Biliary Epithelial-Mesenchymal Transition in Posttransplantation Recurrence of Primary Biliary Cirrhosis

Helen Robertson,[1] John A. Kirby,[1] William W. Yip,[2] David E.J. Jones,[1,2] and Alastair D. Burt[2]

Primary biliary cirrhosis (PBC) recurs in the allograft after liver transplantation. Study of early tissue changes in the time-course of disease recurrence provides a unique insight into the initial stages of the disease process, which, in nontransplant patients, occurs long before clinical presentation. We describe a patient who developed classical clinical, biochemical, immunological, and histological features of PBC within 9 months after transplantation. Use of tissue from this patient before and during the development of PBC allowed us to identify biliary epithelial cell (BEC) epithelial-mesenchymal transition (EMT) as a key pathogenetic process. BEC expression of S100A4 (an early fibroblast lineage marker established as a robust marker of EMT), vimentin, and pSmad 2/3 [a marker of transforming growth factor beta (TGF-β) pathway signaling] were identified immunohistochemically in most BECs in liver tissue from this patient at the point of diagnosis of recurrent disease. BEC expression of S100A4 and pSmad 2/3 was seen as early as 24 days after orthotopic liver transplantation (OLT), although no other features of recurrent PBC were present at this time. *Conclusion:* S100A4, vimentin, and pSmad 2/3 expression in early recurrent PBC after OLT suggests that BEC EMT is occurring (potentially explaining BEC loss) and that this process is driven by TGF-β. S100A4 expression by BEC appears to occur before the development of any other features of recurrent PBC, suggesting that EMT may be an initiating event. (HEPATOLOGY 2007;45:977-981.)

See Editorial on Page 837.

Primary biliary cirrhosis (PBC) is a chronic cholestatic condition of presumed autoimmune etiology[1,2] characterized by loss of the biliary epithelial cells (BECs) in small intrahepatic bile ducts and portal/periportal fibrosis. The mechanisms of BEC loss and progressive portal fibrosis remain unclear. Data suggest that apoptosis is an important mechanism for BEC loss in PBC,[3] but this is thought to predominate in the middle stages (II-III) of the disease and may be relatively infrequent in the earlier stages when BEC injury is at its height.[4] PBC has often been present in patients for many years before they present clinically. It therefore can be difficult to study the very earliest stages of disease pathogenesis when the key originating damage to BECs is occurring. One of the settings in which very early disease may be studied is in the context of recurrence after orthotopic liver transplantation (OLT).[5-7] We describe a patient who developed recurrent PBC within 9 months of transplantation. The study of tissue from this liver affected, by definition, by very early PBC provides us with important insights into a novel, potentially pathogenetic, mechanism that may predominate in very early PBC: epithelial-mesenchymal transition (EMT).[8]

Methods

Diagnostic biopsy tissue blocks were obtained from the tissue archives of Newcastle upon Tyne Hospitals NHS Foundation Trust in accordance with local Ethics Com-

Abbreviations: αSMA, alpha smooth muscle actin; ALP, alkaline phosphatase; BEC, biliary epithelial cell; EMT, epithelial-mesenchymal transition; OLT, orthotopic liver transplantation; PBC, primary biliary cirrhosis; TGF-β, transforming growth factor Beta.

From the [1]Applied Immunobiology and Transplantation Research Group and [2]Liver Research Group, Institute of Cellular Medicine, Newcastle University, Newcastle-upon-Tyne, NE2 4HH, UK.

Received October 4, 2006; accepted December 13, 2006.

Supported by Roche Organ Transplantation Research Foundation, Medical Research Council.

Address reprint requests to: Alastair D. Burt, Dean of Clinical Medicine's Office, Peacock Hall, Royal Victoria Infirmary, Newcastle upon Tyne, NE1 4LP. E-mail: a.d.burt@ncl.ac.uk; fax: (44) 0-191-282-0702.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.21624

Potential conflict of interest: Nothing to report.

Supplementary material for this article can be found on the HEPATOLOGY website (http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html).

mittee approval. Written informed consent was obtained from the patient.

Four-micrometer sections from formalin-fixed liver tissue samples were dewaxed, rehydrated, and subjected to heat-induced antigen retrieval in citrate buffer, pH 6.0, before immunostaining to detect S100A4, an early fibroblast lineage marker previously used for demonstration of EMT,[9-11] vimentin, and alpha smooth muscle actin (αSMA; a myofibroblast marker). Sections were incubated with primary antibody overnight at 4°C: S100A4, 1/400, vimentin, 1/100, cytokeratin 19, 1/50 (all obtained from Dako, UK) and αSMA (Sigma-Aldrich, UK), 1/4000 all in antibody diluent (Dako, UK). Envision Alkaline Phosphatase (Dako, UK) was used to detect bound antigen, the color was developed with Vector Red (Vector Laboratories, UK) substrate, and sections were counterstained with Mayer's hematoxylin. A similar protocol was employed to detect phospho-Smad 2/3 (pSmad 2/3). Briefly, the primary antibody, polyclonal rabbit anti-human pSmad 2/3 (Santa Cruz), was incubated overnight at 4°C at 1/200 in 20% normal swine serum after blocking endogenous biotin (Vector Laboratories, UK). The rabbit antibody was detected using biotinylated swine anti-rabbit immunoglobulins (Dako, UK) followed by avidin-biotin-peroxidase complex and nickel-enhanced diaminobenzidine (Vector Laboratories, UK). The sections were counterstained with alcoholic Fast Green.

Results

Case. The patient presented at age 38 years with itch and profound fatigue. She had cholestatic liver function tests, was antimitochondrial antibody positive (titer 1:640) and anti-M2 positive, and on liver biopsy, was found to have stage III PBC. She was started on high-dose ursodeoxycholic acid, which improved her liver function tests (synthetic function remained normal throughout her illness) but was ineffective in controlling symptoms. She subsequently underwent treatment with cholestyramine, rifampicin, and naltrexone for her pruritus but none were successful. In the light of her uncontrollable itch and poor quality of life, she underwent OLT. Histological examination of her explanted liver confirmed Scheuer stage III PBC with ductopenia, cholate stasis, and portal fibrosis but no definite nodule formation (Fig. 1A). A time-zero baseline biopsy was taken at the time of reperfusion; this served as an inherent "normal" control. At the time of OLT, her alkaline phosphatase (ALP) was 455 U/l (normal, ≤120), ALT 91 U/l (normal, ≤40), albumin 41 g/l, prothrombin time (PT) 11 seconds, and bilirubin 11 μM. She received the standard unit early immunosuppression regimen (at the time) of tacrolimus, azathioprine, and prednisolone. Within 1 week, she had dramatic improvement of fatigue and itch. Nine days after operation, a significant rise in both aminotransferases and ALP was noted; liver biopsy showed moderate acute cellular rejection. Immunosuppression was boosted with 6 doses of methylprednisolone. At 24 days after transplantation, although the aminotransferase elevation had settled there was persistent elevation of ALP; she remained antimitochondrial antibody positive (1/320) and M2 positive. Liver biopsy showed ballooning of perivenular hepatocytes and focal bilirubinostasis but no evidence of significant portal tract inflammation or bile duct injury (Fig. 1B). Over the subsequent 8 months she described progressively increasing fatigue, returning eventually to pretransplantation levels. A biopsy at 9 months after transplantation showed features of recurrent PBC (Fig. 1C). At this point, the patient had an ALP level of 195, an ALT level of 22, an IgM level of 4.5 g/l (normal, ≤2.3) and was antimitochondrial antibody positive (1/320). Immunosuppression was modified to replace tacrolimus with cyclosporine. Within 4 weeks of this change, her symptoms had returned to baseline and her fatigue improved; serum biochemistry parameters also returned to normal. She remains well 9 months later with no features of progressive recurrent disease.

Immunohistochemistry. Sections of the explant liver showed clear expression of S100A4 within BECs of small (Fig. 2A) and medium-sized bile ducts; by contrast, S100A4 was not expressed by BECs in normal (time-zero) liver (Supplementary Fig. 1). Many epithelial cells within areas of ductular reaction in the explant liver also expressed S100A4, as did hepatocytes adjacent to portal tracts. S100A4 was not expressed at 9 days after transplantation in bile duct epithelium but was present in bile duct epithelium and some infiltrating immune cells in 4 of 5 portal tracts in the biopsy taken at 24 days after OLT (Fig. 2B). At this time, intense nuclear accumulation of pSmad 2/3 was seen in a proportion of the cells within the small bile ducts (Fig. 2C). BECs were negative for vimentin in time-zero and day 9 specimens but were immunoreactive at 24 days (Supplementary Fig. 2)

By 9 months after OLT, in the biopsy showing recurrent PBC, there was a dense immune cell infiltrate in all portal tracts, most of which was S100A4-positive. In addition, the small bile ducts expressed S100A4 (Fig. 2D) and vimentin (Supplementary Fig. 2). At no stage did bile ducts express αSMA. However, in the 9-month biopsy several αSMA-positive myofibroblasts lay adjacent to small bile ducts (Fig. 2E), and there was intense nuclear

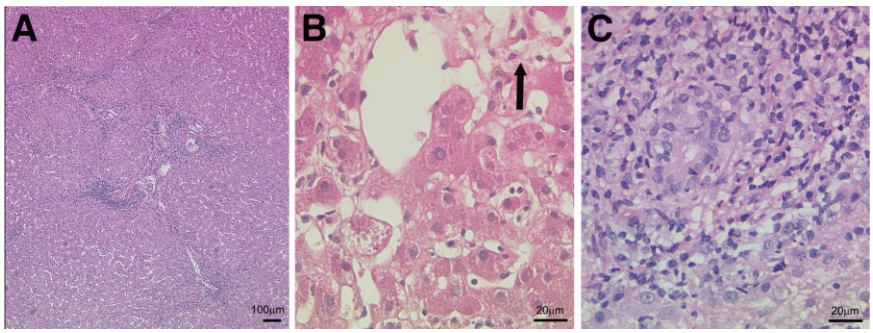

Fig. 1. Light microscopy assessment of liver tissue (hematoxylin-eosin) from (A) explant, demonstrating the classic features of stage III PBC with septum formation but no nodules. (B) At 24 days after transplantation, abnormalities in this biopsy are limited to minimal bilirubinostasis and ballooning of perivenular hepatocytes (arrow). (C) At 9 months after transplantation, this biopsy reveals features of early recurrent PBC; there is a lymphocytic and macrophage infiltrate around and within a medium-sized bile duct in the absence of other features of allograft rejection.

accumulation of pSmad2/3 in both infiltrating leukocytes and BECs (Fig. 2F).

Negative immunohistochemical controls (no primary antibody) showed no labeling with the chromagen (Supplementary Fig. 2).

Discussion

We describe a patient who developed recurrent PBC 9 months after OLT. In sequential liver biopsies, we demonstrated expression of S100A4 within BECs; this is a key marker of early fibroblast lineage development.[9,10,12] This indicates that these cells are undergoing EMT and suggests that this phenotypic shift may explain the "loss" of BECs and "gain" of portal tract fibroblasts, which characterizes a ductopenic disease such as PBC.[11,13] The nuclear accumulation of pSmad 2/3 observed in BECs is consistent with a local response to active transforming growth factor beta (TGF-β)[14] and suggests that this cytokine is playing an important role in EMT induction. Critically, these features were also present at 24 days after OLT, before the expression of any other histological or biochemical features suggestive of recurrent disease (other than mildly cholestatic liver function tests). S100A4 expression was notably absent from BECs in time-zero tissue and at 9 days after transplantation, excluding the possibility that changes were present in the donor organ before transplantation.

EMT has been highlighted as an important mechanism in fibrotic renal disease[9,10,12] and, more recently, as a potentially important effector mechanism in human ductopenic disease (including PBC)[11,13] and murine experimental cholestatic disease.[15] *In vitro* studies using cultured human BECs[11,15] and *in situ* studies using tissue from patients with both PBC and primary sclerosing cholangitis[13] have suggested that EMT is a generalized feature of these diseases (and not restricted to recurrent disease after OLT). Cultured human BECs develop a fibroblast-like phenotype (including the development of

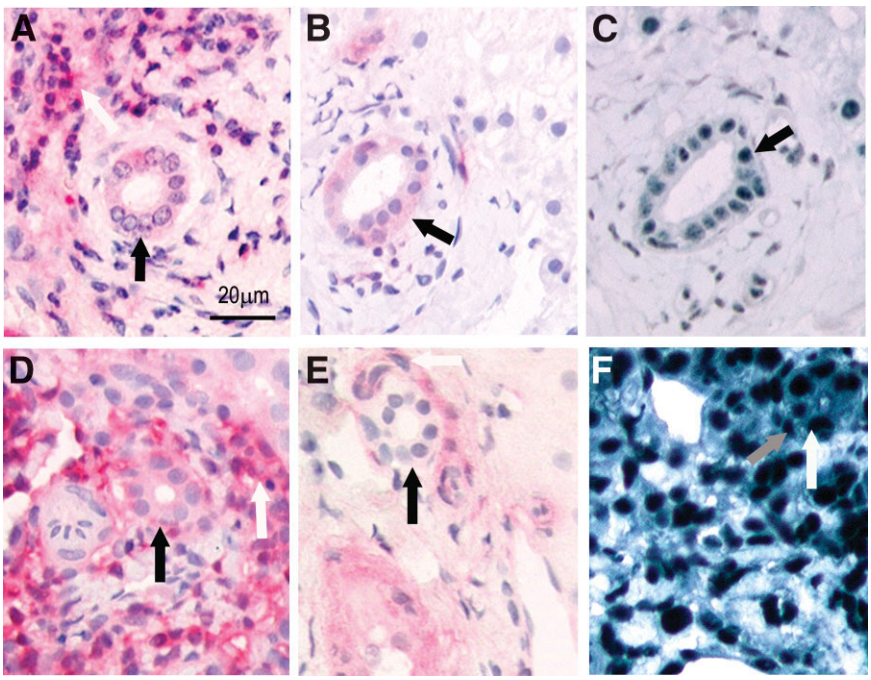

Fig. 2. Immunohistochemical assessment of liver tissue from (A) explant. Immunoreactivity for S100A4 is seen in both BECs (black arrow) and cells of the portal tract infiltrate (white arrow). (B) Liver tissue 24 days after transplantation; note S100A4 staining of the BEC lining a small intrahepatic bile duct (black arrow). (C) At 24 days after transplantation, in the same bile duct there is strong nuclear accumulation of pSmad 2/3 in all cells. (D) At 9 months after transplantation, there is strong staining for S100A4 in both BECs (black arrow) and portal tract infiltrating cells (white arrow). (E) αSMA is seen in myofibroblasts in close proximity to a surviving bile duct (arrow). (F) Nuclear accumulation of pSmad 2/3 is intense in both bile duct (white arrow) and infiltrating cells (gray arrow).

invasive properties) when cultured in the presence of TGF-β, express S100A4 and subsequently αSMA and fibronectin.[11] The coexpression of S100A4 with pSmad 2/3 staining in affected BECs in liver biopsies from PBC and primary sclerosing cholangitis patients suggest that TGF-β is also driving BEC EMT *in vivo*[13] (manuscript in preparation). Previous studies have demonstrated the presence of elevated levels of TGF-β in the livers of PBC patients.[14]

Our observations that BEC EMT appears to occur in early PBC, a disease in which BEC apoptosis is known to play a role, gives rise to an apparent paradox given observations that EMT may protect cells from apoptosis.[16] One potential explanation comes from emerging data suggesting that although apoptosis is an important factor in BEC loss, it does not appear to play a significant role in the very earliest stages of the disease.[4] A model compatible with all of the findings is that BEC EMT represents an extremely early process (supported by the kinetics of change in the case described here) that gives rise to early cholestasis. The effects of hydrophobic bile acids retained in the cholestatic environment then override the apoptosis-protective effects of EMT and drive a secondary phase of apoptosis-driven BEC loss[17]; this model can be tested in *in vitro* culture systems.

Several agents appear to reverse EMT in animal models. Importantly, bone morphogenetic protein-7, a TGF-β family member that blocks the TGF-β activation pathway, has been demonstrated to be effective in a renal fibrosis model.[18] More recently, hepatocyte growth factor has been shown to both reverse EMT *in vitro* and attenuate liver fibrosis induced by bile duct ligation.[15] Of particular relevance to the treatment of PBC, especially recurrent disease, is the demonstration that rapamycin is able to reverse EMT through inhibition of TGF-β and to attenuate renal fibrosis in the unilateral ureteric obstruction renal fibrosis model.[19] Rapamycin also has been shown to be effective at reversing fibrosis in the bile duct–ligated rat via a TGF-β–directed mechanism, although the role played by reversal of EMT in this effect was not studied.[20] The potential for PBC reversal in the earliest disease stages is of real potential therapeutic importance and requires further study.

Our patient appeared to undergo biochemical remission from her recurrent PBC after a change from tacrolimus to cyclosporine-based immunosuppression. Emerging data suggest that recurrent PBC is strongly associated with the use of tacrolimus-based immunosuppression.[5] The underlying mechanisms are unclear, and the implications for post-OLT therapy of patients with PBC need to be explored. Although the apparent improvement after the switch to cyclosporine-based immunosuppression may be a simple coincidence, the events would be compatible with either tacrolimus playing some role in promotion of EMT or conferring susceptibility to a factor that promotes EMT, or cyclosporine playing a role in reversing the process. If the switch to cyclosporine therapy did have a benefit in terms of BEC EMT, it is likely to have exerted its effects at the level of altering immune-cell TGF-β release rather than at the level of BECs themselves, given the demonstration that cyclosporine when directly applied to cultured epithelial cells promotes rather than reverses EMT.[21] At present, PBC recurrence after OLT represents a growing clinical problem for which there is no consensus regarding potential therapeutic approaches; our observations suggest that there may be benefit in addressing whether change in immunosuppression regimens might be of benefit in this situation.

References

1. Kaplan MM, Gershwin ME. Primary biliary cirrhosis. New Eng J Med 2005;353:1261-1273.
2. Jones DEJ. Pathogenesis of primary biliary cirrhosis. J Hepatol 2003;39: 639-648.
3. Harada K, Nakanuma Y. Molecular mechanisms of cholangiopathy in primary biliary cirrhosis. Med Mol Morphol 2006;38:55-61.
4. Alvaro D, Floreani A, Onori R, Franchitto A, Mancino MG, Guido M, et al. The activation of IGF1 system is a requisite for cholangiocyte survival during the progression of primary biliary cirrhosis (PBC). J Hepatol 2006; 44:S121-S122.
5. Garcia RFL, Garcia CE, McMaster P, Neuberger J. Transplantation for primary biliary cirrhosis: Retrospective analysis of 400 patients in a single center. HEPATOLOGY 2001;33:22-27.
6. Guy JE, Qian PQ, Lowell JA, Peters MG. Recurrent primary biliary cirrhosis: peritransplant factors and ursodeoxycholic acid treatment post-liver transplant. Liver Transpl 2005;11:1252-1257.
7. Van de Water J, Gerson LB, Ferrell LD, Lake JR, Coppel RL, Batts KP, et al. Immuno-histochemical evidence of disease recurrence after liver transplantation for primary biliary cirrhosis. HEPATOLOGY 1996;24:1079-1084.
8. Lee JM, Dedhar S, Kalluri R, Thompson EW. The epithelial-mesenchymal transition:new insights in signaling, development and disease. J Cell Biol 2006;172: 973-981.
9. Robertson H, Ali S, McDonnell BJ, Burt AD, Kirby JA. Chronic renal allograft dysfunction: the role of T cell-mediated tubular epithelial to mesenchymal cell transition. J Am Soc Nephrol 2004;15:390-397.
10. Tyler JR, Robertson H, Booth TA, Burt AD, Kirby JA. Chronic allograft nephropathy: intraepithelial signals generated by transforming growth factor-beta and bone morphogenetic protein-7. Am J Transplant 2006;6: 1367-1376.
11. Rygiel KA, Robertson H, Burt AD, Jones DEJ, Kirby JA. Demonstration of the transition of intrahepatic biliary epithelial cells to fibroblasts during chronic inflammatory liver diseases. J Hepatol 2006;45:S241 (abstract).
12. Vongwiwatana A, Tasanarong A, Rayner DC, Melk A, Halloran PF. Epithelial to mesenchymal transition during late deterioration of human kidney transplants: the role of tubular cells in fibrogenesis. Am J Transplant 2005;5:1367-1374.
13. Robertson H, Xu X, Burt AD, Kirby JA. Epithelial to mesenchymal transition in chronic cholestatic liver disease. J Pathol 2005;207:30A (abstract).

14. Mishra B, Tang Y, Katuri V, Fleury T, Said AH, Rashid A, et al. Loss of cooperative function of transforming growth factor-beta signaling proteins, smad3 with embryonic liver fodrin, a beta-spectrin, in primary biliary cirrhosis. Liver Int 2004;24:637-645.
15. Xia J-L, Dai C, Michalopoulos GK, Liu Y. Hepatocyte growth factor attenuates liver fibrosis induced by bile duct ligation. Am J Pathol 2006; 168:1500-1512.
16. del Castillo G, Murillo MM, Alvarez-Barrientos A, Bertran E, Fernandez M, Sanchez A, et al. Autocrine production of TGF-beta confers resistance to apoptosis after an epithelial-mesenchymal transition process in hepatocytes: Role of EGF receptor ligands. Exp Cell Res 2006; 312:2860-2871.
17. Rodrigues CMP, Fan GS, Ma XM, Kren BT, Steer CJ. A novel role for ursodeoxycholic acid in inhibiting apoptosis by modulating mitochondrial membrane perturbation. J Clin Invest 1998;101:2790-2799.
18. Zeisberg M, Hanai J, Sugimoto H, Mammoto T, Charytan D, Strutz F, et al. BMP-7 counteracts TGF-B1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. Nat Med 2003;9:964-967.
19. Wu M-J, Wen M-C, Chiu Y-Y, Shu K-H, Tang M-J. Rapamycin attenuates unilateral ureteral obstruction-induced renal fibrosis. Kidney Int 2006;69:2029-2036.
20. Biecker E, Gottardi AD, Neef M, Unternahrer M, Schneider V, Ledermann M, et al. Long-term treatment of bile-duct-ligated rats bwith rapamycin (sirolimus) significantly attenuates liver fibrosis: analysis of the underlying mechanisms. J Pharmacol ExpTher 2005;313:952-961.
21. Slattery C, McMorrow T, Ryan MP. Overexpression of E2A proteins induces epithelial-mesenchymal transition in human renal proximal tubular epithelial cells suggesting a potential role in renal fibrosis. FEBS Lett 2006;580:4021-4030.