

# Role of transforming growth factor $\beta$ 1 on hepatic regeneration and apoptosis in liver diseases

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## Abstract

**Aims**—To investigate the effects of transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) on regeneration and induction of apoptosis of liver cell and bile duct in various liver diseases.

**Methods**—Formalin fixed paraffin wax sections of 18 liver tissue samples were obtained by needle biopsy, surgery, or necropsy; these included six liver cirrhosis, three obstructive jaundice; five fulminant hepatitis, one subacute hepatitis, and three normal liver. Expression of TGF- $\beta$ 1, apoptosis related Le<sup>y</sup> antigen, Fas antigen, a receptor for tumour necrosis factor, and biotin nick end labelling with terminal deoxynucleotidyl transferase mediated dUTP (TUNEL) for locating DNA fragmentation, was investigated histochemically.

**Results**—TGF- $\beta$ 1 was expressed in areas of atypical bile duct proliferation, where bile duct continuously proliferated from liver cells. In occlusive jaundice and fulminant hepatitis, TUNEL was positive in nuclei and cytoplasm of metaplastic cells which formed incomplete bile ducts, and these cells appeared to extend from TGF- $\beta$ 1 expressing liver cells. Fas antigen was found only on the cell membrane of proliferated bile duct in fulminant hepatitis, which differed from TGF- $\beta$ 1 and TUNEL positive areas. Le<sup>y</sup> antigen was expressed in liver cell and bile duct at the areas with atypical bile duct proliferation, but its coexpression with TUNEL was rare.

**Conclusions**—TGF- $\beta$ 1 plays a role in the arrest of liver cell regeneration and atypical bile duct proliferation, and in areas of rapidly progressing atypical bile duct proliferation, such as in fulminant hepatitis or bile retention. Apoptosis appears to be induced by TGF- $\beta$ 1. This phenomenon may account for the inadequate hepatic regeneration that occurs with liver disease.

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Keywords: TGF- $\beta$ 1, apoptosis, hepatic regeneration.

Transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1), a peptide widely distributed in tissues, is known to have diverse biological activities. TGF- $\beta$ 1 may regulate the proliferation of epithelial and mesenchymal cells as well as immuno-

competent cells. TGF- $\beta$ 1 also exerts stimulatory effects on the deposition of extracellular matrix and the proliferation of fibroblasts; these effects are typically shown in wounded tissue repair.<sup>1</sup>

One of the important biological activities of TGF- $\beta$ 1 is inhibition of liver cell regeneration.<sup>2-8</sup> However, it also promotes the proliferation of fibroblasts, which seems to cause fibrosis of the liver.<sup>9-12</sup> Therefore recent research on the role of TGF- $\beta$ 1 in liver diseases has been concentrated chiefly on the progression of fibrosis in this disease,<sup>13-15</sup> although fibrosis is not a simple phenomenon—it should be recognised as a series of events, including fibril production and liver cell regeneration.

Recently, TGF- $\beta$ 1 has been shown to induce liver cell apoptosis through the inhibition of DNA synthesis by liver cells.<sup>16-21</sup> Apoptosis—programmed cell death—plays a key role in developmental biology and in the maintenance of the steady state in continuously renewing tissue. Cell condensation and fragmentation into a number of membrane bound “apoptotic bodies”, initially containing well preserved organelles and often condensed chromatin, are hallmarks of apoptotic cell death when identified by electron microscopy. So far, the pathological significance of this phenomenon in liver disease remains unclear. In order to clarify the role of TGF- $\beta$ 1 on regeneration and apoptosis of liver cells and bile duct, we conducted an immunohistochemical analysis of the expression of TGF- $\beta$ 1, Le<sup>y</sup> antigen,<sup>22,23</sup> an apoptosis related marker, and Fas antigen,<sup>24-26</sup> one of the tumour necrosis factor (TNF) receptors, in various types of liver disease. The terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labelling method (TUNEL),<sup>27-29</sup> which recognises DNA fragmentation, was also used to analyse the site of apoptosis.

## Methods

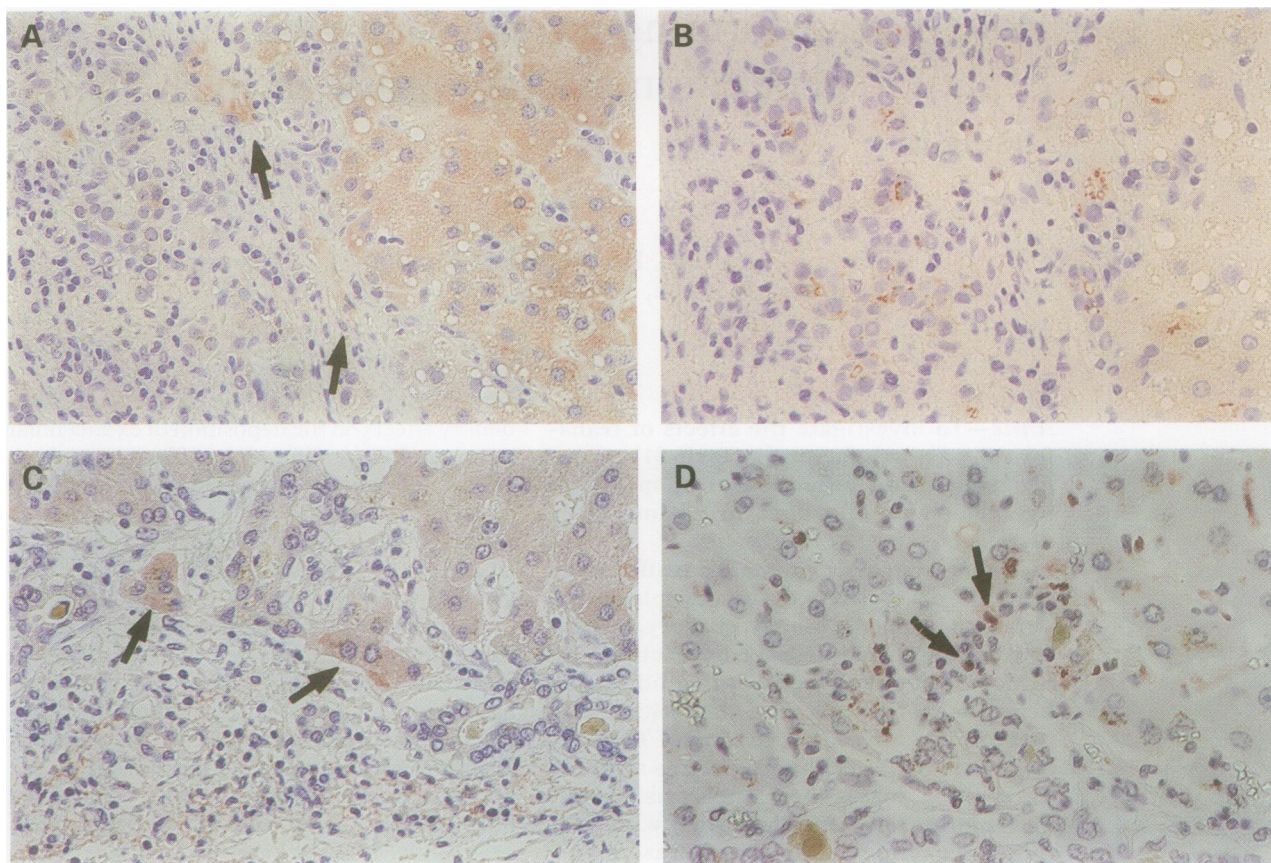
The study was conducted on 18 liver tissue samples obtained by needle biopsy, surgery, or necropsy. These consisted of five cases of fulminant hepatitis (type B), one of subacute hepatitis (type B), six of active cirrhosis of the liver (type C), three of obstructive jaundice (cancer of the pancreatic head), and three of normal liver (normal part of the surgically resected liver with benign tumour). Type B and type C hepatitis were serologically diagnosed by the presence of hepatitis B surface antigen and hepatitis C virus antibody (assessed with a

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**Figure 1** TGF- $\beta$ 1 was expressed in the cytoplasm of liver cells and the metaplastic bile ducts in regenerative nodules of active cirrhosis of the liver (indicated by arrows) (A), AEC ( $\times 100$ ). Le<sup>y</sup> antigen also gave positive findings in some liver cells and proliferated bile ducts (B), AEC ( $\times 100$ ). Morphologically incomplete bile duct continuous with TGF- $\beta$  positive liver cells was observed in obstructive jaundice (indicated by arrows) (C), AEC ( $\times 100$ ). Bile duct nuclei were positive for TUNEL (shown by arrows) (D), AEC ( $\times 100$ ).

second generation assay, Abbott Laboratories), respectively.

The tissue was fixed for 24 hours with 10% buffered formalin solution, embedded in paraffin wax, and thin sections measuring 2–3  $\mu$ m in thickness were prepared. Deparaffinisation was carried out with xylene, and the sections were then hydrated using an alcohol series. They were thoroughly rinsed with purified water. Immunohistochemical staining was conducted by the following method. Intrinsic peroxidase was inactivated for 10 minutes with 0.3% H<sub>2</sub>O<sub>2</sub>, and rinsed with phosphate buffered saline (PBS, 1/15 mol/l, pH 7.2). The sections were incubated with properly diluted rabbit or mouse serum for 30 minutes at room temperature to avoid non-specific binding of second antibodies. Sections were reacted overnight at 4°C with anti-human TGF- $\beta$ 1 rabbit polyclonal antibody (4  $\mu$ g/ml, King Brewing), antihuman Le<sup>y</sup> mouse monoclonal antibody (20  $\mu$ g/ml, BM-1, Japan Immunoresearch Laboratory Co),<sup>29</sup> or anti-human Fas mouse monoclonal antibody (20  $\mu$ g/ml, CH-11, Medical and Biological Laboratory Co) followed by a reaction for 30 minutes at 20°C using a biotinylated second antibody kit (IMMUNON immunostaining systems, Shandon Lipshaw Inc). The reaction product was visualised using a mixed solution of sodium acetate buffer including 0–3% H<sub>2</sub>O<sub>2</sub> and 3-amino-9-ethylcarbazole (AEC). Sections were further stained with haematoxylin and observed with Olympus BH-2 microscope.

TUNEL was carried out according to the method of Gavrieli *et al.*<sup>27,29</sup> After fixing with 10% formalin buffer, sections were deparaffinated and hydrated. Sections were reacted with proteinase K (20  $\mu$ g/ml) at room temperature for 20 minutes in 100 mM trisHCl buffer (pH 7.4), and rinsed in purified water including 3% H<sub>2</sub>O<sub>2</sub> in order to inactivate intrinsic peroxidase activity. After rinsing with terminal deoxynucleotidyl transferase (TdT) buffer (potassium cacodylate 100 mM, CoCl<sub>2</sub> 2 mM, dithiothreitol 0.2 mM, pH 7.2), sections were incubated for 60 minutes at 37°C with TdT solution (TdT 0.3 equivalent U/ $\mu$ l biotinylated uridine triphosphate 0.04 nmol/ $\mu$ l). They were rinsed in buffer solution (sodium citrate 30 mM, NaCl 300 mM) for 15 minutes and then in purified water and PBS. Non-specific reaction was blocked with 10% normal rabbit serum. Sections were then reacted with streptavidin labelled with peroxidase for 30 minutes at room temperature. Positive reaction was visualised using AEC mixed solution, and the nuclei were stained with haematoxylin. As a positive control, a reaction with DNase 1 (0.7  $\mu$ g/ml potassium cacodylate buffer, pH 7.2) was conducted before the addition of TdT reaction solution, and as a negative control, TUNEL was conducted using a solution without TdT.

For the observation with a confocal laser scanning microscope (LSM 410, Carl Zeiss), fluorescent (FITC) labelled streptavidin was used instead of peroxidase conjugate.



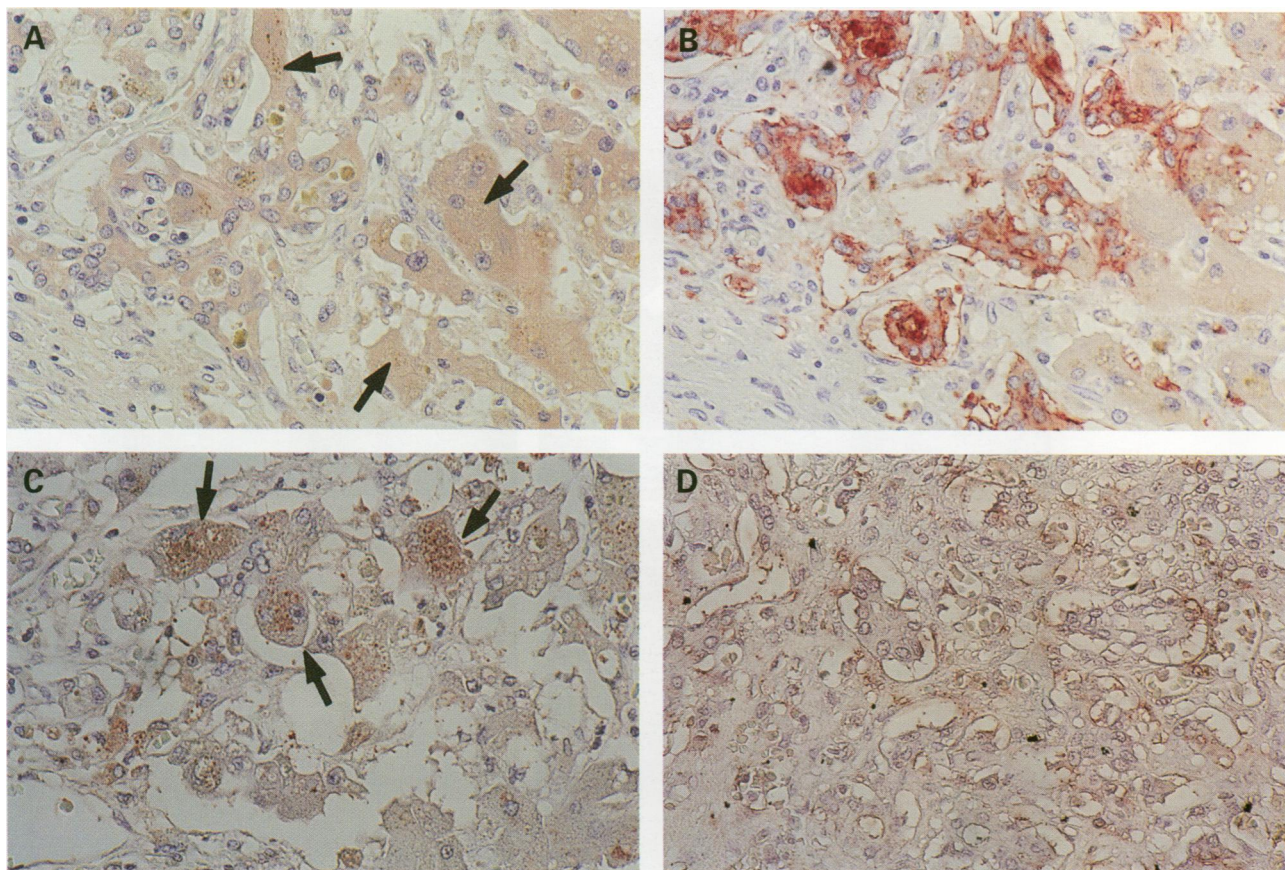


Figure 2 Bile duct proliferation extending from TGF-β1 expressing liver cells showed incomplete morphology in fulminant hepatitis. Some of the nuclei were destroyed (indicated by arrows) (A), AEC (×100). Le<sup>y</sup> antigen was seen in the same area (B) (×100). Cytoplasm of liver cell was positive for TUNEL (arrows) (C) (×100). Fas antigen showed positive in the cell membrane of bile ducts which may proliferate at the other sites described above (D), AEC (×100).

**Results**

In the normal part of the liver, TGF-β1, Fas antigen, and TUNEL were negative in all cases. Le<sup>y</sup> antigen was also negative in all cases with the exception of inflammatory cells. In the area of regenerative nodules in active cirrhosis of the liver, TGF-β1 was observed not only in the cytoplasm of liver cells, but also of the metaplastic bile duct epithelium (fig 1A), where proliferative changes of liver cells to bile duct epithelial cells were noted. Le<sup>y</sup> antigen was also expressed in the cytoplasm of some liver cells and in metaplastic bile duct epithelium, although the bile duct structure was recovered (fig 1B). Histochemistry for Fas antigen and TUNEL tests conducted at the same time showed negative results.

In cases with obstructive jaundice, TUNEL was positive in the nuclei of metaplastic cells, which formed incomplete bile ducts (fig 1D) and these cells appeared to extend from TGF-β1 expressing liver cells (fig 1C). Le<sup>y</sup> antigen was positive in the cytoplasm of metaplastic bile duct forming cells, but Fas antigen was negative.

In cases of fulminant hepatitis, TGF-β1 was expressed both in liver cells and in adjacent bile duct forming cells, which were highly metaplastic (fig 2A). Some nuclei of cells in these areas showed morphological changes with an expression of Le<sup>y</sup> antigen (fig 2B). TUNEL was positive in the cytoplasm of these cells (fig 2C).<sup>28</sup> Fas antigen was, however, positive only

on the cell membrane of bile duct forming cells at the sites of inflammatory cell phagocytosed bilirubin (fig 2D). Proliferated bile duct forming cells were negative for TUNEL, but positive for Le<sup>y</sup> antigen in the cytoplasm.

Confocal laser scanning microscopy revealed various stages of apoptosis. In an early stage, morphology of nucleolus and nucleoli was relatively preserved, and the FITC positive sites showed the finding of DNA fragmentation (fig 3A). As apoptosis progressed, the following findings were observed: outflow of the nuclear contents (fig 3B), migration of the TUNEL positive substance from the nucleus to the cytoplasm (fig 3C), and disappearance of the nucleus. TUNEL was positive only in the

Summary of expression of TGF-β 1, Le<sup>y</sup> Ag, Fas Ag, and TUNEL in liver cell and bile duct in various conditions of liver disease

	TGF-β1	Le <sup>y</sup> Ag	Fas Ag	TUNEL
Active cirrhosis liver cell—bile duct (atypical)	+	+	-	-
Obstructive jaundice liver cell— incompletely formed bile duct (atypical)	+	+	-	+
Fulminant hepatitis liver cell— incompletely formed bile duct (atypical)	+	+	-	+
Proliferated bile duct	± ~ -	+	+	-

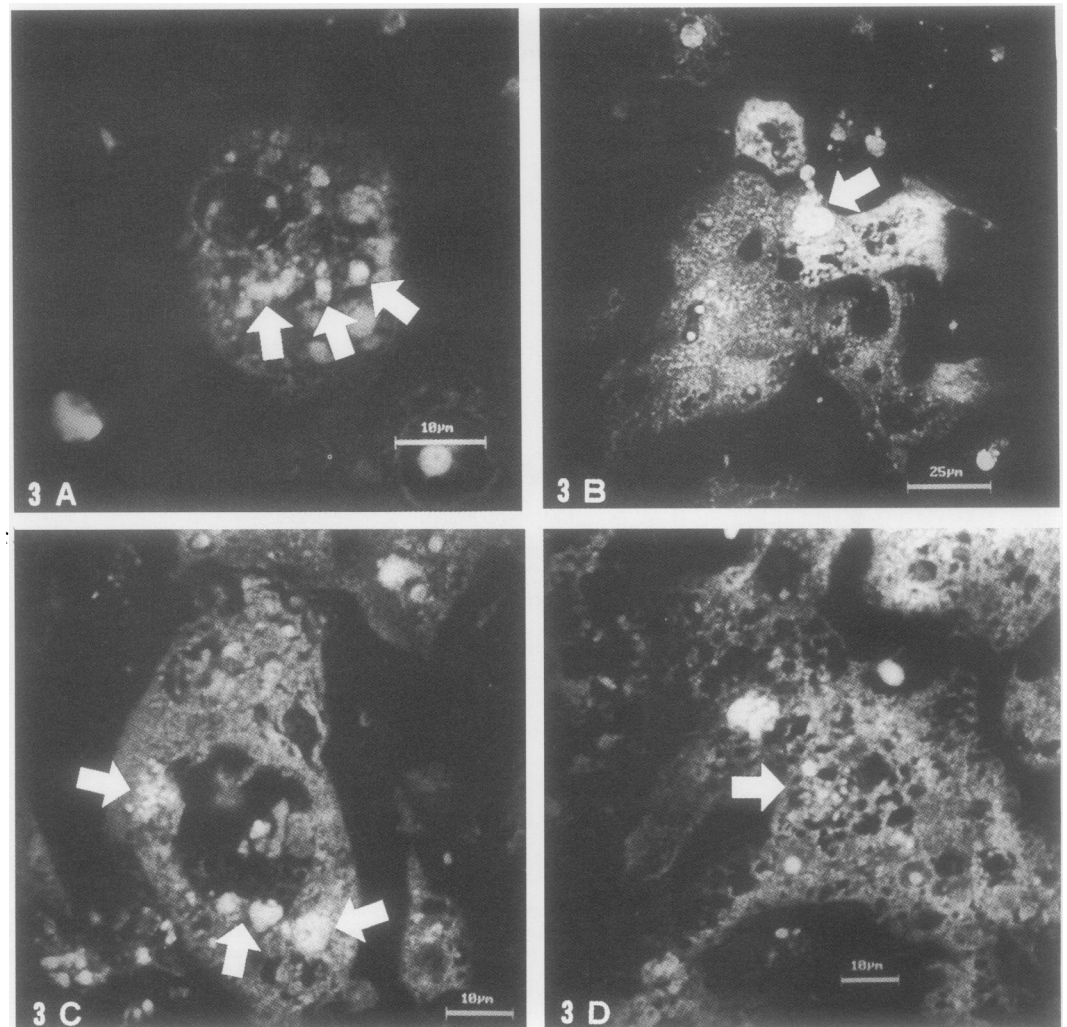


Figure 3 Areas shown in panels (A), (B), and (C) are the same as in the corresponding panels in fig 2. Fragmentation of DNA was noted in fulminant hepatitis under confocal laser scanning microscopy with TUNEL (shown by arrows) (A). Outflow of nuclear contents is indicated with arrows (B). Migration of TUNEL positive substances to cytoplasm is shown by arrows (C). TUNEL positive findings in the cytoplasm of cells with destroyed nuclei are indicated with arrows in (D).

cytoplasm of liver cells (fig 3D), which migrated into the area with incomplete formation of bile ducts.

### Discussion

In the lesions with marked bile duct proliferation, which was typically seen in the regenerative nodules of active cirrhosis of the liver, obstructive jaundice, and fulminant hepatitis, two possible origins of the cells forming bile ducts have been described, namely atypical ductular proliferation, in which liver cells transform to the bile duct epithelium, and typical ductular proliferation, in which the remaining bile duct cells proliferate and form bile ducts.<sup>30-32</sup> The fact that liver cells transform into the bile duct forming cells in an early stage is confirmed experimentally. Tomoyori *et al* reported that in the hepatectomised rat, liver cells showed marked expansion of the bile canaliculus and formed an acinar arrangement.<sup>33</sup> A similar transformation has also been described in experimental transplantation of the liver into the spleen in rats.<sup>34</sup>

Our study showed that TGF- $\beta$ 1 was expressed on bile duct forming cells and liver

cells in areas of atypical ductular proliferation (table). This suggests that TGF- $\beta$ 1 is an important factor modulating the regeneration mechanism of the bile duct system as well as the parenchymal cells of the liver. However, the sites of TGF- $\beta$ 1 detection may not be the sites of synthesis. For example, the cells other than those detected immunohistochemically (for example, the endothelial cell) may be the true sites of synthesis; this can be proved by techniques such as in situ hybridisation.

Atypical ductular proliferation was observed when the number of remaining small bile duct cells was insufficient to proliferate in situations that result in a decrease in the absolute number of liver cells, destruction of liver architecture, and marked bile retention. With an increase in bile retention, more rapid proliferation of bile duct cells is required.<sup>35</sup> When pronounced destruction of the liver architecture takes place, such as in fulminant hepatitis, regeneration of liver cell should in theory be coordinated with that of bile duct system. However, the latter appeared strikingly enhanced in our study. It is conceivable that the large amounts of TGF- $\beta$ 1 noted are necessary to inhibit excessive bile duct proliferation.



In the present study, we could detect the morphological occurrence of apoptosis by laser scanning microscopy at the sites where metaplastic bile duct cells had contact with liver cells. Le<sup>y</sup> antigen was expressed in the cytoplasm of liver cells in active cirrhosis of the liver together with TGF- $\beta$ 1, and it was also expressed in metaplastic cells seen in atypical bile duct proliferation. However, concomitant positive findings of Le<sup>y</sup> antigen and TUNEL were only observed in metaplastic cells with incomplete bile duct formation which had contact with TGF- $\beta$ 1 positive liver cells in obstructive jaundice and fulminant hepatitis. On the basis of these findings, we conclude that the detection of Le<sup>y</sup> antigen is a useful method of recognising the possible induction of apoptosis, but it was difficult to confirm the process by this expression alone, as display of the Le<sup>y</sup> antigen does not always represent chromatin condensation and DNA fragmentation of the nuclei.

Expression of Fas antigen was not observed at the sites where TUNEL was positive. In addition, since Fas antigen was not detected at TGF- $\beta$ 1 positive sites around the area of atypical bile duct proliferation where apoptosis was found, induction of apoptosis might not be mediated by the Fas antigen. It has been reported that the bile duct, which is formed by cell proliferation and differentiation in experimental obstructive jaundice, disappears due to the induction of apoptosis after the obstruction is relieved.<sup>36,37</sup> In the present study, TGF- $\beta$ 1 was not detected in the mature, differentiated metaplastic bile duct, and in the stage when apoptosis was detected. This finding is at variance with published reports.

From these results, we suggest that TGF- $\beta$ 1 plays a role in the arrest of liver cell regeneration and in atypical bile duct proliferation. In severe necrosis of liver tissue with marked destruction of liver architecture, seen typically in fulminant hepatitis or bile retention, apoptosis was observed at the site of rapid atypical bile duct proliferation. This could be the result of the inhibition of DNA synthesis by TGF- $\beta$ 1 and this phenomenon may account for the regenerative phase of liver disease.

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