in bile. Second, extracellular ATP profoundly damages hepatocytes and experimentally produces cholestasis (Che *et al.*, 1995). The abundant bile canalicular ectonucleotidases rapidly degrade ATP to adenosine. The dual processes of nucleotide breakdown and nucleoside conservation are important physiologically and pathologically.

Time-dependent expression of SPNT after BDL/S surgery

Since one of major role of SPNT was reported to prevent

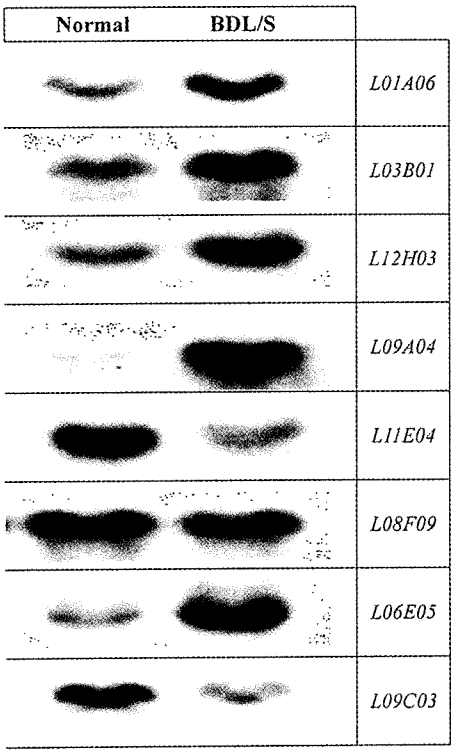

Fig. 1. Northern blot analysis for the levels of specific genes among GSs appeared 5 times or more in cirrhotic liver. Northern blots were performed with total RNA extracted from the normal and cirrhotic rat livers induced by BDL/S for 4 weeks. The same total RNA was hybridized with *Hind*III/*Kpn* I fragment of clone pUC19 coding for each GSs. The GSs used probes are indicated on the right side of each autoradiograms.

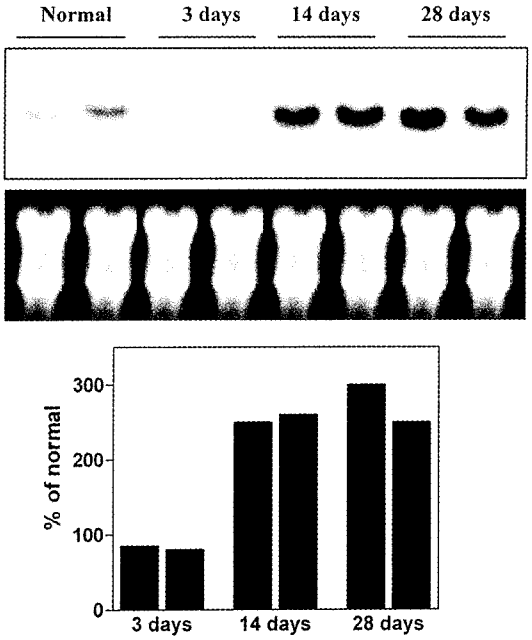

Fig. 2. Time-dependent expression and densitometric analysis of SPNT mRNA in total rat liver RNA after BDL/S. Total RNA was isolated from 2 experimental rats, which were sacrificed as indicated days after BDL/S. The *Hind*III/*Kpn*I fragment of clone pUC19 coding for SPNT was used as probe. The biochemical parameters of rat livers used in the current studies are shown in Table III.

cholestasis in the liver as mentioned above, it was of interest that the accumulation of bile juice in the liver could affect the expression of SPNT mRNA. We planned to determine the change of SPNT mRNA expression levels at 3, 14, 28 days after BDL/S operation by use of northern blots. The biochemical parameters of livers used in the current studies are shown in Table III.

As shown in Fig. 2, any change in SPNT expression was not observed in rat liver after 3 days of surgery. In contrast, there were significant increases in SPNT expression after 14 and 28 days of surgery, respectively. Therefore, it can be suggested that bile juice *per se* has less effect on the expression of SPNT mRNA, since the acute exposure of liver under cholestatic condition did not induce the SPNT mRNA.

An aspect of SPNT expression in various cirrhotic model

Next, we examined whether chronic liver damage *per*

Table III. Characterization of the rats used in the current studies

	Sham	Normal		BDL/S						CCl_4	DMN
				3 days		14days		28days			
ALT (IU/L)	45	45	50	732	838	139	145	172	163	633	199
AST(IU/L)	85	80	95	1394	1600	578	610	700	600	2680	307
ALP (IU/L)	290	300	280	708	585	897	987	616	780	2690	1500
t-Bilirubin (mg/dL)	0.2	0.2	0.3	8.6	10.5	9.8	10.2	8.4	9.0	3.0	0.9
Albumin (g/dL)	4.7	4.5	5.0	2.7	2.7	3.2	2.9	3.2	3.0	4.0	3.0
Hydoxyproline (μg/g liver)	400	380	368	447	460	797	680	1047	1160	1752	1443

Rats were bile duct ligation and scission as described in Materials and Methods. After surgery, the rats were studied at the time points indicated in the table.

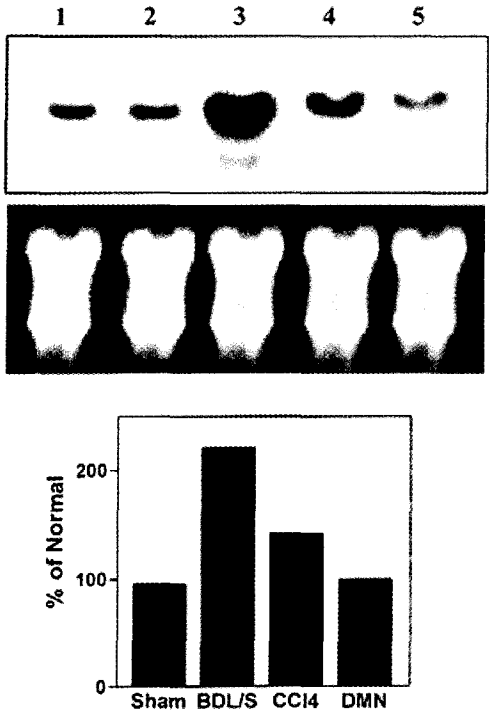

Fig. 3. SPNT mRNA expression and densitometric analysis in different models of cirrhosis. Lane 1, normal liver; Lane 2, sham liver; Lane 3, BDL/S liver (induced for 4 weeks); Lane 4, CCl_4 liver; Lane 5, DMN liver. The *Hind*III/*Kpn*I fragment of clone pUC19 coding for SPNT was used as probe. Each rat cirrhosis were induced as described in Table III.

se causes to alter the expression of SPNT mRNA. Therefore, another two animal models induced by the chronic exposure to carbon tetrachloride (CCl_4) (Hernandez-Munoz *et al.*, 1990; Brenner *et al.*, 1993) and dimethylnitrosamine (DMN) (Jezequel *et al.*, 1987; Yasuda *et al.*, 1999), respectively, was chosen to determine the SPNT mRNA levels in the liver. The biochemical parameters of these experimental model used in the current study are summarized in Table III.

As shown in Fig. 3, SPNT mRNA levels were significantly increased in BDL/S-induced fibrotic rat liver for 4 weeks, while it showed no significant differences in CCl_4- and DMN-induced fibrotic livers, respectively, as compared to normal liver. On the contrary to our expectation that the cholestasis or hepatic fibrosis can affect the expression in SPNT mRNA, the expression pattern was increased only in the fibrotic liver induced by bile duct ligation and scission. Therefore, it can be suggested that long term hepatic injury and fibrosis have no relationship with the expression of SPNT mRNA. Also, this phenomenon could not be explained by the correlation studies between the mRNA expression and various biochemical parameters induced by cholestatic exposure or hepatotoxins such as carbon tetrachloride and dimethylnitrosamine (data not shown).

From the above results, although further study might be needed to examine the clinical relevance as well as to understand the exact mechanism, it was considered that the increment of SPNT mRNA in BDL/S liver morphological compatibility to human (Kountouras *et al.*, 1984) was remarkable.

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