Characterization of Histopathological Features that Differentiate Hepatitis B Virus Infection from Acute Cellular Rejection

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Background : Differentiation of viral hepatitis from acute cellular rejection (ACR) after liver transplantation can be difficult because of overlapping histological features. Here we investigated clinicopathologic characteristics of 311 liver allograft biopsies and searched for characteristic histopathological features that would facilitate the differential diagnosis between hepatitis B virus (HBV) infection and ACR. **Methods :** A retrospective clinicopathologic examination of 311 liver allograft biopsies consisting of clinically proven ACR or HBV infection was performed. Immunohistochemical staining for HBcAg and HBsAg was done for 64 allograft biopsies showing HBV infection. **Results :** Moderate to severe bile duct damage, diffuse centrilobular necrosis and centrilobular inflammation (p<0.000, for each) were more frequently observed in cases of ACR, whereas diffuse acidophilic bodies and spotty necrosis (p<0.000, for each) were more prevalent in cases of HBV infection. Immunopositivity for HBcAg (n=60, 93.8%) was higher than that for HBsAg (n=14, 21.9%). **Conclusions :** The presence of moderate to severe bile duct damage, diffuse centrilobular necrosis and centrilobular inflammation was a characteristic feature of ACR, whereas diffuse distribution of acidophilic bodies or spotty necrosis was the only characteristic feature of HBV infection. HBcAg was a more sensitive immunohistochemical marker than HBsAg for detecting HBV infection in liver allograft biopsies.

Key Words : Liver; Transplantation; Graft rejection; Hepatitis B virus; Infection

Liver transplantation (LT) is a therapeutic modality for patients with various end-stage liver diseases. Because hepatitis B virus (HBV) infection is prevalent in Asian countries including Korea, Hong Kong, and China, patients with liver failure from HBV infection represent a large pool of candidates for LT in these countries.[1,2] In the past, LT in Asians with chronic hepatitis B was thought to pursue a rapid and virulent course with high rates of HBV recurrence and mortality.[3] However, outcomes of LT for HBV-associated patients have improved significantly due to the use of long-term immunoprophylaxis with hepatitis B immunoglobulin (HBIG) and antiviral agents.[2,4-6]

Acute cellular rejection (ACR) and recurrent viral hepatitis B often occur in liver allografts early after LT.[7] On the other hand, late-onset ACR occurring more than 1,000 days after LT has also been reported.[8] HBV infection can occur as acute hepatitis within six months after LT, with a mild lobular activity and variable degrees of portal inflammation.[7] Because the clinical manifestations are similar but the treatments are quite different, differentiation of HBV infection from ACR is very important in practice.

Diagnosis of ACR in liver allografts has typically been made based on the Banff schema.[9] The grade of ACR is determined by scoring severities of mixed portal inflammation, bile duct damage, and venous subendothelial inflammation of portal venules or terminal hepatic venules.[9] However, mild subendothelial inflammation and bile duct damage are not specific for the diagnosis of ACR and are also observed in cases of chronic viral hepatitis B.[10] This kind of overlap of morphologic characteristics can render the differential diagnosis between HBV infection and ACR more difficult, especially when the degree of portal and lobular inflammation is similar. Moreover, mild subendothelial inflammation and bile duct damage can lead to a mistaken primary diagnosis of mild ACR, and if strong immunosuppression is then implemented, the result will be worsening of liver function, as has been described in cases of recurrent viral hepatitis C.[11]

In our retrospective study, we investigated the clinicopathologic characteristics of 311 liver allograft biopsies consisting of clinically proven ACR or HBV infection and tried to identify the characteristic histopathological features in liver allograft

biopsies that would differentiate HBV infection from ACR.

MATERIALS AND METHODS

Materials and clinical data

A total of 311 follow-up liver allograft biopsies were obtained from 184 patients who had undergone LT for various end-stage liver diseases at Asan Medical Center, Seoul, Korea from October 1992 to December 2006 and who had received follow-up liver allograft biopsies one or more times. We grouped the biopsies by the clinical diagnoses, classifying them as ACR (n=248), ACR with concomitant HBV infection (n=10) and HBV infection (n=53). ACR was clinically diagnosed based on the presence of abnormal liver function test profiles and subsequent clinical improvement following augmented immunosuppressive treatment. HBV infection was clinically diagnosed based on the presence of abnormal liver function test profiles, an abnormally low titer of anti-HBsAg antibody, and the presence of HBsAg, HBV DNA probe, or HBeAg in the blood samples of patients. ACR with concomitant HBV infection was clinically diagnosed based on the presence of abnormal liver function test profiles and a subsequent clinical response to augmented immunosuppressive treatment, as well as clinical evidence of HBV infection as described above. Survival parameters were included for clinical analysis: duration of follow-up, and time and cause of death.

Histological analysis of 311 liver allograft biopsies

A total of 311 follow-up liver allograft biopsies in cases of ACR and HBV infection were analyzed for the presence of histological features occurring in the portal, periportal, and lobular areas. The following parameters were included for portal and periportal changes and are summarized in Table 1: portal inflammation (mild, moderate, severe); inflammatory cells (lymphocytic, mixed); bile duct damage (absent, mild, moderate, severe); venous endothelial inflammation (absent, mild, moderate, severe); rejection activity index (RAI) by the Banff schema (equivocal [RAI; 1-3], mild [RAI; 4-5], moderate to severe [RAI; 6-9])[9]; interface hepatitis (absent, focal, diffuse); fibrosis (portal, periportal, septal, cirrhosis). The degree of portal inflammation, bile duct damage, and venous endothelial inflammation was assessed based on the Banff schema.[9] The following parameters were included for lobular changes: acidophilic bodies (absent, focal, diffuse); spotty necrosis (absent, focal, diffuse); centrilobular necrosis (absent, focal, diffuse); centrilobular inflammation (absent, present); cholestasis (absent, focal, diffuse). A focal lesion was considered to involve less than half of the entire lobular area, and a diffuse lesion was considered to involve more than half of the entire lobular area.

Immunohistochemistry for HBV

Immunohistochemical staining for HBcAg (1:100; Signet, Dedham, MA, USA) and HBsAg (1:100; Signet) was done for 54 allograft biopsies for HBV infection and 10 allograft biopsies for

Table 1. Histological parameters for the analysis of portal and periportal changes

Category		Criteria
Portal inflammation	Mild	Expansion of a minority of portal tracts
	Moderate	Expansion of most or all of portal tracts
	Severe	Marked expansion of most or all of portal tracts with spillover into the periportal parenchyma
Bile duct damage	Mild	A minority of bile ducts are infiltrated by inflammatory cells and show only mild reactive changes such as increased nuclear: cytoplasmic ratio
	Moderate	Most or all of bile ducts are infiltrated by inflammatory cells and occasionally show degenerative changes such as nuclear pleomorphism, disordered polarity, and cytoplasmic vacuolization
	Severe	Most or all of bile ducts show degenerative changes or focal lumenal disruption
Venous endothelial inflammation	Mild	Subendothelial lymphocytic infiltration involving a minority of the portal and/or hepatic venules
	Moderate	Subendothelial infiltration involving most or all of the portal and/or hepatic venules
	Severe	Moderate or severe perivenular inflammation with extension into the perivenular parenchyma and/or perivenular necrosis.
Interface hepatitis	Focal	Piecemeal necrosis in less than 50% of the entire portal tracts
	Diffuse	Piecemeal necrosis in more than 50% of the entire portal tracts
Fibrosis	Portal	Fibrous portal expansion
	Periportal	Periportal fibrosis with intact lobular architecture
	Septal	Fibrous septa reaching adjacent portal tracts and terminal hepatic venules
	Cirrhosis	Diffuse nodular formation

HBV infection with concomitant ACR. All immunohistochemical tests were done using a Benchmark automatic immunostaining device (Ventana Medical System, Tucson, AZ, USA) using formalin-fixed, paraffin-embedded tissue sections. Five-micrometer-thick sections were obtained using a microtome, transferred onto adhesive slides, and dried at 62°C for 30 min. After dewaxing and rehydrating, antigen retrieval was carried out. After incubation with primary antibodies, sections were incubated with biotinylated antimouse immunoglobulins, followed by peroxidase-labeled streptavidin in LSAB kits (DAKO) and the 3,3´-diaminobenzidine chromogen as a substrate. Negative controls were obtained by omitting primary antibodies for all slides. Slides were counterstained with Harris hematoxylin. Positive immunostaining was defined as any nuclear or cytoplasmic staining for HBcAg and any cytoplasmic staining for HBsAg.

Statistical analysis

Paired t-tests and chi-square tests were used to determine whether there was any difference in histological variables and immunohistochemical results. Survival was calculated from the time of transplantation to death, loss of follow-up, or the last follow-up. The Kaplan-Meier method was used to compute an overall patient survival curve. The Log rank test was used to identify histological parameters that predicted overall patient survival. Statistical analyses were done using SPSS version 10.0 (SPSS, Inc., Chicago, IL, USA), and p-values of less than or equal to 0.05 were considered statistically significant.

RESULTS

Analysis of portal and periportal changes occurring in liver allograft biopsies from patients with ACR, ACR and concomitant HBV infection, and HBV infection

Histological features analyzed in portal and periportal areas are summarized in Table 2. A total of 248 cases of ACR were reclassified into 107 cases of mild ACR and 141 cases of moderate to severe ACR based on rejection activity index (RAI) of the

Table 2. Analysis of portal and periportal changes occurring in acute cellular rejection (ACR) and HBV infection in 311 liver allograft biopsies

Category		Mild ACR n=107 (%)	Moderate to severe ACR n=141 (%)	ACR and HBV infection n=10 (%)	HBV infection n=53 (%)
Portal inflammation	Mild	54 (50.5)	6 (4.2)	1 (10.0)	11 (20.8)
	Moderate	52 (48.6)	93 (66.0)	5 (50.0)	9 (17.0)
	Severe	1 (0.9)	42 (29.8)	4 (40.0)	33 (62.2)
Inflammatory cells	Lymphocytic	3 (2.8)	1 (0.7)	1 (10.0)	21 (39.6)
	Mixed	104 (97.2)	140 (99.3)	9 (90.0)	32 (60.4)
Bile duct damage	Absent	0	0	0	5 (9.4)
	Mild	24 (22.4)	0	0	25 (47.2)
	Moderate*	81 (75.7)[a]	63 (44.7)[a]	6 (60.0)[a]	18 (34.0)[b]
	Severe*	2 (1.9)[a]	78 (55.3)[a]	4 (40.0)[a]	5 (9.4)[b]
Venous endothelial inflammation	Absent	0	0	0	11 (20.8)
	Mild	84 (78.5)	9 (6.4)	5 (50.0)	32 (60.4)
	Moderate	22 (20.6)	96 (68.1)	3 (30.0)	10 (18.8)
	Severe	1 (0.9)	36 (25.5)	2 (20.0)	0
Rejection activity index by Banff	Equivocal	0	0	0	12 (22.6)
	Mild	107 (100.0)	0	4 (40.0)	20 (37.8)
	Mod-severe	0	141 (100.0)	6 (60.0)	21 (39.6)
Interface hepatitis	Absent	105 (98.2)	99 (70.2)	6 (60.0)	20 (37.7)
	Focal	1 (0.9)	35 (24.8)	2 (20.0)	13 (24.6)
	Diffuse	1 (0.9)	7 (5.0)	2 (20.0)	20 (37.7)
Fibrosis	Portal	97 (90.7)	92 (65.2)	6 (60.0)	23 (43.4)
	Periportal	10 (9.3)	43 (30.5)	2 (20.0)	15 (28.3)
	Septal	0	6 (4.3)	2 (20.0)	13 (24.5)
	Cirrhosis	0	0	0	2 (3.8)

Moderate to severe bile duct damage* was more frequently observed in cases of ACR[a] than in HBV infection[b] regardless of rejection activity index (p <0.000, χ^2 test).
ACR, acute cellular rejection; HBV, hepatitis B virus.

Banff schema.[9]

Moderate to severe portal inflammation was less frequently observed in cases of mild ACR, (49.5%) compared with cases of moderate to severe ACR (95.8%), ACR with concomitant HBV infection (90.0%), and HBV infection (79.2%, p<0.000, for each), whereas it was observed to a similar extent in cases of moderate to severe ACR (95.8%), ACR with concomitant HBV infection (90.0%), and HBV infection (79.2%). Mixed inflammatory cells were frequently observed in cases of ACR of any grade (mild ACR, 97.2%; moderate to severe ACR, 99.3%) compared with those of HBV infection (60.4%, p<0.000, for each). Although cases of ACR with concomitant HBV infection showed mixed inflammatory cells frequently (90.0%), the difference from those of HBV infection (60.4%, p=0.123) was not statistically significant.

Moderate to severe bile duct damage was frequently observed in cases of mild ACR (77.6%, p<0.000), moderate to severe ACR (100.0%, p<0.000), and ACR with concomitant HBV infection (100.0%, p=0.001) compared with cases of HBV infection (43.4 %). Although cases of HBV infection showed variable bile duct damage (mild bile duct damage, 47.2%; severe bile duct damage, 9.4%), the absence of bile duct damage in association with moderate to severe portal inflammation was observed even in 5 cases of HBV infection (9.4%, p<0.000, Fig. 1A, arrow head).

Moderate to severe venous endothelial inflammation was significantly more frequent in cases of moderate to severe ACR (93.6%, p<0.000) and ACR with concomitant HBV infection (50.0%, p=0.003) compared with cases of HBV infection (18.8 %). However, venous endothelial inflammation was not observed in 11 cases of HBV infection in spite of significant inflammation in the portal tracts (20.8%, p<0.006, Fig. 1A, arrows). Similar to cases of HBV infection with mild bile duct damage (47.2%), 60.4% of cases of HBV infection showed mild venous endothelial inflammation.

Focal to diffuse interface hepatitis was frequently observed in cases of HBV infection (62.3%, p<0.000, for each) compared with cases of mild ACR (1.8%) and moderate to severe ACR (29.8%). However, interface hepatitis was also observed in 40% of cases of ACR with concomitant HBV infection, with no statistically significant difference (p=0.194) between cases of moderate to severe ACR (29.8%) and ACR with concomitant HBV infection (40%). The degree of fibrosis showed no statistically significant differences between the four groups.

Among portal and periportal changes, moderate to severe bile duct damage was the only significant histopathological feature differentiating pure ACR and even ACR with concomitant HBV infection from HBV infection, regardless of RAI.

Analysis of lobular changes occurring in liver allograft biopsies from patients with ACR, ACR and concomitant HBV infection, and HBV infection

Histological features analyzed in lobular areas are summarized in Table 3. Diffuse acidophilic bodies were frequently observed in cases of HBV infection (43.4%, p<0.000, Fig. 1B, arrow heads) and ACR with concomitant HBV infection (60.0%, p<0.000) compared with those of mild ACR (0.9%) and moderate to severe ACR (3.5%). Similarly, diffuse spotty necrosis was frequently observed in cases of HBV infection (28.3%, p<0.000, Fig. 1B, arrow head) and ACR with concomitant HBV infection (10.0%,
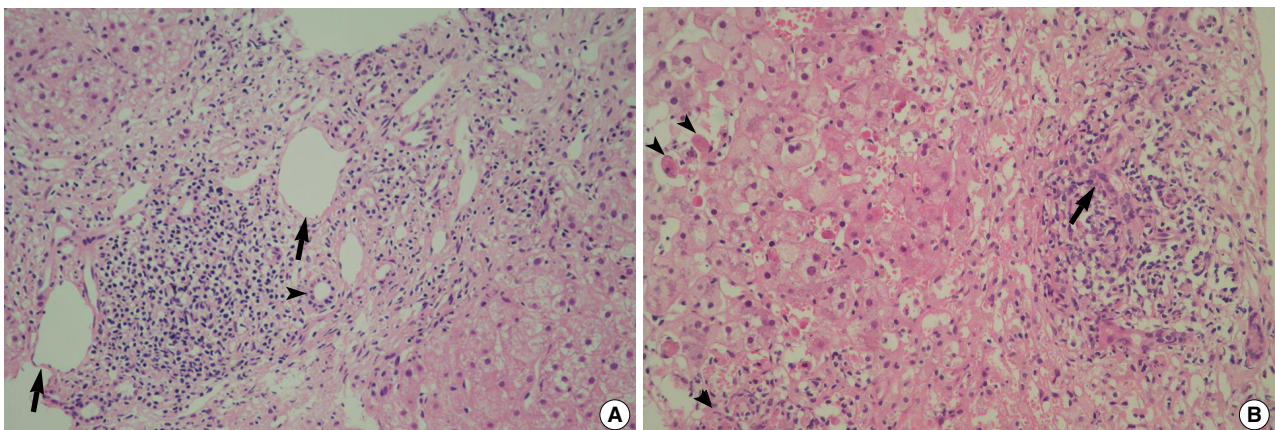
Fig. 1. Hepatitis B virus (HBV) infection after liver transplantation (LT). (A) A case of HBV infection after LT shows relatively absence of bile duct damage (arrow head) and venous subendothelial inflammation (arrows) compared to adjacent lymphocytic portal inflammation. (B) A case of HBV infection after LT shows severe interface hepatitis with moderate bile duct damage (arrow), frequent acidophilic bodies (arrow heads, left upper area), and foci of spotty necrosis (arrow head, left lower area) in the lobular area.

p<0.000) compared with cases of mild ACR (0%) and moderate to severe ACR (0.7%).

Diffuse centrilobular necrosis was frequently observed in cases of moderate to severe ACR (12.1%, p<0.000) and ACR with concomitant HBV infection (20.0%, p=0.001) compared with cases of HBV infection (0%). Similarly, centrilobular inflammation was frequently observed in cases of moderate to severe ACR (52.5%, p<0.000) and ACR with concomitant HBV infection (50.0%, p=0.022) compared with that of HBV infection (17.0%). The degree of cholestasis showed no statistically significant differences between the four groups.

Among lobular changes, diffuse acidophilic bodies and spotty necrosis were much more characteristic histopathological features for differentiating pure HBV infection and even HBV infection with concomitant ACR from ACR, regardless of RAI. Diffuse centrilobular necrosis and centrilobular inflammation were much more characteristic histopathological features for differentiating moderate to severe ACR and even ACR with concomitant HBV infection from HBV infection.

Expression of HBV antigen in the HBV infection group and in the ACR with concomitant HBV infection group

Results of immunohistochemical tests for HBcAg and HBsAg in 64 liver allograft biopsies with HBV infection are summarized in Table 4. Positive immunoreactivity for HBcAg (Fig. 2A) was higher than that for HBsAg (Fig. 2B) in both groups (98.1% vs 16.7% and 70.0% vs 50%, respectively). The most common

Table 3. Analysis of lobular changes occurring in acute cellular rejection (ACR) and HBV infection in 311 liver allograft biopsies

Category		Mild ACR n=107 (%)	Moderate to severe ACR n=141 (%)	ACR and HBV infection n=10 (%)	HBV infection n=53 (%)
Acidophilic body	Absent	94 (87.9)	110 (78.0)	1 (10.0)	13 (24.5)
	Focal	12 (11.2)	26 (18.5)	3 (30.0)	17 (32.1)
	Diffuse*	1 (0.9)[a]	5 (3.5)[a]	6 (60.0)[b]	23 (43.4)[b]
Spotty necrosis	Absent	97 (90.7)	133 (94.3)	4 (40.0)	11 (20.8)
	Focal	10 (9.3)	7 (5.0)	5 (50.0)	27 (50.9)
	Diffuse*	0[a]	1 (0.7)[a]	1 (10.0)[b]	15 (28.3)[b]
Centrilobular necrosis	Absent	103 (96.3)	106 (75.1)	8 (80.0)	53 (100.0)
	Focal	3 (2.8)	18 (12.8)	0	0
	Diffuse	1 (0.9)	17 (12.1)	2 (20.0)	0
Centrilobular inflammation	Absent	90 (84.1)	67 (47.5)	5 (50.0)	44 (83.0)
	Present	17 (15.9)	74 (52.5)	5 (50.0)	9 (17.0)
Cholestasis	Absent	72 (67.3)	103 (73.0)	8 (80.0)	41 (77.4)
	Focal	31 (29.0)	31 (22.0)	2 (20.0)	8 (15.1)
	Diffuse	4 (3.7)	7 (5.0)	0	4 (7.5)

Diffuse acidophilic bodies* and spotty necrosis* were more frequently observed in cases of HBV infection[b] than in ACR[a] regardless of rejection activity index (p<0.000, χ^2 test).
ACR, acute cellular rejection; HBV, hepatitis B virus.
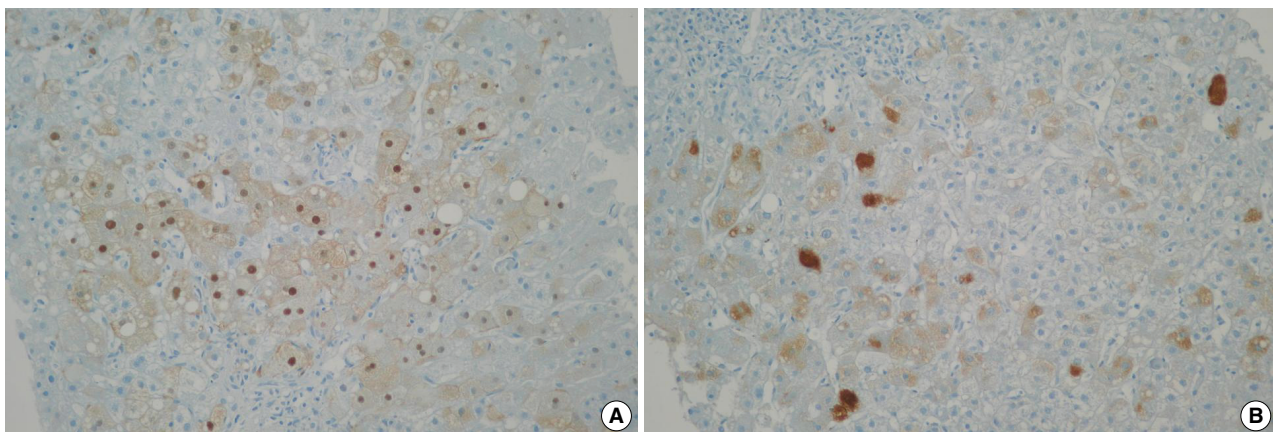
Fig. 2. Immunohistochemistry for hepatitis B virus (HBV) in a case of HBV infection. (A) Hepatocytes show nuclear and cytoplasmic immunopositivity for HBcAg. (B) Hepatocytes show cytoplasmic immunopositivity for HBsAg.

Table 4. Comparison of expression of hepatitis B virus (HBV) antigen between the HBV infection group and the acute cellular rejection with concomitant HBV infection group

Expression	HBV infection	Acute cellular rejection with HBV infection
HBcAg (+) HBsAg (−)	45/54 (83.3%)	5/10 (50%)
HBcAg (+) HBsAg (+)	8/54 (14.8%)	2/10 (20%)
HBcAg (−) HBsAg (+)	1/54 (1.9%)	3/10 (30%)

HBV, hepatitis B virus; HBcAg, hepatitis B core antigen; HBsAg, hepatitis B surface antigen.

expression pattern was that of HBcAg (+) and HBsAg (−) in both groups (83.3% and 50%, respectively). Two cases of HBV-associated fibrosing cholestatic hepatitis showed positive expression only for HBcAg. One case of HBV infection and three cases of ACR with concomitant HBV infection revealed an expression pattern of HBcAg (−) and HBsAg (+). No statistically significant difference between the two groups was identified for the expression of HBcAg or HBsAg.

Overall patient survival rates according to pathologic diagnoses of 311 liver allograft biopsies

According to pathologic diagnoses of 311 liver allograft biopsies, overall patient survival rates from the date of the first transplant at 1-year, 3-year and 5 years were 88.7%, 81.6%, and 79.9% for mild ACR, 84.0%, 76.3%, and 73.4% for moderate to severe ACR, and 92.5%, 64.8%, and 64.8% for HBV infection, respectively. There was no statistically significant difference for overall patient survival rates among the three groups by log rank test (p=0.475).

In the mild ACR group, diffuse cholestasis was significantly correlated with a decrease in overall patient survival rate (p=0.001), whereas other histological parameters showed no statistically significant difference. In the moderate to severe ACR group, all histological parameters showed no statistically significant difference regarding overall patient survival rates. In the HBV infection group, only diffuse interface hepatitis had a significant association with the decrease in overall patient survival rates (p=0.006).

DISCUSSION

In the present study, we analyzed the histopathologic features of liver allograft biopsies, the diagnoses of which were clinically confirmed, in order to identify pathognomonic features that

would allow the differential diagnosis between ACR and HBV infection. In portal and periportal areas, we emphasize only moderate to severe bile duct damage as a significant histopathological feature differentiating pure ACR and even ACR with concomitant HBV infection from HBV infection regardless of RAI. Histological features of mild ACR in the portal areas based on RAI of the Banff schema (37.8%), including frequent mild bile duct damage (47.2%) and mild venous endothelial inflammation (60.4%) in the HBV infection group of the present study, could lead to over-diagnosis of ACR in cases of recurrent HBV infection in liver allograft biopsies.[10] Mild bile duct damage is not a specific feature of ACR and has been reported in cases of recurrent viral hepatitis B after LT[12] and even in chronic HBV infection in non-transplant livers.[10] In lobular areas, we emphasize that diffuse centrilobular necrosis or inflammation are the characteristic histopathological features for differentiating moderate to severe ACR and even ACR with concomitant HBV infection from HBV infection. Diffuse centrilobular necrosis and inflammation are well known to be characteristic features of ACR.[9] Moreover, late-onset ACR can manifest as isolated perivenular inflammation and hepatocyte dropout, which is known as central perivenulitis.[13] Although the Banff schema[9] have limitations in the diagnosis of central perivenulitis as ACR in cases where other portal features of ACR are absent or non-diagnostic,[14] centrilobular necrosis in the presence of cholestasis and lobular inflammation has been reported to represent ACR even when portal changes in ACR are absent or non-diagnostic.[15] Thus, the presence of diffuse centrilobular necrosis or inflammation suggests the possibility of concomitant ACR even in cases showing classic histological features of HBV infection.

For HBV infection, we emphasize diffuse distribution of lobular activity such as acidophilic bodies and spotty necrosis as the significant histopathological features differentiating pure HBV infection as well as HBV infection with concomitant ACR from ACR regardless of RAI. Late-onset ACR and even chronic rejection can also manifest with variable lobular activity. However, if present, lobular activity is usually concentrated in perivenular regions without diffuse distribution in entire lobules.[7,13]

Complete positive immunoreactivity (100.0%) for either HBcAg or HBsAg in 64 cases of liver allograft biopsies with HBV infection demonstrates the usefulness of combined immunohistochemical staining as an ancillary method to confirm HBV infection. As the first sign of recurrent HBV infection, focal nuclear or cytoplasmic staining for core antigen was reported in liver allograft biopsies obtained in the initial incubation phase (first 3 months after LT),[7,16] consistent with higher imm-

unopositivity for HBcAg than HBsAg found in the present study. In cases of fibrosing cholestatic hepatitis, extensive expression of both HBcAg and HBsAg was reported in hepatocytes, suggesting a direct cytopathic effect of HBV.[17-21] However, in the present study, only HBcAg was expressed in 2 cases of fibrosing cholestatic hepatitis.

The presence of diffuse cholestasis had a clinical impact on overall patient survival in the mild ACR group of the present study. Although the presence of central necrosis was reported to be associated with subsequent occurrence of ACR, development of chronic rejection, and graft loss,[15,21] our data revealed no clinical impact of central necrosis on overall patient survival.

In summary, moderate to severe bile duct damage is the characteristic histopathological feature of ACR regardless of RAI, whereas mild bile duct damage or venous subendothelial inflammation are overlapping features of HBV infection and create a risk of over-diagnosis of ACR. Diffuse centrilobular necrosis or inflammation are the characteristic histopathological features for differentiating moderate to severe ACR and even ACR with concomitant HBV infection from HBV infection. Diffuse distribution of acidophilic bodies or spotty necrosis is a helpful features for the differential diagnosis of HBV infection from ACR. In addition, immunostaining for both HBcAg and HBsAg should be recommended for the differential diagnosis between ACR and HBV infection, when HBV infection is clinically suspected.

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