Extracellular matrix metabolism-related gene expression in bile duct-ligated rats

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Abstract. Bile duct-ligated rats have been widely used as a model of cholestatic liver fibrosis. The aim of this study was to analyze the sequential expression of extracellular matrix (ECM) metabolism-related genes in these rats. We analyzed the intrahepatic messenger RNA (mRNA) expression of several ECM metabolism-related genes: transforming growth factor ß1 (TGF-ß1), connective tissue growth factor (CTGF), procollagen-α1 (collagen-I), matrix metalloproteinase (MMP)-2, MMP-13 and tissue inhibitor of metalloproteinases (TIMP)-1 on days 10, 21 and 42 after bile duct ligation (BDL). A DNA microarray was used to evaluate the expression of genes related to early fibrogenesis on day 10 following BDL; the grade of hepatic fibrosis was found to gradually progress from day 10 to 42. Collagen-I mRNA expression significantly increased from day 10 to day 42, as did TGF-ß1 and CTGF mRNA. On the other hand, MMP-13 and -2 mRNA expression increased maximally from day 10 to 21, but tended to decrease by day 42. TIMP-1 mRNA expression was significant on day 21 and was sustained until day 42. The DNA microarray revealed genes significantly increased on day 10, including calgranulin B, solute carrier family 34, thymosin and tubulin, but not fibrogenesis-related cytokines or MMPs/TIMPs. In conclusion, the enhanced gene expression of collagen-I, TGF-ß1, CTGF and TIMP-1 and the decreased gene expression of MMP-13 and -2 was noted on day 42 in BDL-induced liver fibrosis.

Introduction

Hepatic fibrosis is the disorganized over-accumulation of extracellular matrix (ECM) components in the liver. In a normal liver, these components are constantly remodeled by matrix-degrading enzymes, leading to a controlled deposition of matrix components. Increased production of ECM components is responsible for the altered ECM metabolism of the fibrotic liver (1). Transforming growth factor ß1 (TGF-ß1) has a prominent role in the pathogenesis of liver fibrosis and is the most important cytokine involved in the activation of hepatic stellate cells (HSCs). Acting via both paracrine and autocrine pathways, TGF-ß1 promotes ECM production and inhibits production of the matrix metalloproteinases (MMPs) for effective ECM deposition in the liver (2). On the other hand, MMPs have degrading activity against ECM components and could potentially initiate the remodeling of the fibrotic liver. The activities of MMPs are regulated by means of the action of a family of inhibitory proteins, known as tissue inhibitors of metalloproteinases (TIMPs). In order to inhibit MMP activity, TIMPs interact with MMPs with a 1:1 stoichiometry (3,4). Deregulation of the enzymatic machinery involved in ECM degradation may also be an important contributing factor in the pathogenesis of hepatic fibrosis and cirrhosis.

Many animal models are used to investigate hepatic fibrosis. The common bile duct-ligated rat has been widely used as a model of human cholestatic hepatic fibrosis, including primary biliary cirrhosis and primary sclerosing cholangitis. We previously reported the preventive and therapeutic effects of antifibrotic agents using this model (5,6), for which it is important to know the precise sequential dynamics of ECM metabolism. However, there are few reports analyzing ECM metabolism-related gene expression in a bile duct ligation (BDL) model in detail (7-10).

The aim of the present study was to assess the gene expression levels of ECM metabolism-related genes such as TGF-ß1, connective tissue growth factor (CTGF), procollagen-α1 (collagen-I), MMP-2, MMP-13 and TIMP-1 at serial time points following BDL in rats.

Materials and methods

Animals. Wistar male rats were obtained from Japan SLC (Shizuoka, Japan) and maintained in a room at a controlled temperature of 24±2˚C with a 12-h light-dark cycle. Animals were given a standard pellet chow and water *ad libitum*. Vitamin K (5 mg/kg) was injected intramuscularly on days 7, 14, 21, 28 and 35. Anesthesia was performed by intraperitoneal injection of pentobarbital (Dainippon Pharmaceutical,

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Osaka, Japan) at a dose of 50 mg/kg. All experiments were carried out in accordance with the Animal Experimentation Guidelines of Tottori University.

Establishment of the bile duct ligation model. Wistar male rats weighing approximately 250 g were used. BDL was carried out as previously described (11). The common bile duct was double-ligated and cut between the ligatures. On days 10 (n=9), 21 (n=6) and 42 (n=5), the rats were sacrificed under pentobarbital anesthesia. On day 21, 5 untreated rats were also sacrificed as normal controls. Blood was collected from the vena cava inferior. Serum samples were frozen and stored at -80˚C. The liver was washed with saline and weighed. Liver specimens were snap frozen and stored at -80˚C, or fixed in 10% buffered formalin and embedded in paraffin for histological analysis.

Histological analysis. Sections (4-μm) of formalin-fixed, paraffin-embedded livers were processed according to routine hematoxylin and eosin and Azan-Mallory staining.

Measurement of hepatic hydroxyproline content. Hepatic tissue (200 mg wet weight) was hydrolyzed in 4 ml of 6 N HCl at 105˚C overnight. The hydrolysate was evaporated under vacuum four times. The sediment was redissolved in distilled water, mixed with activated charcoal for decolorization, and then filtered. The solution was kept in the acidic range by adjustment to pH 5.0 and evaporated under vacuum, and the sediment was redissolved in distilled water, supplemented with 2 ml of isopropanol and incubated with 1 ml of 7% chloramine-T solution for 5 min at room temperature. Ehrlich's solution (2 ml) consisting of 1.76 g of *p*-dimethylamino-benzaldehyde dissolved in 4.08 ml of 60% perchloric acid and 95.5 ml of isopropanol was added, and the mixture was incubated at 60˚C for 10 min. After cooling, absorbance was measured at 562 nm. Hydroxyproline (Hyp) levels were expressed as micrograms of Hyp per gram of liver (12).

Measurement of hepatic TGF-ß1 protein. Following acid-ethanol extraction of TGF-ß1 from hepatic tissue (13), TGF-ß1 protein was measured with an enzyme-linked immunosorbent assay (ELISA) kit that uses anti-human TGF-ß1 antibody (Quantikine Human TGF-ß1 Immunoassay; R&D Systems, Minneapolis, MN, USA), which detects only the active form of TGF-ß1. The assay for TGF-ß1 concentration was conducted in duplicate. Linearity between the amount of TGF-ß1 and absorbance at 492 nm was demonstrated.

RNA extraction and reverse-transcription. Tissue samples were homogenized, and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA concentration was determined by measuring absorbance at 260 nm, and the quality of RNA was verified by electrophoresis on an ethidium bromide-stained 1% agarose gel. Total RNA (~2 μg) was reverse transcribed (RT) in a solution with a final volume of 11.5 μl containing 4 μl of 5X standard buffer, 2 μl of 0.1 MdTT, 1 μl of SuperScript II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA, USA), 2 μl of 10 M MdNTPs (Promega, Madison, WI, USA), 1 μl of 50 pmol/μl Random Primer (Promega), 0.5 μl of 100 pmol/μl Oligo(dt)15 Primer (Promega) and 1 μl of 40 U/μl ribonuclease inhibitor (Wako Pure Chemical Industries Ltd., Osaka, Japan). Samples were incubated at 37˚C for 60 min, then at 95˚C for 5 min, and were subsequently cooled to 4˚C for 5 min.

Real-time polymerase chain reaction (PCR). Quantitative real-time PCR was performed in a final volume of 10 μl containing PCR grade water 4.1 μl, Universal ProbeLibrary probe 1 μl (Roche, Tokyo, Japan), forward primer (10 μM) 0.2 μl, reverse primer (10 μM) 0.2 μl, Light Cycler TaqMan Master 2 μl (Roche) and cDNA sample 2.5 μl of the RT samples. The mRNA levels of TGF-ß1, CTGF, collagen-I, MMP-2, MMP-13 and TIMP-1 were assessed by real-time PCR assay using ß-actin as a housekeeping gene. The forward and reverse primer sequences used are shown in Table I. Thermal cycler conditions were as follows: hold at 95˚C for 10 min, repeat 45 cycles of 95˚C for 30 sec and 60˚C for 1 min.

DNA microarray analysis. After total RNA was extracted from liver tissue, it was reverse transcripted to cDNA using oligo(dT)24 primer with a T7 promoter sequence (Affymetrix, Santa Clara, CA, USA) and reverse transcriptase (Invitrogen). Subsequently, double-stranded cDNA was obtained using DNA polymerase (Invitrogen) and DNA ligase (Invitrogen). Biotin-labeled cDNA was obtained using T7 RNA polymerase (Enzo Life Sciences, Farmingdale, NY, USA). The cDNA of each serial group was mixed with the respective samples of the same group. This cDNA was hybridized using the GeneChip Rat Expression Array 230A (Affymetrix) for 16 h. Biotin-labeled portions were stained with fluorescent dye and evaluated using a GeneChip system for scanning the micro-array. Data were analyzed using Microarray Suite software (Affymetrix) and Microsoft Excel.

Statistical analysis. The Mann-Whitney test was used to assess the statistical significance between groups. The Kruskal-Wallis test was used to assess the statistical significance between groups on different days. All statistical tests were carried out using Stat View for Windows (SAS Institute, Cary, NC, USA). $P<0.05$ was considered significant.

Results

Biochemical analysis and liver fibrosis. Biochemical liver test results increased gradually in conjunction with time following BDL (Table II). Hepatic Hyp content was significantly increased on days 21 ($P<0.05$) and 42 ($P<0.01$) in bile duct-ligated rats versus control rats (Fig. 1). Values were as follows: control rats, 2.05±0.04 μg/g wet liver; bile duct-ligated rats on day 10, 1.69±0.9 μg/g wet liver; on day 21, 9.65±4.87 μg/g wet liver; on day 42, 25.5±12.5 μg/g wet liver. Histological liver findings stained with Azan-Mallory exhibited fibrosis and bile duct proliferation 10 days after BDL, and cirrhotic liver 42 days after BDL (Fig. 2A-D).

Collagen-I, TGF-ß1 and CTGF expression. Collagen-I mRNA levels in bile duct-ligated rats were significantly increased on days 10, 21 and 42 compared to the control ($P<0.01$; Fig. 3A). TGF-ß1 mRNA expression levels were increased in bile duct-ligated rats compared to the control from day 10. This

Table I. Primers used for real-time PCR.

Gene	Accession no.	Primers
ß- actin	BC063166	Forward: 5'-CCCGCGAGTACAACCTTCT-3' Reverse: 5'-CGTCATCCATGGCGAACT-3'
TGFß-1	X52498	Forward: 5'-CCTGGAAAGGGCTCAACAC-3' Reverse: 5'-CAGTTCTTCTCTGTGGAGCTGA-3'
Collagen-I	Z78279	Forward: 5'-ATGTTCAGCTTTGTGGACCTC-3' Reverse: 5'-GCAGCTGACTTCAGGGATGT-3'
CTGF	ABO23068	Forward: 5'-GCTGACCTAGAGGAAAACATTAAGA-3' Reverse: 5'-CCCGGTAGGTCTTCACACTG-3'
TIMP-1	U06179	Forward: 5'-CAGCAAAAGGCCTTCGTAAA-3' Reverse: 5'-TGGCTGAACAGGGAAACACT-3'
MMP-2	X71466	Forward: 5'-GCGCTTTTCTCGAATCCAT-3' Reverse: 5'-GGGTATCCATCTCCATGCTC-3'
MMP-13	M60616	Forward: 5'-GGACAAGCAGCTCCAAAGG-3' Reverse: 5'-GGTCCAGACCGAGGGAGT-3'

TGFß-1, transforming growth factor ß1; CTGF, connective tissue growth factor; TIMP-1, tissue inhibitor of metalloproteinases-1; MMP, matrix metalloproteinase.

Table II. Biochemical data.

	Control (n=5)	BDL day10 (n=9)	BDL day 21 (n=6)	BDL day 42 (n=5)
T-Bil (mg/dl)	0.05±0.01	3.5±1.3	6.5±1.3[a]	7.9±0.1[ac]
AST (IU/l)	139±11	290±41[a]	588±186	1116±512[b]
ALT (IU/l)	47±2	77±8[a]	141±42	198±89
ALP (IU/l)	1042±51	1845.3±174.2[a]	2005±151[a]	1262±80[abc]

BDL, bile duct ligation; T-Bil, total bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase. Values are the means ± SD. [a]p<0.05 vs. control; [b]p<0.05 vs. BDL day 10; [c]p<0.05 vs. BDL day 21.

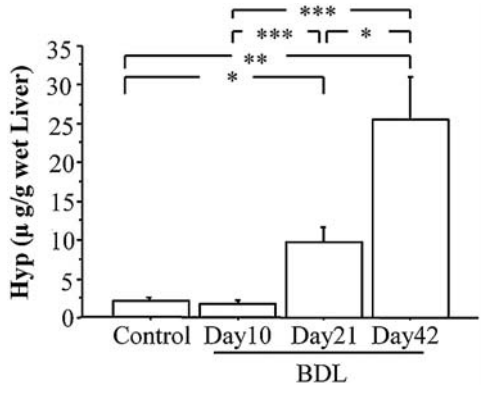

Figure 1. Hepatic hydroxyproline content. Hepatic Hyp content in control and bile duct-ligated rats 10 days after bile duct ligation (BDL) (day 10), 21 days after BDL (day 21) and 42 days after BDL (day 42). The hepatic Hyp content increased significantly over each period following BDL compared to the control and to the previous period (not including day 10 vs. controls). Values are the means ± SD. *P<0.05; **P<0.01; ***P<0.001.

increase was sustained until day 42, but the differences were not statistically significant (P=0.63, Kruskal-Wallis test; Fig. 3B). Following the increase in TGF-ß1 gene expression, protein levels of TGF-ß1 were significantly increased in bile duct-ligated rats on days 10, 21 and 42 versus the control (P<0.01), and also on day 10 as compared to days 21 and 42 (P<0.01). A less significant difference was observed between levels on days 21 and 42 (P=0.045) (Fig. 3C). Values were as follows: control rats, 1.04±0.7 ng/g wet liver; bile duct-ligated rats on day 10, 8.15±5.27 ng/g wet liver; on day 21, 38.3±22.1 ng/g wet liver; on day 42, 67.5±37.5 ng/g wet liver. Levels of CTGF mRNA were significantly increased in bile duct-ligated rats on day 21 (P=0.029). These increases were sustained until day 42 (P=0.009) compared to the control (Fig. 3D).

MMP-13, MMP-2 and TIMP-1 expression. Levels of MMP-13 mRNA significantly increased from day 10 in bile duct-ligated

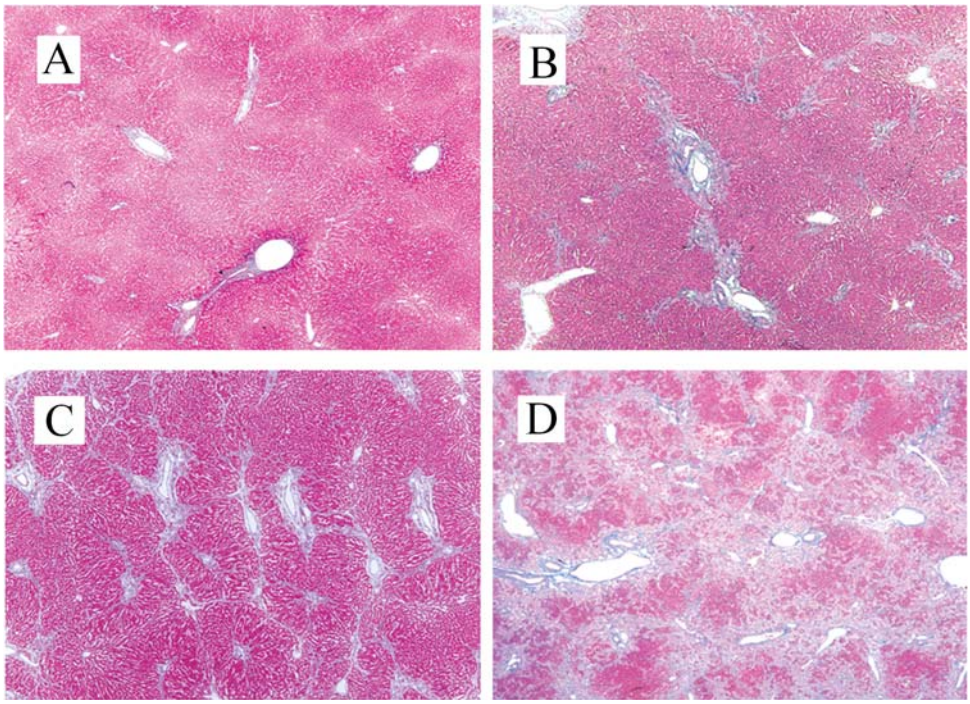

Figure 2. Histological liver findings. Azan-Mallory staining shows progressive fibrosis and bile duct proliferation. (A) There was no fibrosis in the liver of control rats. (B) Fibrosis was observed only in periportal regions of the liver of bile duct-ligated rats on day 10. (C) The fibrotic septa were thin and restricted to periportal regions on day 21. (D) Cirrhosis on day 42. Original magnification x40.

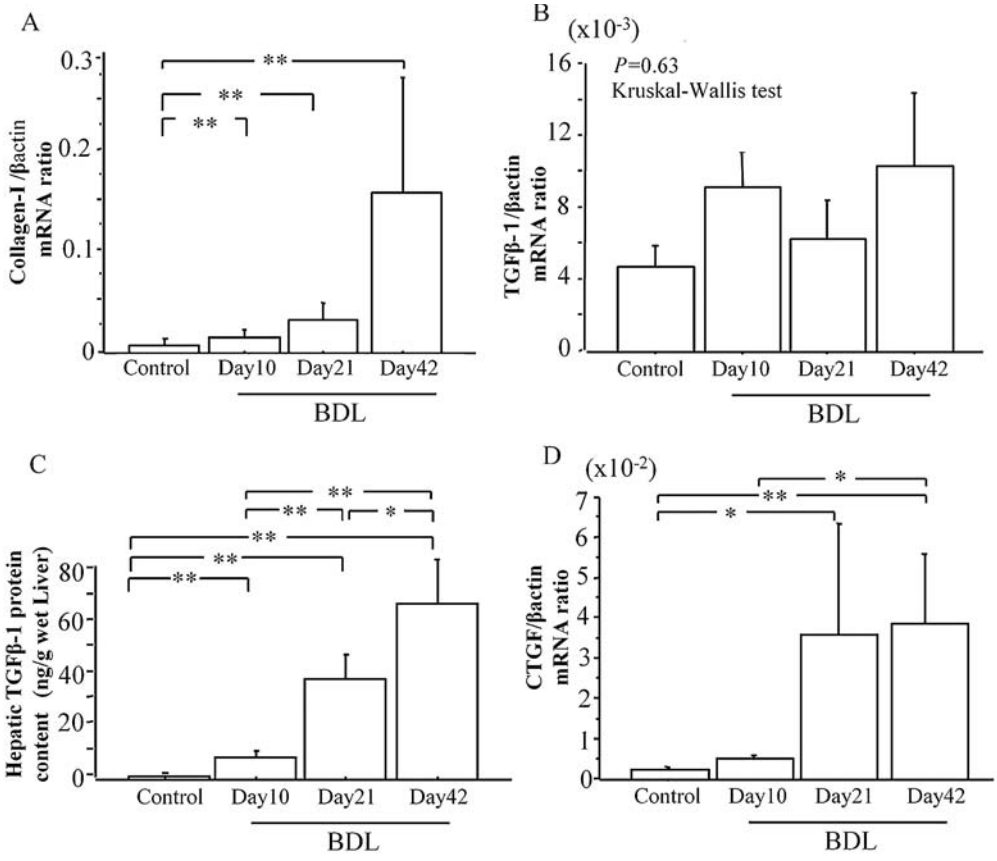

Figure 3. Sequential changes in collagen-I mRNA, TGF-ß1 mRNA, TGF-ß1 protein and CTGF mRNA. (A) Sequential data of intrahepatic mRNA levels of collagen-I after bile duct ligation (BDL). Intrahepatic mRNA levels of collagen-I were significantly increased over time after BDL compared to the control. (B) Sequential data of intrahepatic mRNA levels of TGF-ß1 after BDL. Intrahepatic mRNA levels of TGF-ß1 mRNA expression were increased in BDL rats compared to the control from day 10. This increase was sustained until day 42, but the differences were not statistically significant. (C) Sequential changes in TGF-ß1 protein content after BDL. Liver contents of TGF-ß1 protein were significantly increased from day 10 to 42, occurring after the changes in expression of TGF-ß1 mRNA. (D) Sequential data of intrahepatic mRNA of CTGF. Levels of CTGF significantly increased from day 21 and were sustained until day 42. Each bar represents the mean ± SD. *P<0.05; **P<0.01.

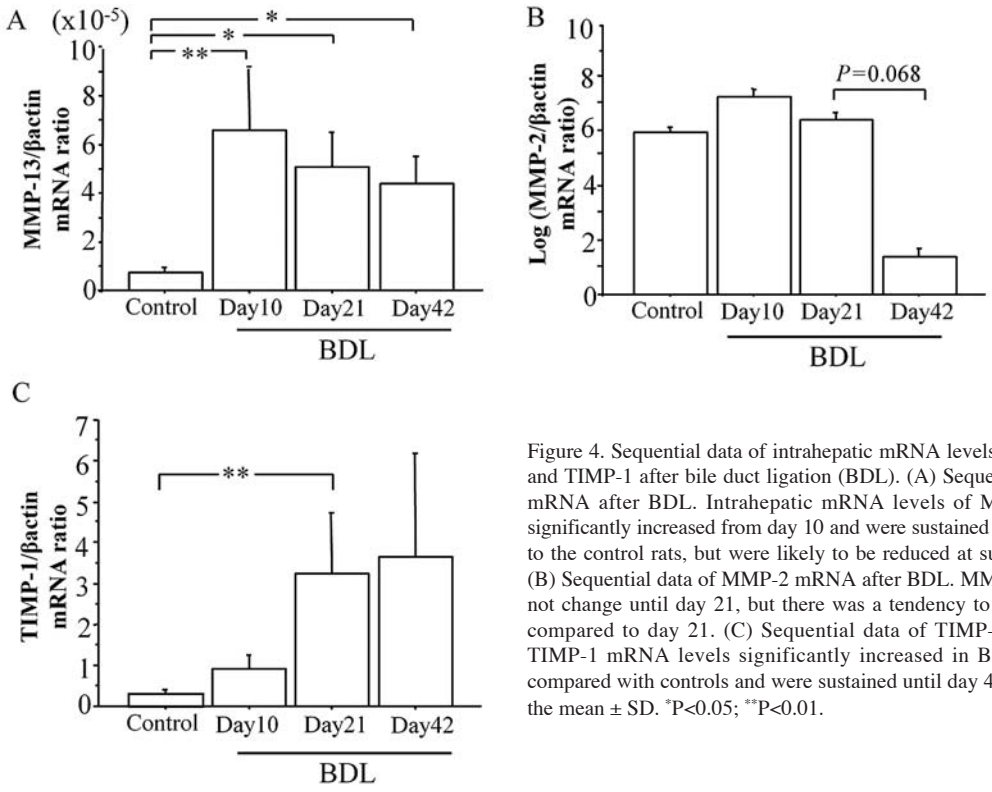

Figure 4. Sequential data of intrahepatic mRNA levels of MMP-13, MMP-2 and TIMP-1 after bile duct ligation (BDL). (A) Sequential data of MMP-13 mRNA after BDL. Intrahepatic mRNA levels of MMP-13 mRNA were significantly increased from day 10 and were sustained until day 42 compared to the control rats, but were likely to be reduced at subsequent time points. (B) Sequential data of MMP-2 mRNA after BDL. MMP-2 mRNA levels did not change until day 21, but there was a tendency to decrease after day 42 compared to day 21. (C) Sequential data of TIMP-1 mRNA after BDL. TIMP-1 mRNA levels significantly increased in BDL rats from day 21 compared with controls and were sustained until day 42. Each bar represents the mean ± SD. *P<0.05; **P<0.01.

rats compared to the control (P=0.004). These increases were sustained on day 21 and day 42 compared to the control (P<0.05; Fig. 4A). Levels of MMP-2 mRNA did not change until day 21, but had the tendency to be decreased on day 42 compared to day 21 (P=0.068; Fig. 4B). TIMP-1 mRNA levels significantly increased in bile duct-ligated rats from day 21 (P=0.006) compared to the control, and were sustained until day 42 (Fig. 4C).

DNA microarray analysis on day 10 after bile duct ligation. DNA microarray analysis revealed that 11 genes, including calgranulin B, solute carrier family 34, thymosin and tubulin, were significantly increased (>30-fold) in bile duct-ligated rats on day 10 compared to the control (Table III). None of the genes related to collagen production and degradation increased >30-fold after BDL.

Discussion

In this study, we examined ECM metabolism-related gene expression over 42 days following BDL in rats. Previous reports assessed findings within 30 days of BDL in rats (7-10). Histological findings and Hyp contents in this study revaled that fibrosis progressed periodically over time after BDL (days 10, 21 and 42). On day 42, the specimens exhibited liver cirrhosis.

Iredale *et al* (7) examined collagen-I mRNA expression for 3 days in bile duct-ligated rats. Collagen-I mRNA was not detected until 3 days after BDL, at which point a high level of expression was observed compared to the control. Our results indicate that collagen-I mRNA significantly increased with time from day 10 to day 42 following BDL, as compared to the control. Taken together, these data indicate that collagen-I mRNA expression is higher in the BDL liver than in the control liver for at least 3 days after BDL, and that hepatic fibrosis progresses with increasing collagen-I gene expression until day 42.

Napoli *et al* (8) examined the mRNA expression of TGF-ß1 in bile duct-ligated rats from day 2 to day 21, and observed a statistically significant increase in TGF-ß1 on days 14 and 21. Makino *et al* (10), using a biliary obstruction (BO) rat model, also reported that TGF-ß1 mRNA increased significantly on days 14 and 21 after BO. Our results, which show that mRNA levels of TGF-ß1 were increased as of day 10 and sustained until day 42, are in agreement with these previous findings, while hepatic TGF-ß1 protein contents were significantly increased in a time-dependent manner. CTGF, a potent fibrogenic cytokines, is secreted by HCSs and is a downstream mediator of TGF-ß1. It was previously reported that, under normal conditions, the basal expression of CTGF is low, but that it becomes highly up-regulated during wound healing or fibrogenesis (14). CTGF was reported to be a candidate marker of ongoing fibrogenesis in human chronic liver disease (15). Sedlaczek *et al* (14) reported that CTGF was up-regulated in close relation to TGF-ß1 in a cholestatic rat model. Paradis *et al* (16) also reported that CTGF was strongly expressed along the septa, with a significant increase around areas of ductular proliferation in bile duct-ligated rats. Our study demonstrated that CTGF mRNA was up-regulated from days 21 to 42 along with TGF-ß1 mRNA. Taken together, the results suggest that TGF-ß1 and CTGF are involved in cholestasis-induced hepatic fibrosis.

Progressive liver fibrosis results from reduced matrix degradation, that is, a reduction in MMPs and/or an increase in TIMPs in addition to an increase in ECM products. Iredale *et al* (7) showed that TIMP-1 mRNA expression was increased at 6 h, 24 h and 3 days following BDL. Furthermore, interstitial collagenase mRNA expression did not change until 3 days

Table III. DNA microarray analysis.

Gene symbol	Accession no.	Gene title	Inferred gene ontology/molecular function
S100a9	NM_053587	S100 calcium binding protein A9 (calgranulin B)	i) signal transducer activity, ii) calcium ion binding
Slc34a2	NM_053380	Solute carrier family 34 (sodium phosphate), member 2	i) symporter activity, ii) sodium-dependent phosphate transporter activity, iii) sodium ion binding
Tmsb10	NM_021261	Thymosin, ß 10	i) actin monomer binding, ii) actin binding
Tubb6	BI274903	Tubulin, ß 6	i) structural molecule activity, ii) nucleotide binding, iii) GTPase activity, iv) GTP binding
Fabp4	NM_053365	Fatty acid binding protein 4, adipocyte	i) fatty acid binding ii) protein binding, iii) transcriptional repressor activity, iv) binding, v) lipid binding
Lcn2	NM_130741	Lipocalin 2	i) binding, ii) transporter activity
Wnt4	NM_053402	Wingless-related MMTV integration site 4	i) receptor binding, ii) extracellular matrix structural constituent, iii) protein binding, iv) signal transducer activity
Cldn7	AJ011811	Claudin 7	i) identical protein binding, ii) protein binding, iii) structural molecule activity
Aqp4	AI235942	Aquaporin 4	i) water channel activity, ii) porin activity, iii) transporter activity
RT1-CE5	AJ243338	RT1 class I, CE5	MHC class I receptor activity
LOC500040	AI177869	Similar to Testis derived transcript	Zinc ion binding

after BDL. The authors concluded that increased expression of TIMP-1 relative to interstitial collagenase would promote the progression of early-stage liver fibrosis. Kossakowska *et al* (9) demonstrated that MMP-2 activity was increased as of day 2 and continued to increase steadily until day 10 following BDL, although they could not detect MMP-2 mRNA expression with Northern hybridization. Our data reveal that MMP-2 mRNA expression was maintained at normal levels until day 21, and tended to be reduced by day 42. Although the reason for this discrepancy is unclear, mRNA expression of MMP-2 may be inconsistent with MMP-2 activation. However, MMP-2 mRNA expression is reported to be lower in cirrhotic human livers than in normal livers (17), in agreement with our finding of liver cirrhosis on day 42 of this study.

We also evaluated the sequential change in MMP-13 mRNA expression. MMP-13 mRNA expression was significantly up-regulated in bile duct-ligated rats from day 10 compared to the control rats. These higher values were maintained and were likely to be reduced at subsequent time points. The beneficial effect of collagenase during the resolution of hepatic fibrosis has been well documented in previous studies (18,19). Uchinami *et al* (20) reported that expression of MMP-13 mRNA increased significantly in the BDL livers of mice until 3 weeks, and that MMP-13 accelerated fibrogenesis in BDL livers by mediating the initial inflammation. In addition, various cytokines including IL-1 and TGF-ß1 stimulate MMP-13 production in HSCs (21,22). The ECM serves as a binding reservoir for several cytokines, and MMPs release soluble cytokines through ECM degeneration (23-25). These interactions between MMPs and cytokines may be involved in the fibrogenesis of BDL livers.

Our study showed that TIMP-1 mRNA expression was significantly up-regulated from day 21 in bile duct-ligated rat livers compared to control rats. Kossakowska *et al* (9) reported that TIMP-1 mRNA appeared on day 2 following BDL in rats, increased until day 10, and remained stable thereafter. Similar findings have also recently been reported in other experimental fibrotic models, such as CCl_4-induced and immune-induced models (26). TIMP-1 regulates hepatic fibrogenesis through the inhibition of MMPs. Enhanced expression of TIMP-1 relative to MMPs has been reported to promote the progression of hepatic fibrosis in early bile duct-ligated rat livers (7). TIMP-1 seems to be closely involved in the progression of hepatic fibrosis following BDL.

In conclusion, enhanced gene expression of collagen-I, TGF-ß1, CTGF and TIMP-1 and decreased gene expression of MMP-13 and MMP-2 were noted on day 42 in BDL-induced liver fibrosis.

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