The human Delta-like 1 homologue is implicated in the progression of liver fibrosis in biliary atresia

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Abstract

Advanced liver cirrhosis frequently occurs in infants with biliary atresia despite early surgical correction. The aetiology is unknown, but may involve many cytokines and liver cells including hepatic stellate cells (HSCs). A cytokine expression array and real-time quantitative reverse transcription-polymerase chain reaction (QRT-PCR) were used to study cytokine expression during the progression of liver fibrosis in biliary atresia. A Delta-like 1 homologue (DLK1) gene was identified and this gene was up-regulated during the early stage, and down-regulated during the late stage, of biliary atresia, similar to the expression pattern of the procollagen α1(I) gene. Further characterization with immunohistochemistry, confocal microscopy, and in situ hybridization revealed that the DLK1 protein was mainly present in the cytoplasm of smooth muscle actin-positive mesenchymal cells that were morphologically and immunohistochemically identical to activated HSCs/myofibroblasts, whereas DLK1 mRNA was present only in hepatocytes. As DLK1 is a negative regulator of adipocyte differentiation and may control cell fate during differentiation, overexpression of DLK1 protein in HSCs in the early stage of biliary atresia suggests that DLK1 may be implicated in the transformation of HSCs from fat-storing cells to myofibroblasts and in fibrogenesis associated with biliary atresia. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords: biliary atresia; liver fibrosis; DLK1; cytokine expression array; real-time quantitative reverse transcription-PCR; immunohistochemistry; confocal microscopy; in situ hybridization

Introduction

Biliary atresia (BA) is the major cause of prolonged cholestasis in infancy. It is characterized by idiopathic and complete obliteration of the extrahepatic bile duct and ongoing inflammation in the hepatobiliary system. Kasai’s porto-enterostomy is the only effective procedure for correction of BA. However, two-thirds of patients who undergo the Kasai procedure develop liver cirrhosis despite bile flow being adequate and the disorder accounts for more than 50% of children receiving liver transplantation [1–3].

The degree of hepatic fibrosis determines the outcome of most patients who undergo the Kasai operation for BA. Histological features of hepatic fibrosis in BA include loss of intrahepatic bile ducts, increased mast cell population, activation of hepatic stellate cells (HSCs), and increased synthesis but decreased degradation of extracellular matrix [4–6]. These processes involve many cytokines, proteases, and their inhibitors, but only a few, such as TGF-β1, TIMP-1, PAI-1, MMP-1, and MMP-3, have been identified [7,8].

To investigate the potential regulatory mechanism associated with the progression of liver fibrosis in BA, we used a cytokine expression array containing 375 cDNA sequences to compare the cytokine expression profiles in the liver between the early and late stages of BA. The differential expression of a gene encoding the Delta-like 1 homologue (DLK1), which is known to be an inhibitor of adipogenesis [9–13], was confirmed by real-time quantitative reverse transcription-polymerase chain reaction (QRT-PCR) and further characterized by in situ hybridization, immunohistochemistry, and confocal microscopy.

Materials and methods

This study, including the use of fresh and paraffin wax-embedded tissue specimens, was approved by the Medical Ethics and the Human Clinical Trial Committee at Chang Gung Memorial Hospital.
Patients and samples
Snap-frozen tissues were obtained from three wedge liver biopsy samples from early BA patients undergoing the Kasai procedure (referred to as the KP group) and three liver explants from late BA patients undergoing liver transplantation (referred to as the LT group). Informed consent was obtained from the patients’ parents. Patients in the KP group were 1.3, 1.6, and 1.7 months old, and those in the LT group were 10 months, 22 months, and 15 years old, respectively.

RNA isolation and generation of ³²P-labelled cDNA
Total RNA was isolated from frozen liver tissue using REZOL™ C&T (Protech Technology, Taipei, Taiwan), purified by phenol–chloroform extraction, and then quantified by spectrophotometry with absorbance at 260 nm. Fifteen to twenty micrograms of total RNA was annealed with 1 μg of oligo(dT)₁₅ primers (Promega, Madison, WI, USA) and then incubated with 100 μCi of [³²P]dCTP, 800 units of M-MLV reverse transcriptase (Promega), 80 units of RNasin, and dNTP mixture (0.5 mM each of dATP, dGTP, and dTTP) at 42 °C for 3 h to generate ³²P-labelled cDNA. Unincorporated nucleotides were removed by using a Sephadex G-25 gel-filtration spin column (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Hybridization and cytokine expression array analysis
The denatured ³²P-labelled cDNA probes were incubated at 65°C overnight with the Human Cytokine Expression Array GA001 (R&D Systems, Minneapolis, MN, USA), which contains 375 cDNA sequences with duplicate spots for each gene on a nylon membrane. After intensive washing, the membrane was air-dried, autoradiographed onto a Kodak Low Storage Phosphor Screen, and analysed with a Phosphoimager (Molecular Dynamics Storm 840).

The images were analysed using ImageQuant software (Molecular Dynamics). The background intensity value was defined as the average reading value of the whole non-signal area on the array membrane and was subtracted from each spot. The average value of a pair of duplicate spots of each gene was calculated. The relative value of each gene on each array was normalized by dividing the average value of the gene by the mean value of nine different housekeeping genes that showed no change in various samples. After normalization, the mean relative value of each gene was analysed to determine whether the expression of a gene was up- or down-regulated when the disease progressed from early stage (KP) to late stage (LT).

Real-time QRT-PCR
Real-time QRT-PCR was performed to confirm the results obtained from the cytokine expression array. Since both the procollagen α₁(I) and the α-smooth muscle actin (α-SMA) genes are known to be involved in fibrogenesis, the expression of these two genes were also examined. Ten liver specimens from the KP group, ten from the LT group, and five from normal controls (CO), snap-frozen in liquid nitrogen, were analysed. Normal liver tissues were obtained from patients with hiatus hernia with incarcerated herniated liver tissue, or from patients with liver laceration. Total RNA was extracted and converted to cDNA by reverse transcription as described above. Real-time QRT-PCR primers were designed using the Primer Express software (Perkin-Elmer Life Sciences). These primers included 5'-AGC ACC GCA TCC TGA AGG G-3' and 5'-TTC CGC AGC ATG TGG TTG TA-3' for the DLK1 gene, 5'-CGT GGG TGA CGA AGC ACA G-3' and 5'-GGT GGG ATG CTC TCC AGG G-3' for the α-SMA gene, 5'-CAC CAA TCA CCT GCG TAC AGA-3' and 5'-CGT CAT CGC ACA ACA CCT TG-3' for the procollagen α₁(I) gene, and 5'-TCA CCC ACA CTG TGC CCA TCT ACG A-3' and 5'-CAG CGG AAC CGC TCA TTG CCA ATG G-3' for the β-actin gene. The sizes of the amplified products were 193 bp for the DLK1 gene, 151 bp for the α-SMA gene, 111 bp for the procollagen α₁(I) gene, and 295 bp for the β-actin gene. The β-actin gene served as the internal control to normalize the relative mRNA concentration.

Real-time QRT-PCR was performed in a total reaction volume of 50 μl including 25 μl of the SYBR Green PCR master mix (Applied Biosystems), 0.2 μl of forward and reverse primers, and approximately 30 ng of cDNA using an ABI 7700 Sequence Detection System (Perkin-Elmer Life Sciences). After heating at 95°C for 10 min, the samples were subjected to 40 cycles of 95°C for 15 s, 62°C for 20 s, and 72°C for 15 s, followed by melting curve analysis according to the dissociation protocol of the ABI 7700 instrument. The threshold cycle (C_T) value for each sample was the cycle number at which the fluorescence exceeded the threshold limit, which was set at ten times the standard deviation of the baseline. Samples were considered negative for the real-time PCR if C_T values exceeded 40 cycles. PCR products were electrophoresed on a 1.5% agarose gel to confirm the sizes of the products.

Pathological evaluation and grading of liver fibrosis
Forty-two formalin-fixed, paraffin wax-embedded liver specimens from 40 patients with BA from 1997 to 2001 were retrieved from the archives of the Department of Pathology, Chang Gung Memorial Hospital, Kaohsiung, Taiwan. These specimens included 28 KP and 14 LT. Paraffin wax-embedded liver tissues that corresponded to those used for the cytokine expression array and real-time QRT-PCR studies were also included. Eight autopsy liver specimens from children without liver disorders were used as normal controls (CO).
The histology of each specimen was reviewed and Masson’s trichrome staining was performed to evaluate liver fibrosis. The degree of liver fibrosis was graded as described previously [14–16]. Briefly, score 0 was normal; score 1 had periportal fibrosis; score 2 had incomplete septa; score 3 had complete septa without obvious cirrhosis; and score 4 was cirrhosis.

**Immunohistochemistry**

Two-micrometre-thick liver sections were treated with 3% hydrogen peroxide for 10 min, followed by microwave treatment for 10 min in 10 mM citrate buffer. After incubation for 1 h with goat anti-human DLK1 antibody (SC-8624, 1:50 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), the sections were incubated with HRP-rabbit anti-goat IgG (H + L) (Zymed, South San Francisco, CA, USA) according to the manufacturer’s instructions and then with DAB chromogen. Since the DLK1 protein is expressed in neuroendocrine tumours [17–20], a neuroblastoma was used as a positive control for the DLK1 antibody. Omission of the primary antibody was employed as a negative control.

**Confocal microscopic study with dual immunofluorescence**

Confocal microscopy was performed to co-localize DLK1 protein and α-SMA in the non-parenchymal cells of the liver. Consecutive sections of paraffin wax-embedded liver tissues with early BA were incubated with antibody against the DLK1 protein (SC-8624, 1:25) for 2 h, followed by incubation for 30 min with mouse anti-human α-SMA antibody (NCL-SMA, 1:50 dilution) (Vector Laboratories Ltd, Peterborough, UK). Texas Red-conjugated rabbit anti-goat antibody was used to detect the DLK1 protein and fluorescein-conjugated horse anti-mouse antibody (Vector Laboratories Inc, Burlingame, CA, USA) was used to detect α-SMA. The sections were then incubated with these secondary antibodies for 1 h in the dark. The double-stained tissue sections were examined using a Leica TCS SP2 laser scanning microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) with simultaneous excitation wavelengths of 488 and 535 nm.

**In situ hybridization**

pGEM-DLK1 containing a portion (nucleotides 391–783) of the DLK1 cDNA was used to prepare riboprobes by in vitro transcription using the DIG RNA labelling kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions. To avoid any non-specific detection of other genes, the sequence of this DLK1 cDNA clone was checked on the website of Nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) (R̄: 1053402635-028252-15596) and shown to have less than 50% similarity with any other transcripts including all the growth factors.

Consecutive sections (4 µm thick) of formalin-fixed, paraffin wax-embedded liver tissues were digested with 20 µg/ml proteinase K (GIBCO, Gaithersburg, MD, USA) at 37°C for 10 min. Then in situ hybridization was performed and was followed by signal amplification with DAKO GenPoint kit as previously reported [21] except that the hybridization condition was at 50°C overnight. For a negative control, the sense probe was used for hybridization instead of the anti-sense probe.

**Statistical analysis**

Comparisons of gene expression between the groups in both cytokine expression array and real-time QRT-PCR were performed by one-way ANOVA. Correlation of real-time QRT-PCR results between the different genes was assessed by the Pearson correlation coefficient. The degree of fibrosis in the different groups was compared by the Spearman rank correlation coefficient. A p value less than 0.05 was considered significant in all statistical analyses.

**Results**

Gauging liver fibrosis during the early and late stages of biliary atresia

Twenty-eight KP, 14 LT, and eight CO liver specimens were selected for histological evaluation. The mean ages of these patients were 1.9 ± 0.8, 13.1 ± 5.8, and 4.9 ± 5.6 months, respectively. Among the 28 liver specimens in the KP group, two had no fibrosis, six had score 1 fibrosis, 14 had score 2 fibrosis, and six had score 3 fibrosis. Among the 14 liver specimens in the LT group, two had score 3 fibrosis and 12 had score 4 fibrosis. No fibrosis was observed among the eight specimens in the control group.

Eight specimens in the control group. The degree of fibrosis was assessed by the Pearson correlation coefficient. The degree of fibrosis in the different groups was compared by the Spearman rank correlation coefficient. A p value less than 0.05 was considered significant in all statistical analyses.

The DLK1 gene is overexpressed during the early stage of biliary atresia

Using the Human Cytokine Expression Array containing 375 cDNA sequences, we found 25 genes with a lower expression level and four genes with a higher expression level in KP than in LT. Among the four genes that had a higher expression level in KP, the DLK1 gene was unique, in that the mean intensity in LT was only one-quarter (0.28593) of that in KP (Figure 1).

Consistent with the array data, real-time QRT-PCR showed that the expression level of the DLK1 gene was significantly higher in KP than in LT (p = 0.001). Expression of procollagen α1(I) was also higher in
DLK1 gene is differentially expressed between KP and LT in the Human Cytokine Expression Array. The intensity of the hybridization signal of the DLK1 (also known as Pref-1) gene (left lower corner) is much higher in KP than in LT, whereas the intensities of the hybridized spots corresponding to the follistatin, IGF binding protein-1 (IGFBP-1), IGFBP-4, and IGFBP-5 genes are approximately the same in both groups KP than in LT, but the difference was not statistically significant ($p = 0.178$). Interestingly, expression of the DLK1 gene correlated positively with expression of the procollagen $\alpha_1(I)$ gene ($r = 0.452$, $p = 0.023$). In contrast, the expression level of the $\alpha$-SMA gene was significantly higher in LT than in both CO and KP ($p = 0.013$ and 0.001, respectively) (Figure 2).

DLK1 protein is expressed in HSCs and in some other non-parenchymal cells

Immunohistochemical staining showed that DLK1 protein was mainly localized in the cytoplasm of non-parenchymal cells within hepatic lobules. Most of the DLK1-containing cells were morphologically identical to HSCs with the feature of a star shape with cytoplasmic processes (Figure 3). DLK1 was also present in some Kupffer cells and sinusoidal endothelial cells, but to a much lesser extent than in HSCs. Hepatocytes in most cases showed weak staining with the DLK1 antibody. Interestingly, the bile retained in the bile ductules was strongly positive with the DLK1 antibody, forming a ring pattern along the luminal border. Bile ductal epithelial cells in the portal areas were only weakly stained with the antibody.

Identification of HSCs as DLK1 protein-containing cells

Activated HSCs have been known to express $\alpha$-SMA [22,23]. Confocal microscopy with DLK1 and $\alpha$-SMA dual immunofluorescent staining revealed that positive
Figure 3. Immunohistochemical staining of representative liver tissues with the DLK1 antibody. (A) In CO without fibrosis, intracytoplasmic staining is noted in scattered non-parenchymal cells (arrow) located between the hepatocytes, while the latter are only faintly stained with a few small dots. (B) In KP with score 2 fibrosis, many more non-parenchymal cells (arrows) show intracytoplasmic staining. In addition to the weak staining in hepatocytes and bile ductal epithelium, a punctate staining pattern is also present in the hepatocytes. Static bile and bile plugs are strongly stained with the DLK1 antibody, forming a ring pattern (arrow-heads) in the portal triads. (C) High-power view of the liver tissue in KP shows DLK1-positive HSCs (arrows) with the typical feature of cytoplasmic processes. The nucleus of one HSC is discernible (asterisk). (D) In LT with score 4 fibrosis, non-parenchymal cells (arrows), hepatocytes, and bile plugs (arrow-heads) are still stained with the DLK1 antibody, but staining is generally weaker than in KP.

Figure 4. Co-localization of DLK1 and α-SMA in hepatic stellate cells (HSCs) by dual immunofluorescent staining, detected by confocal microscopy. (A) DLK1 immunoreactivity detected in liver tissue using rabbit anti-goat Ab conjugated with Texas Red shown as red fluorescence. (B) α-SMA immunoreactivity of HSCs detected using horse anti-mouse Ab conjugated with fluorescein shown as green fluorescence. (C) Co-localization of DLK1 with α-SMA in the same non-parenchymal liver cells, indicating that DLK1 is present in HSCs. Scale bar = 20 µm.

signals with the DLK1 antibody co-localized in most α-SMA-positive cells (Figure 4), suggesting that the DLK1 protein was present mainly in activated HSCs. Notably, the intensity of the fluorescent signal for the DLK1 antibody was consistently lower in hepatocytes than in the adjacent HSCs.

**DLK1 mRNA is present in hepatocytes, but not in non-parenchymal cells**

*In situ* hybridization with the *DLK1* riboprobes was performed to identify cells that express *DLK1* mRNA. To increase the sensitivity of *in situ* hybridization to...
Figure 5. In situ hybridization of representative liver tissues with DLK1 riboprobes. (A) No signal is detected with the DLK1 sense probes in a normal control. (B) Signals are predominantly present in hepatocytes but not in non-parenchymal cells in a normal control with the DLK1 anti-sense probe. Weak signals are also present in the bile ductal epithelial cells. (C) In KP with score 2 fibrosis and (D) in LT with score 4 fibrosis, in situ hybridization with the DLK1 anti-sense probe shows signals mainly in hepatocytes without a significant difference in staining intensity.

Discussion

The major consequence of BA is inflammation in the liver and the biliary system, leading to liver fibrosis. We used a cytokine expression array to elucidate the progress of liver fibrosis in BA and identified a novel gene, DLK1, among the few that were highly expressed during the early stage of BA. This expression pattern is very similar to that of the procollagen α1(I) gene, suggesting that DLK1 overexpression may be an early event in the progression of BA.

DLK1 is a transmembrane protein of the EGF-like homeotic superfamily with six tandem EGF-like repeats in the extracellular domain, highly homologous to invertebrate Delta and Notch proteins. The protein is termed Delta-like or DLK protein [9,20], as it does not have the DSL (Delta, Serrate, and LAG2) domain mediating Notch–Delta interactions [24–26]. DLK1 is also known as the preadipocyte factor-1 (pref-1) and is a negative regulator of adipocyte differentiation [9]. It is expressed in tumours with neuroendocrine features and in the adrenal gland and placenta [20]. Recently, it was found that DLK, pref-1, pG2 (differentially expressed in human phaeochromocytomas and neuroblastomas), and FA-1 (a gene isolated from human amniotic fluid) are identical or polymorphic products of a single gene [19,27]. DLK1 has been shown to participate in cell-to-cell interactions that control cell fate during differentiation [13,17].
In this study, we found that the DLK1 protein is present in HSCs, which are the most important cells involved in hepatic fibrogenesis [28,29]. HSCs are vitamin A-storing cells and contain lipid droplets within the space of Disse [22,30–32]. In chronic liver injury, these cells are activated, lose the phenotype of fat-storing cells, and are transformed into myofibroblast-like cells that express a high level of α-SMA and various levels of desmin [22,32]. This process is putatively reversible. Since DLK1 inhibits adipogenesis and is highly expressed during the early stage of BA, its presence in HSCs may promote their conversion to myofibroblasts, leading to liver fibrosis. DLK1 was highly expressed in uterine myometra, suggesting an association with smooth muscle differentiation [33]. The observation that expression of the DLK1 gene is decreased during the late stage of BA suggests that DLK1 may only be required for initiation, but not for perpetuation, of liver fibrosis.

Besides HSCs, DLK1 is also present in some Kupffer cells and sinusoidal endothelial cells. Activated Kupffer cells have been shown to produce TGF-β1, which is fibrogenic and can activate HSCs [34,35]. The presence of DLK1 in bile plugs indicates that DLK1 protein can be excreted into the bile through bile canaliculi and condensed in the bile plugs in BA. One surprising finding in this study is that DLK1 mRNA is only detectable in hepatocytes even using very sensitive in situ hybridization with a catalysed reporter deposition system to amplify the hybridization signals. This suggests that the DLK1 protein may be predominantly produced in hepatocytes and then transported into non-parenchymal cells via a paracrine mechanism. On the other hand, it cannot be excluded that these non-parenchymal cells also express DLK1 mRNA at a level that is below the limit of the detection method employed. Further investigation including semi-quantitative analysis in isolated cells and cell co-culture models could provide the resolution to clarify the role of DLK1 in interactions between different cell types in the liver.

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