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CASE REPORT

The challenge of acute rejection in intestinal transplantation

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Abstract Early diagnosis and treatment of acute cellular rejection (ACR) after intestinal transplantation (ITx) is challenging. We report the outcome of three patients: two presented mild ACR improved with steroids. One presented steroid-resistant severe rejection, improved after rabbit anti-thymocyte globulin (r-ATG), but unfortunately died for encephalitis caused by opportunistic infections.

Keywords Intestinal transplantation · Acute cellular rejection · Antibody-mediated rejection · Immunosupression

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Abbreviations

ACR	Acute cellular rejection
AMR	Antibody-mediated rejection
CHDF	Continuous hemodiafiltration
CIIPS	Chronic idiopathic intestinal pseudo-obstruction syndrome
CIT	Cold ischemic time
CMV	Cytomegalovirus
CRBI	Catheter-related blood infection
DDIT	Deceased-donor intestinal transplantation
DSA	Donor-specific antibody
GF	Growth failure
HD	Hemodiafiltration
HHV-6	Human herpesvirus-6
HLA	Human leucocyte antibody
ITx	Intestinal transplantation
IVC	Inferior vena cava
LDLT	Living-donor liver transplantation
MP	Methylprednisolone
NID	Neuronal intestinal dysplasia
PE	Plasma exchange
PN	Parenteral nutrition
POD	Postoperative day
r-ATG	Rabbit anti-thymocyte globulin
SMA	Superior mesenteric artery
SMV	Superior mesenteric vein
TPN	Total parenteral nutrition
WIT	Warm ischemic time

Introduction

The survival rate after intestinal transplantation has improved worldwide in virtue of technical advances [1]. The early detection and prompt treatment of ACR is pivotal

to avoid graft and patient loss, and probably is the most important factor that improved the post-transplant outcome [2, 3]. We herein present the three primary deceased-donor intestinal transplantations (DDIT) performed in the Kyoto University Hospital since 2009.

Materials and methods

A historical cohort of three patients submitted to DDIT was reported.

In terms of the operative procedure of DDIT, donor's surgery consisted in the dissection of superior mesenteric artery (SMA) and vein (SMV). Systemic heparinization was performed and the jejuno–ileal graft was removed. On the back table, the graft was perfused with cold lactated Ringer's solution and then with University of Wisconsin (UW) solution. For the recipient, the remnant small bowel was removed and the graft's SMA was anastomosed to the recipient's SMA (end-to-end) or aorta (end-to-side). The donor's SMV was anastomosed to the recipient's SMV (end-to-end) or IVC (end-to-side). The oral side of graft jejunum was anastomosed directly to the remnant recipient's duodenum or jejunum and the anal side was used as ileostomy.

Daclizumab (cases 1 and 2) or basiliximab (case 3) were used for induction therapy, and intravenous tacrolimus (initial target level of 25–30 ng/ml) and methylprednisolone (MP 10 mg/kg after reflow, 1.25 × 4, 1 × 4, 0.75 × 4, and 0.5 × 4 mg/kg, each 6 h and each 12 h thereafter and tapered off gradually) were used as maintenance therapy for immunosupression. For each episode of rejection, 10–20 mg/kg/day of MP (steroid pulse) was used for 3 days and gradually tapered off. Tacrolimus blood level was checked once or twice a day, and it was transitioned to oral application in 2 weeks. Donor-specific antibody (DSA) was analyzed through single antigen beads method and was performed when antibody-mediated rejection was suspected in the immunohistochemistry (presence of C4d+ in the endothelium).

Daily endoscopies and pathological examinations were performed for the first 2 weeks and gradually spaced to every other day for the next 2 weeks and to once a month thereafter. Histological analysis was performed with hematoxylin–eosin stain and immunohistochemistry. The immunostaining was performed using the CSA system (DAKO, Gost-rup, Denmark). In this study, we used frozen section samples. For primary reagents, we used monoclonal antibodies against C4d, FasL, CD3, CD4 and CD8 (DAKO), and DAB staining was performed for signal visualization. Negative controls for primary antibodies were IgG1, IG2a and IgG2b (Cat no. X0931, X0943 and X0944; DAKO).

Case 1

A 12-year-old female with neuronal intestinal dysplasia (NID) type B complicated by repeated catheter-related blood infection (CRBI), was submitted to deceased-donor intestinal transplantation (DDITx). The inferior vena cava (IVC) was obliterated just distal to the renal veins and the only remnant central vein access for parenteral nutrition was the left internal jugular vein. She presented severe growth failure (GF) with 130 cm (−4.1 SD) of height (H) and 24.4 kg (−2.9 SD) of body weight (BW). The donor was cytomegalovirus (CMV) IgG(+) and the recipient was CMV IgG(−). Cold ischemic time (CIT) was 438 min and warm ischemic time (WIT) was 43 min. She presented an episode of indeterminate ACR with the presence of apoptotic bodies (Fas-L positive cells) and lymphocyte infiltration on the sixth postoperative day (POD 6) (Fig. 1a). She was given steroid pulse and the target concentration of tacrolimus was increased to 30∼35 ng/ml. On POD 11 CMV antigenemia was positive despite ganciclovir prophylaxis, and was successfully treated by valganciclovir. She is doing well in an outpatient basis 2 years and 4 months after DDIT.

Case 2

An 18-year-old male developed short bowel syndrome due to midgut volvulus. He had a severe GF (BW 27.6 kg, −3.4 SD) and needed total parenteral nutrition (TPN). ITx was considered due to TPN-related liver dysfunction. While on the waiting list for DDIT, his liver function deteriorated, and he received living-donor liver transplantation (LDLT). Nine months later, DDIT was performed. The graft had 300 cm, CIT was 374 min and WIT was 39 min. The donor's superior mesenteric artery (SMA) was anastomosed to the recipient Aorta and the donor's superior mesenteric vein (SMV) was anastomosed to the recipient's IVC, in an end-to-side fashion. Three episodes of indeterminate or mild rejection (POD 9, 81 and 141) were diagnosed by the presence of apoptotic bodies (Fig. 1b, c), and were successfully treated by steroid pulse. One year eight months after DDIT and 2 years 5 months after LDLT, liver biopsy showed cirrhosis associated with steatohepatitis. A month later, spontaneous bacterial peritonitis lead to deterioration of liver function, and another LDLT was performed. The intestinal graft's SMV was anastomosed to the liver graft's portal vein using an interposition graft from the recipient's right common iliac vein. Two years and two months after DDIT, he presented no further episodes of rejection (both liver and intestine) and is doing relatively well.

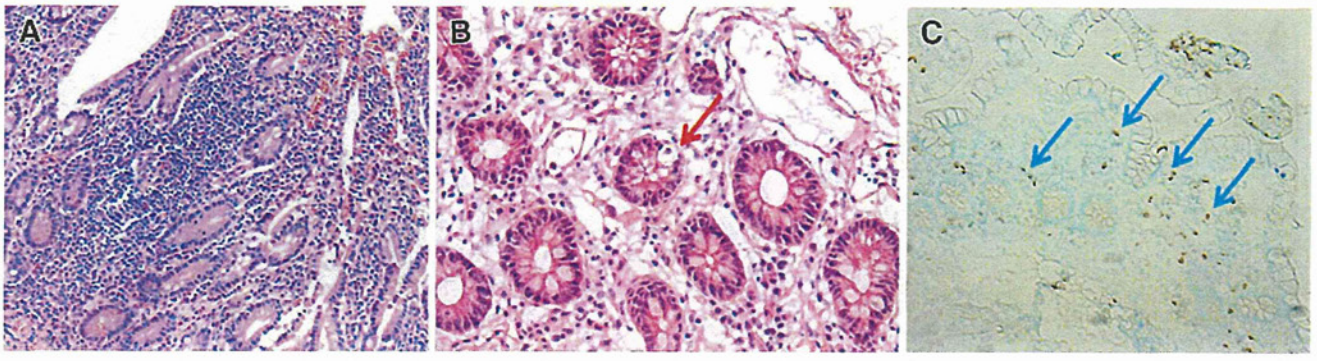

Fig. 1 **a** Case 1, rejection was suspected for lymphocyte infiltration on POD 6 (hematoxylin/eosin H/E); **b** case 2, detection of rejection through the presence of apoptosis in the crypt (*red arrow*, H/E); **c** case 2, presence of Fas-L positive cells (*blue arrows*, immunohistochemistry for FasL). Steroid pulse therapy was successfully performed for these two cases

Case 3

A 10-year-old female with hypoganglionosis received ITx for severe GF (H 106.7 cm (−4.9 SD) and BW 13.9 kg (−2.8 SD)) and repeated CRBI. She received an intestinal graft of 180 cm, CIT was 439 min and WIT was 29 min. Initially, tacrolimus was given intravenously, but its levels were extremely variable (Fig. 3). On POD 6, antibody-mediated rejection (AMR) was suspected for the presence of C4d+ endothelium in the intestinal immunohistochemistry (Fig. 2a) associated with the presence of serum donor-specific antibody (DSA). Although we aimed a target level of 25–30 ng/ml, the actual level of tacrolimus became low to 8 ng/ml (POD 15) during conversion from intravenous to oral application (Fig. 3). The fever increased on POD 18 and severe rejection was diagnosed on POD 19, with the presence of CD8+ cells in the crypt (Fig. 2b) associated with a strong inflammatory reaction and destruction of mucosal structure (Fig. 2c). Steroid pulse was not effective and rabbit anti-thymocyte globulin (r-ATG, 1.5 mg/kg/day) was administered for 1 week (POD 23–30). Tacrolimus target was increased to 30–35 ng/ml and MP was maintained 2 mg/kg/day after three sets of steroid pulses. Gradual improvement of the intestinal mucosa was observed, and steroid dose was tapered off carefully. She developed acute renal failure on POD 29 and thereafter she needed continuous hemodiafiltration (CHDF). Although she had no signs of liver dysfunction before the ITx, liver dysfunction associated with jaundice appeared and plasma exchange (PE) was needed as liver support. Despite the use of broad-spectrum antibiotics, antifungal agents and antiviral agents, high fever and drowsiness appeared on POD 75 without endoscopic signs of rejection. On POD 78 CT showed cerebral edema associated with encephalitis. She died on POD 90 with the detection of aspergillus and human herpes virus 6 (HHV-6) on post-mortem cerebral specimen. Aspergillus was also detected in the pericardium and the endocardium (Table 1).

Discussion

Recently, the short- and long-term results of intestinal transplantation have improved in virtue of surgical advances, development of new immunosuppressive strategies, and the use of prophylactic drugs against viral, fungal and bacterial infections [1, 2]. In the United States, the 1-year survival rates improved from 53.2 % in 1997 to 78.4 % in 2011 and the 5-year survival rate increased from 31.5 % in 1997 to 58.1 % in 2011, being a good option to treat patients with terminal intestinal failure [3, 4].

In Japan, intestinal transplantation was first performed in 1996 with 20 cases up to October 2010; 1- and 5-year patient survival rates of 87 and 69 %, respectively. The relatively few cases resulted from the scarcity of deceased donations, which had recently been overcome after the creation of a new Act on Japanese transplantation legislation, started on July, 2010 [5]. Since then, the total number of ITx has reached 24 cases for 21 patients by March, 2012, 11 from deceased donors and 13 from living-donors.

Acute cellular rejection has been the major cause of intestinal graft failure [2, 3]. Early recognition through frequent endoscopic and histological evaluation is essential to avoid severe rejection. The presence of apoptosis is one of the established pathological findings of typical rejection [6], and in our hospital, the detection of FasL-positive lymphocytes (apoptotic bodies) by the immunostaining has been considered as an early marker of rejection, thereby allowing an earlier approach to avoid the development of severe rejection. Cases 1 and 2 received prompt steroid pulse therapy with the detection of apoptotic bodies. Consequently, they did not develop severe rejection.

On the contrary, case 3 had developed severe and steroid-resistant ACR. Preceding the ACR, antibody-mediated rejection was suspected. Kato T et al. [7] reported the association between the HLA antibody with ACR in ITx and multivisceral transplantation recipients. Therefore, we

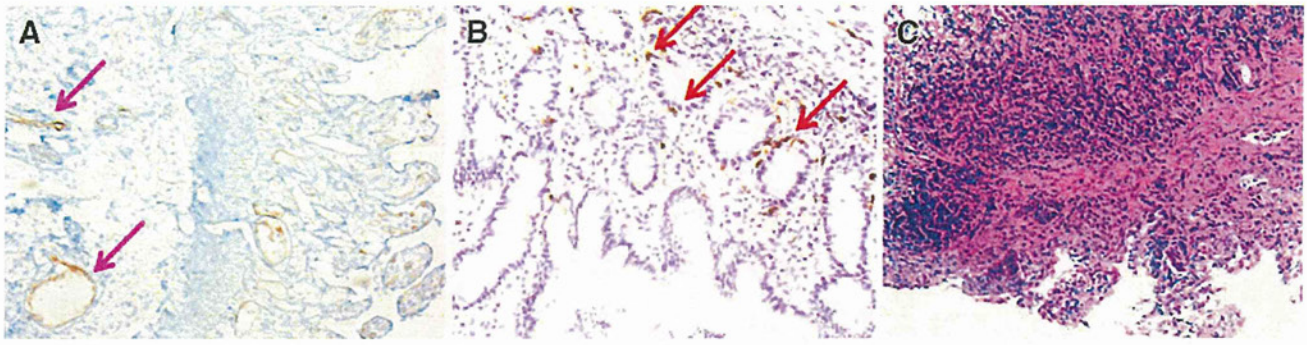

Fig. 2 **a** Case 3 Antibody-mediated rejection (AMR) was suspected for the presence of endothelial C4d positive cells (*pink arrows*, POD 6); **b** ACR was suspected for CD8 positive lymphocytes in the crypt (*red arrows*); **c** severe rejection with a strong inflammatory reaction and destruction of mucosal structure was diagnosed on POD 19

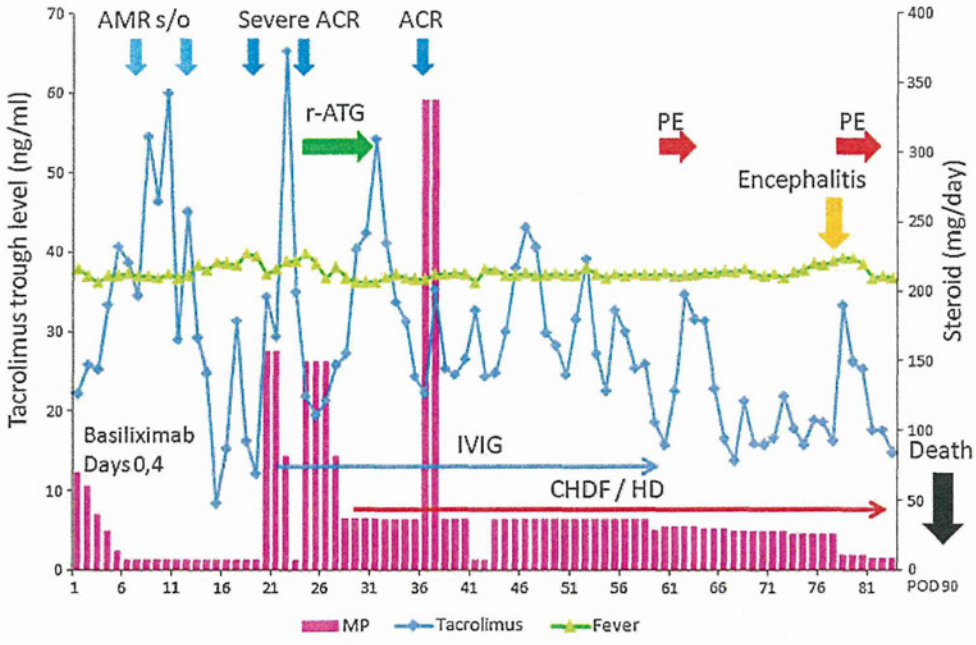

Fig. 3 Case 3, levels of tacrolimus were extremely variable. Antibody-mediated rejection (AMR) was suspected before severe acute cellular rejection (ACR). Steroid pulse therapy (MP, methylprednisolone) was not effective and r-ATG (rabbit anti-thymocyte globulin) was given. Intravenous immunoglobulin (IVIG) was used in attempt to control AMR. The patient died on the 90th POD secondary to encephalitis caused by aspergillus and human herpes virus-6, associated with generalized aspergillosis

Table 1 Patients demographics

Case	Age (years), gender	Diagnosis	Indications	Graft (cm)	ACR	Infection	Outcome
1	12, F	NID type B	CRBI, GF, CV 1/6	320	I	CMV	Alive 2 y 4 m
2	18, M	Midgut volvulus, SBS	TPN-related liver failure SBS, GF	300	I	CMV aspergillus	Alive 2 y 2 m
3	10, F	Hypoganglionosis	CRBI GF	180	S	Aspergillus, HHV-6	Died 90th POD encephalitis

M male, *F* female, *NID* neuronal intestinal dysplasia, *SBS* short bowel syndrome, *CRBI* catheter-related blood infection, *GF* growth failure, *CV* Central vein access remnant, *I* indeterminate, *S* severe, *CMV* cytomegalovirus, *HHV-6* Human herpesvirus-6, *MRCNS* Methicillin-resistant coagulase-negative staphylococcus, *TPN* total parenteral nutrition, *y* years, *m* months, *d* days, *POD* postoperative day

should pay more attention to patients with AMR since they have a higher risk of developing severe ACR. In our case, the decrease in tacrolimus levels may have also contributed to the development of severe ACR. Tacrolimus levels must be strictly controlled during the first weeks after ITx. In attempt to rescue the intestinal graft, strong

immunosupression was utilized, and the patient became extremely vulnerable to opportunistic infections. After the use of thymoglobulin and subsequent improvement of ACR, tacrolimus and steroid dose reduction should be attempted to avoid infectious complications. Liver and renal failure also contributed to the patient's vulnerability.

In conclusion, an early recognition and the prompt treatment of ACR are essential for ITx. The first two cases demonstrated that the presence of Fas-L positive apoptotic bodies may be used to detect early phases of rejection. A closer follow-up is needed for patients who develop AMR since they are prone to develop severe rejection. A delicate immunologic balance must be maintained between treatment of ACR and the vulnerability to opportunistic infections.

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Original Paper

Once-Daily Prolonged-Release Tacrolimus in *De Novo* Liver Transplantation: A Single Center Cohort Study

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Key Words: Tacrolimus; Once-daily formula; Liver transplant; Dose adjustment; Biopsy-proved acute cellular rejection.

Abbreviations: Biopsy-Proven Acute Cellular Rejection (BPAR); Estimated Glomerular Filtrating Rate (eGFR); Living Donor Liver Transplantation (LDLT); Postoperative Day (POD); Tacrolimus (TAC); Tacrolimus Prolonged Release (TAC-PR).

ABSTRACT

Background/Aims: The feasibility of oral administration of once-daily prolonged-release tacrolimus (TAC-PR) in *de novo* liver transplantation is not clear and therefore was investigated further. **Methodology:** The clinical profiles of 16 consecutive primary living donor liver transplantation (LDLT) recipients, who received oral TAC-PR once daily (TAC-PR group) between January 2009 and August 2010, were compared with those of 14 consecutive liver transplantation recipients given twice-daily tacrolimus (TAC; TAC group) between August 2006 and January 2009. Of the 14 patients in the TAC group, 9 received LDLT (TAC-L subgroup). **Results:** Patient characteristics were similar between groups. Trough levels of TAC during the first 3 months after liver transplantation were well-adjusted in both groups. Dose adjustment was more frequently required (31.3%) in the TAC-PR group and the total amount of TAC was significantly higher in the TAC-PR group (181.1±75.3mg) than in the TAC-L group (100.2±53.8mg, p=0.014). The incidence of biopsy-proven acute cellular rejection, renal dysfunction, other morbidities and hospital stay length were similar between groups. **Conclusions:** Oral administration of TAC-PR for *de novo* liver transplantation recipients was well tolerated with similar safety and efficacy profiles as traditional twice-daily TAC with closely controlled adjustment of the TAC-PR dose.

INTRODUCTION

Calcineurin inhibitors, which emerged in the 1970s, are the most potent immunosuppressants available. Organ transplant recipients receive calcineurin inhibitors twice daily in an oral formula of either tacrolimus (TAC) or cyclosporine A as a primary immunosuppressive regimen. Recently, a prolonged-release formulation of TAC (Graceptor, Astellas Pharma Japan Ltd, Tokyo, Japan; hereafter referred to as TAC-PR) was developed to provide once-daily dosing with efficacy and safety similar to those of the twice-daily formulation (1). After the initial trial however, there have been no studies reported in the field of liver transplantation. Moreover, it is not clear from the available data whether there are any difficulties in adjusting doses during the early post-transplant period in *de novo* liver transplantation recipients. The aim of the present study was to compare the feasibility, safety and efficacy of a regimen of oral administration of TAC-PR with that of traditional twice-daily TAC in *de novo* living donor liver transplantation (LDLT) recipients.

METHODOLOGY

Sixteen consecutive primary adult-to-adult LDLT recipients during the period between January 2009 and August 2010 received one dose of TAC immediately after the transplant, followed by oral TAC-PR in a once-daily protocol (TAC-PR group) from postoperative day 1 (POD 1). In contrast, 14 consecutive primary adult liver transplantation recipients (TAC group) during the period between August 2006 and January 2009 received TAC twice daily. Nine of these patients underwent LDLT (TAC-L group) and 5 patients underwent deceased donor whole liver transplantation (TAC-D group).

The initial daily dose of TAC, 0.05mg/kg in both the TAC-PR and TAC groups, was given through a nasogastric tube immediately after arrival in the intensive care unit after liver transplantation and then the tube was clamped for 1 hour. The dose of TAC-PR given on POD 1 was calculated using the following formula: TAC-PR POD 1 dose (mg) = 2×amount of TAC on POD 0 (mg) × target trough level (ng/mL) × (0.6-0.8)/trough level on POD 1 (ng/mL).

The dose was adjusted based on the morning trough level from POD 1 to 3 and then the dose of TAC-PR for POD 4 was determined by the trough level on POD 3. The evening dose of TAC was adjusted based on the morning trough level on each day after POD 1. The dose was held when the trough level was over 20ng/mL and additional doses were administered when the trough concentration of TAC was suboptimal. The target trough level of TAC was 8-12ng/mL within 28 days after liver transplantation and 6-10ng/mL between 29 to 90 days after liver transplantation in both groups.

The immunosuppression regimen comprised TAC-PR or TAC and corticosteroids, starting with 1g methylprednisolone during the transplant, then tapering from 100mg/day to 5mg/day in patients with primary biliary

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cirrhosis, primary sclerosing cholangitis or autoimmune hepatitis, or tapering off in recipients with liver failure of other etiologies. Mycophenolate mofetil was added in patients with renal impairment, rejection episodes or others as needed. Patients with hepatitis C virus (HCV) received corticosteroid-free immunosuppression, comprising TAC or TAC-PR, mycophenolate mofetil and basiliximab (anti-CD25 monoclonal antibody, Novartis Pharma K.K., Tokyo, Japan).

An elementary diet (ED) tube (8Fr, silicon, Create Medic Co., Ltd, Yokohama, Japan) was placed into the jejunum during the transplant surgery and an ED was started as soon as possible. Oral intake of medicine including TAC was started when water intake was fully possible and followed oral intake of food. A biliary drainage tube (pancreatic tube, 5Fr, Sumitomo Bakelite, Tokyo, Japan) was placed in all recipients and drained until the serum total bilirubin level was below 3mg/dL after cholangiography using the drainage tube.

The incidence of holding TAC or adding TAC, biopsy-proven acute cellular rejection (BPAR), renal toxicity evaluated by estimated GFR (eGFR) (2), infection and other morbidities during the first 3 months after liver transplantation was compared between TAC-PR group and TAC group or between the TAC-L group and TAC-D subgroups.

Statistical analysis

Continuous data are expressed as mean ±SD and group data sets were compared using Student's *t* test, a Mann-Whitney U test or the Kruskal-Wallis test. Categorical data are presented as percentages and differences between proportions were compared using the chi-squared test. The cumulative risk of BPAR was estimated by Kaplan-Meier analysis (log rank test). A *p* value of less than 0.05 was considered to be significant.

RESULTS

Background and characteristics of recipients were similar between the TAC-PR group and TAC groups, except for recipient age and graft volume (**Table 1**). Age of recipients in the TAC-PR group was 55.3±7.9 years, significantly higher than that of recipients in the TAC group (45.5±14.9 years, p=0.030) and tended to be higher than that of recipients in the TAC-L group (47.4±13.0 years, p=0.071). Graft weight was 554±117g in the TAC-PR group, 892±485g in the TAC group (p=0.014, *vs.* TAC-PR group) and 563±98g in the TAC-L group (n.s. *vs.* TAC-PR group). Preoperative model of end-stage liver disease score and eGFR were similar between the groups. Seven (43.8%) patients with HCV in the TAC-PR group and 3 (21.4%) patients with HCV in the TAC group received steroid-free immunosuppression.

Trough levels of TAC were similar between the TAC-PR and TAC-L groups throughout the study period while those of the TAC-D group were slightly higher than those of the TAC-PR group, although there was no statistical difference between them except on POD7 and POD90 (**Figure 1A**). Trough levels in the TAC-PR group were well controlled within the target level even in the early post-transplantation period.

The incidence of holding TAC was 1/16 (6.3%, POD1) in the TAC-PR group and 3/14 (21.4%, POD1, POD2 and POD6) in the TAC group due to high trough levels. The in-

TABLE 1. Patient characteristics.

	TAC-PR (QD) (n=16)	TAC (BID) (n=14)	TAC-L (BID) (n=9)	TAC-D (BID) (n=5)	*p* value	
					TAC-PR *vs.* TAC	TAC-PR *vs.* TAC-L
Age (y)	55.3±7.9	45.5±14.9	47.4±13.0	42.0±19.1	0.030	0.071
Gender (M/F)	7/9	8/6	4/5	4/1	0.464	0.552
Primary diagnosis						
HCV	7	3	1	2		
HBV	2	2	2	0		
PBC/PSC	2	2	1	1		
Wilson disease	0	1	0	1	0.088	0.170
Badd-Chiari	1	0	0	0		
EtOH	1	0	0	0		
Fulminant	1	1	1	0		
Others	2	5	4	1		
Type of donor						
Deceased	0	5	0	5	0.009	0.999
Living	16	9	9	0		
Type of graft						
Left lobe with caudate	7	4	4	0		
Right lobe	9	3	3	0	0.832	0.303
Right lateral section	0	2	2	0		
Whole liver	0	5	0	5		
Graft weight (g)	554±117	892±485	563±98	1485±250	0.014	0.852
Preoperative MELD score	21.8±10.4	20.1±6.7	23.3±6.0	14.4±3.4	0.603	0.704
Preoperative eGFR (mL/min)	83.4±35.2	70.9±33.5	62.7±34.8	85.8±28.4	0.333	0.171
Operative time (min)	709±87	808±225	819±261	791±176	0.140	0.161
Blood loss (mL)	7303±4645	5014±3128	4190±2461	6498±3925	0.138	0.080

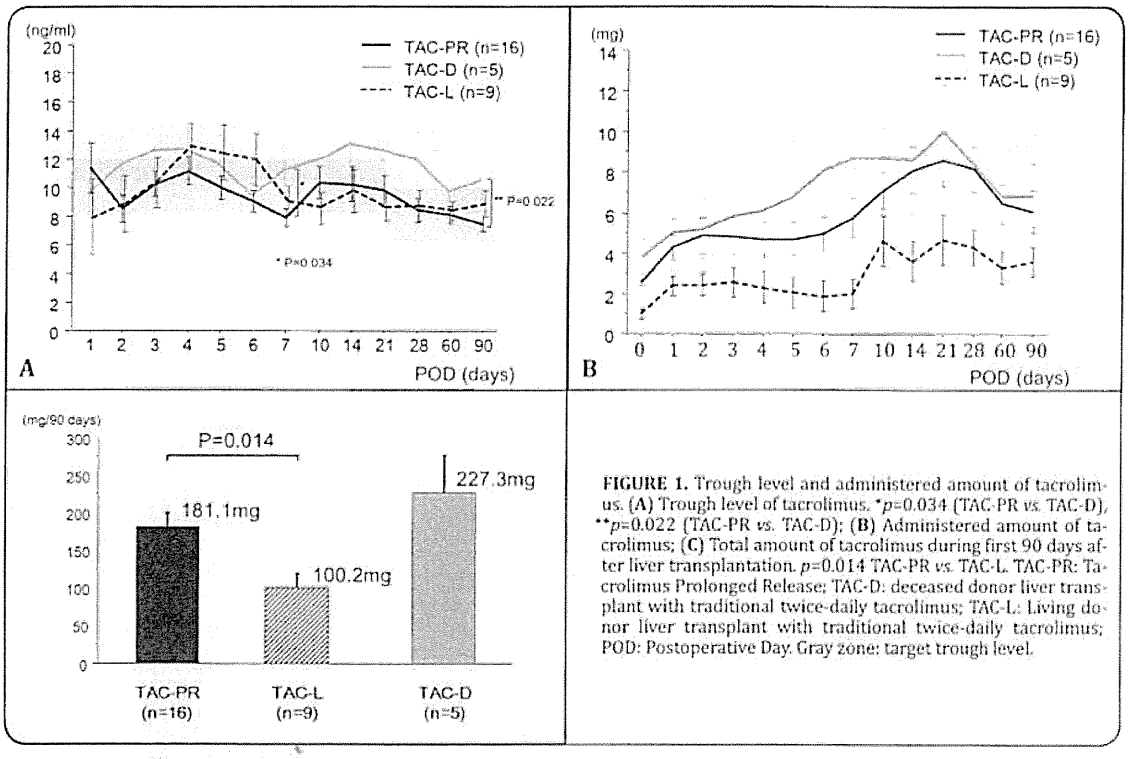

FIGURE 1. Trough level and administered amount of tacrolimus. **(A)** Trough level of tacrolimus. *p=0.034 (TAC-PR *vs.* TAC-D), **p=0.022 (TAC-PR *vs.* TAC-D); **(B)** Administered amount of tacrolimus; **(C)** Total amount of tacrolimus during first 90 days after liver transplantation. p=0.014 TAC-PR *vs.* TAC-L. TAC-PR: Tacrolimus Prolonged Release; TAC-D: deceased donor liver transplant with traditional twice-daily tacrolimus; TAC-L: Living donor liver transplant with traditional twice-daily tacrolimus; POD: Postoperative Day. Gray zone: target trough level.

cidence of additional TAC (**Figure 2**) due to low trough levels was 5/16 (31.3% POD2-7) in the TAC-PR group, while in the TAC group all of the adjustments were managed by increasing the evening dose.

The administered dose of TAC was higher in the TAC-PR group compared with the TAC-L group and similar to that in the TAC-D group (**Figure 1B**). The daily dose of TAC was increased until POD 21 when it peaked, then decreased in both the TAC-PR and TAC groups. The total amount of TAC was 1.8-fold higher in the TAC-PR group than in the TAC-L group and slightly less than that in the TAC-D group (**Figure 1C**).

The start time of the ED after transplant was similar between groups and initiation of oral intake of food was also similar between groups. Gastrointestinal symptoms such as diarrhea and vomiting were uncommon (6.3% in the TAC-PR group, 14.3% in the TAC group) in both groups (**Table 2**).

Cumulative incidence of BPAR by POD 90 was 18.7% in the TAC-PR group and 50.0% in the TAC group. There was no statistically significant difference in the incidence of BPAR within POD 90 (**Figure 3**).

Preoperative eGFR was not different between groups. The ratio of the lowest eGFR within POD 90 to preoperative eGFR was almost identical between groups (75.3% in TAC-PR group, 70.5% in TAC group and 75.2% in TAC-L group) (**Table 2**).

Postoperative surgical morbidities, such as postoperative intra-abdominal bleeding or portal vein thrombosis, were similar between groups (**Table 2**). Postoperative non-surgical morbidities such as infection were not specific to the TAC-PR group, although two recipients developed intracranial bleeding which was not considered to be a side effect of TAC. Duration of hospital stay after liver transplantation was similar between groups (TAC-PR 93.7±58.5 days, TAC 103.7±114.5 days; **Table 2**).

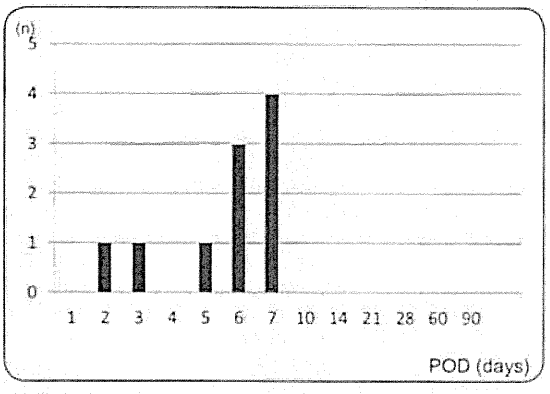

FIGURE 2. Incidence of additional tacrolimus in the TAC-PR group (n=16). Additional TAC-PR was required due to suboptimal levels within the first week after liver transplantation, but there was no need for additional TAC-PR after POD 10. POD: Postoperative Day.

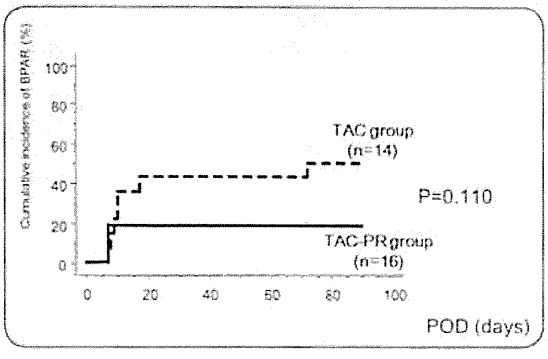

FIGURE 3. Incidence of biopsy-proven acute cellular rejection within 3 months after LDLT. There was no statistical difference between TAC-PR and TAC groups (p=0.110). BPAR: Biopsy-Proven Acute Cellular Rejection; POD: Postoperative Day.

TABLE 2. Postoperative course and morbidities.

	TAC-PR (QD) (n=16)	TAC (BID) (n=14)	TAC-L (BID) (n=9)	TAC-D (BID) (n=5)	*p* value TAC-PR *vs.* TAC	*p* value TAC-PR *vs.* TAC-L
Starting ED diet (POD)	2.8±0.7	3.6±3.0	3.0±1.0	4.0±4.1	0.668	0.343
Starting of oral intake (POD)	14.4±11.2	16.0±13.6	17.9±16.5	13.0±8.0	0.727	0.543
Diarrhea and other abdominal symptoms (%)	1 (6.3%)	2 (14.3%)	0	1 (20%)	0.464	0.444
Acute cellular rejection (%)	3 (18.8%)	7 (50.0%)	4 (44.4%)	3 (60%)	0.07	0.17
Ratio of postoperative-minimal-eGFR/ preoperative-eGFR	75.3%	70.5%	75.2%	62.0%	0.798	0.996
Morbidities						
Postoperative intra-abdominal bleeding	0	3	2	1		
Portal vein thrombus	0	1	0	1		
Hepatic artery stenosis	0	2	1	1		
Acute renal failure	0	1	0	1		
Infection	1	4	3	1		
Intracranial bleeding	2	0	0	0		
Hospital stay (day)	93.7±58.5	103.7±114.5	125.7±150.2	72.8±14.6	0.778	0.486

DISCUSSION

Once-daily administration of TAC is better for recipients because of easy handling with less stress than the mandatory scheduled intake of the twice-daily formula. Oral administration of twice-daily TAC for *de novo* liver transplantation recipients is widely accepted in clinical practice, however, because of its simplicity and efficacy in stable post-transplantation recipients (3-7). In contrast, oral administration of TAC-PR for *de novo* liver transplantation carries a risk of inappropriate control of the drug concentration, which can cause graft damage or graft loss. Therefore, in some programs, intravenous TAC is administered concurrent with oral TAC in the early post-transplantation period due to the fear of suboptimal drug concentration (personal communication). Another regimen for TAC-PR in liver transplantation recipients is oral administration of twice-daily TAC or temporary intravenous administration of TAC at first, then conversion to a TAC-PR formula. As long as the concentration is well controlled, once-daily oral administration of TAC is better for *de novo* liver transplantation recipients than methods that require conversion because of its simplicity and unnecessary conversion of the TAC formula.

To date, there has been only one clinical trial of TAC-PR for *de novo* liver transplantation recipients (1) but the authors did not mention the management of the TAC-PR dose and its clinical outcome. In the present single center cohort study of the use of once-daily TAC-PR in *de novo* liver transplantation, we investigated the feasibility of oral administration of TAC-PR and the incidence of holding or adding TAC-PR early after liver transplantation to evaluate the difference between TAC-PR and the traditional TAC protocol.

We presented a formula for calculating the first dose of TAC-PR on POD1 based on the trough level of TAC after one dose immediately after liver transplantation, which proved to be very accurate. The trough level of early post-transplantation recipients (POD 2 to POD 7) was quite well controlled within the target level of TAC. The incidence of holding the dose of TAC-PR was low. In contrast, dose adjustment by adding a dose due to a suboptimal concentration of TAC was required in 5/16 (31.3%) recipients from POD 2 to POD 7 in the TAC-PR group, while all dose adjustments were managed by increasing the evening dose of TAC in the TAC group. Thus, the dose adjustment for TAC-PR was not difficult but careful attention was required, especially within the first week after liver transplantation. This procedure of "adding a dose" could be prevented if the dose is determined after establishing the TAC trough level of the day; however we chose to determine the scheduled dose for POD 4 and after, based on the trough levels of the day before for fear of missing a dose. Our method may be more acceptable in the clinical setting due to the certainty.

The actual dose of TAC was generally higher in the TAC-PR group than in the TAC group and it was 1.8-fold higher in the TAC-PR than in the TAC group for the first 90 days after liver transplantation, consistent with previous studies (1,8). This is probably due to the reduced absorption of TAC from the intestine. TAC is absorbed mainly from the proximal jejunum, while the prolonged release particles of TAC-PR interfere with absorption, which is important for the once-daily oral administration protocol.

The incidence of acute cellular rejection tended to be higher in the TAC group than in the TAC-PR group. The deviation of the incidence of acute cellular rejection is likely due to the small number of recipients in both groups. The incidence of other morbidities post-transplantation, including infection and renal dysfunction, was also similar.

This study was a non-randomized cohort study comparing the TAC-PR and TAC protocols with only a small number of cases in both groups. The results clearly demonstrate, however, that the TAC-PR protocol is more feasible and effective with strict adjustment compared to the traditional twice-daily TAC protocol. Future studies should clarify the long-term feasibility and efficacy, especially the rate of reduction of non-compliance and reduction of renal dysfunction when using the TAC-PR formula.

In conclusion, oral administration of TAC-PR for *de novo* liver transplantation recipients was well tolerated with a safety and efficacy profile similar to that of the traditional twice-daily TAC with closely controlled adjustment of the TAC-PR dose.

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Significance of Alanine Aminopeptidase N (APN) in Bile in the Diagnosis of Acute Cellular Rejection After Liver Transplantation

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Background. **Allograft dysfunction after liver transplantation requires histopathologic examination for confirmation of the diagnosis, however, the procedure is invasive and its interpretation is not always accurate. The aim of this study was to find novel protein markers in bile for the diagnosis of acute cellular rejection (ACR) after liver transplantation.**

Materials and Methods. **Quantitative proteomic analysis using the ^{18}O labeling method was used to search for bile proteins of interest. Nine recipients were selected who had liver dysfunction, diagnosed by liver biopsy, either with ACR (ACR group, $n = 5$) or without (LD group, $n = 4$). Donor bile samples were obtained from nine independent live liver donors. Enzyme activity in bile samples was assayed and liver biopsy specimens were immunostained for candidate protein of ACR.**

Results. **The analysis identified 78 proteins, among which alanine aminopeptidase N (APN/CD13) was considered a candidate marker of ACR. Comparative analysis of the ACR and LD groups showed high APN enzyme activity in three (60%) of five cases of the ACR group, while it was as low as donor level in all patients of the LD group. APN enzyme activity in bile samples of liver dysfunction liver transplantation (LDLT) recipients of the ACR group collected within 3 d before biopsy-confirmed ACR ($n = 10$) was significantly higher (584 ± 434 U/g protein) than in those of recipients free of ACR ($n = 96$, 301 ± 271 U/g protein) ($P = 0.004$). APN overexpression along bile canaliculi was observed during ACR in all five cases of the ACR group.**

Conclusion. **APN in bile seems to be a useful and noninvasive biomarker of ACR after liver transplantation.**

Key Words: **living donor liver transplantation; bile; alanine aminopeptidase N (APN); acute cellular rejection; proteomic analysis; ^{18}O labeling method.**

INTRODUCTION

Liver transplantation (LT) is a widely accepted as an effective and life-saving treatment for end-stage liver disease or acute liver failure. Despite improvement in immunosuppressive therapy and continuous advances in surgical techniques, the patient and graft survival rates after liver transplantation remain around 80% at 1 y and 70% at 5 years according to the databases of the United Network for Organ Sharing (UNOS) and Japanese Liver Transplant Society. The suboptimal outcome is due to the complexity of management of allograft liver function. Acute cellular rejection, recurrence of viral hepatitis, *de novo* hepatitis, drug-induced hepatitis, and/or other causes of allograft dysfunction occur during the early period after liver transplantation, and some of these pathologies could mimic the histologic and clinical presentation of each other, which emphasizes the need for the development of new techniques for accurate diagnosis of early allograft dysfunction. Acute cellular rejection (ACR) accounts for 7% to 42% of living liver transplant recipients [1–3], but other conditions should be always

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considered in the differential diagnosis. Therefore, a feasible and reproducible diagnostic method for ACR is important in clinical practice.

In kidney transplantation, urinary enzymes or low-molecular weight proteins are considered useful markers of ACR during the early post-transplantation period [4, 5]. Other biomarkers of ACR after renal transplantation are being investigated at present using proteomic analysis of urine [6, 7]. Analogous to kidney transplantation, several studies reported the identification of serum and bile biomarkers of ACR after liver transplantation [8–15]. Our group reported previously that interleukin-6 (IL-6) in bile correlated with ACR after liver transplantation in rats and deceased liver transplantation in human [16, 17]. Another group reported that intercellular adhesion molecule-1 (ICAM-1) in bile was associated with ACR after liver transplantation [8, 9]. However, a subsequent study from another group showed that these protein markers in bile were not associated with ACR [12] after liver transplantation. Thus, no specific biomarker in bile associated with ACR in liver transplantation is yet available.

In the present study, we used ^{18}O labeling-based proteomic analysis of bile after liver transplantation to identify candidate proteins in bile that can predict allograft rejection. The proteomic analysis identified the enzyme alanine aminopeptidase N (APN/CD13) as a candidate marker for ACR after liver transplantation. In the second part of the study, we evaluated APN in clinical samples of the donors and recipients of liver transplantation. The results indicated that APN seems to be a useful and noninvasive marker for ACR after liver transplantation.

MATERIALS AND METHODS

The present prospective study was approved by the Human Ethics Review Committee of Osaka University, and a signed consent form was obtained from each patient.

Quantitative Bile Analysis by ^{18}O Labeling Method to Identify Biomarkers of Acute Cellular Rejection After Liver Transplantation

Bile Samples

To identify novel candidate biomarkers of bile proteins for ACR after liver transplantation, we first analyzed three bile samples [obtained on postoperative day (POD) 1, 4, and 14] from a single liver transplant recipient who developed ACR, in whom ACR was confirmed by histopathology of liver biopsy on POD7. The patient showed improvement of liver function tests on POD14 following steroid recycle therapy, and a repeat liver biopsy on POD14 showed amelioration of ACR. Relative quantitative protein analysis using ^{18}O labeling was applied to this set of bile samples from this recipient.

Preparation of Bile Sample

The crude bile sample (protein concentration: 1.0 mg/mL) was centrifuged at 4°C, 14,000 rpm for 60 min by using microcon YM-10 (MWCO 10 kDa) (Millipore, Bedford, MA) for desalting and concentration. The sample was washed with 100 μL of 100 mM Tris-HCl buffer (pH 7.8) by centrifugation at 4°C, 14,000 rpm for 60 min. The resultant protein fraction was dissolved in lysis buffer [8 M urea and 1 mM ethylenediaminetetraacetic acid (EDTA)] and incubated at 37°C for 30 min, and then reduced with 25 mM dithiothreitol (DTT) at 37°C for 3 h, followed by alkylation of the thiols with 55 mM iodoacetoamide in the dark for 30 min. The protein concentration was determined by the Bradford method (Pierce, Rockford, IL).

SDS-PAGE and In-Gel Digestion

The proteins (200 μg per lane) were separated by 15% sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the gel was stained with Sypro-Ruby solution. Each lane was cut into 20 contiguous slices at the same position in the gel. All gel slices were incubated in a solution of 100 mM NH_4HCO_3 and 30% acetonitrile at 40°C for 30 min, and the supernatant was discarded; this process was repeated twice. All gel slices were placed in 50 mM Tris-HCl, pH 9.0, and then subjected to in-gel digestion with lysylendopeptidase (substrate:enzyme ratio, 50:1) at 37°C, overnight. The protein digest was applied to a reverse-phase resin (InertSep RP-1; GL-Science, Tokyo, Japan) for desalting, and the effluent was dried under vacuum.

Post ^{18}O Labeling

The digested peptides, derived from bile samples harvested on POD1, POD4, and POD14, were re-dissolved in buffer (50 mM Tris-HCl, pH 9.0), prepared with $H_2{}^{16}O$ and $H_2{}^{18}O$, respectively, and incubated overnight with lysylendopeptidase (substrate:enzyme ratio, 200:1) at 37°C. Then, the samples were boiled at 100°C for 10 min for inactivation of the enzyme. Equal amounts of the ^{18}O-labeled (POD4) and non-labeled peptide pools (POD1 or POD14) were mixed and desalted with InertSep RP-1. The mixtures of POD1+POD4 and POD4+POD14 were subjected to strong cation-exchange(SCX)-high performance liquid chromatography(HPLC).

SCX-HPLC, Nano-Flow RP-HPLC, and Protein Identification by MALDI-MS/MS

The peptides were applied to a column (TSK-Gel SP-2SW I.D. 1.0 × 150 mm) in the LC system (Agilent 1100series, Agilent Technologies), and eluted into 20 fractions by using a linear gradient (solution A, 10 mM phosphate, pH 3.2, 10% CH_3CN, *versus* solution B, 1 M NaCl in solution A). Each of the 20 fractions was further separated using a C_{18}Pepmap column (0.075 × 150 mm, Dionex, Sunnyvale, CA) in an Ultimate nano-LC system (Dionex). A linear gradient using solvent A (0.1% trifluoroacetic acid in water) and solvent B (0.1% trifluoroacetic acid in acetonitrile) were used for the separation; the peptides were eluted by increasing solvent B from 5% to 25% over a period of 55 min, and then from 25% to 50% over a period of 20 min, at a flow rate of 200 nL/min. The effluents were monitored at 214 and 280 nm, and directly blotted onto a flat surface of a stainless steel plate [a matrix-assisted laser desorption ionization (MALDI) sample plate]. Thereafter, the matrix solution (5 mg/mL of α-CHCA) was blotted manually onto each sample spot, and then air-dried.

The overall protein identification was carried out by the MALDI-TOF/TOF (4700 proteomics analyzer, Applied Biosystems, Framingham, MA) followed by database search using Mascot ver. 2.0 (Matrix Science, Manchester, UK). Ions were generated by irradiating the sample area with a 200 Hz Nd:YAG laser operated at 355 nm. Calibration was performed using $[M + H]^+$ ions of a mixture of angiotensin I (m/z 1296.6), dynorphin (m/z 1604.0), ACTH (1-24) (m/z 2932.6), and β-endorphin (m/z 3463.8).

Western Blot Analysis

Each bile sample was diluted by 50 mM Tris-HCl (pH 7.8) and 20 μg of bile protein was electrophoresed onto 10% SDS-polyacrylamide gel and transferred electrophoretically to Immobilon PVDF membrane (Millipore). After the addition of a blocking solution (5% nonfat dry milk, 0.1% Tween-20, and PBS), the membrane was incubated overnight with the recommended dilution of gout anti- CD13 (APN) polyclonal antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA). The primary antibodies were washed in a solution of 0.05% Tween-20 and PBS and then incubated with horseradish peroxidase-conjugated secondary antibody. Proteins were visualized using enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Piscataway, NJ) followed by exposure to X-ray film.

Evaluation of APN in Bile Samples After Living Donor Liver Transplantation

Patients

A total of 53 patients underwent living donor liver transplantation between June 2004 and January 2009 at our hospital. The inclusion criteria for the study were living donor liver transplant recipients with liver dysfunction within 3 months after liver transplantation. The latter was confirmed by liver biopsy with the diagnosis of either ACR or liver dysfunction other than ACR (nonACR). Donor bile samples were also obtained from nine live liver donors.

The immunosuppressants used after liver transplantation included tacrolimus or cyclosporine A, with or without mycofenolate mofetil (MMF) and corticosteroids. Following the histopathologic diagnosis of ACR, steroid recycle therapy was started with 1 g of methylprednisolone, followed immediately by steroid tapering to 20 mg/d. After the steroid recycle therapy, a second liver biopsy was obtained to confirm the allograft status and establish the effect of treatment on ACR.

Allograft dysfunction was defined as serum total bilirubin greater than 2.0 mg/dL and/or increased levels of aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT) above the normal ranges. The diagnosis of ACR was based on histopathological examination conducted by two independent expert transplant pathologists. We classified recipients with liver dysfunction into two groups, liver dysfunction with ACR (ACR group) and liver dysfunction without ACR (LD group).

Bile Sample Collection

Bile samples were collected from living donors from the common bile duct *via* 3 Fr tube during surgery. Bile samples were collected from the biliary external drainage tube inserted into the bile duct during liver transplantation. Bile samples from the liver transplant recipients were prospectively collected from the biliary external drainage tube inserted into the bile duct during liver transplantation at POD1 to POD7 daily, POD10, POD14, POD21, POD28, and POD90 (when the external biliary drainage tube was removed). We also collected other bile samples during the course of the liver biopsy procedure. Bile samples were handled under sterile conditions, divided into several corning tubes, and stored at −80°C until analysis.

Enzyme Activity Assay of Alanine Aminopeptidase N (APN)

We used alanine-*p*-nitroanilide hydrochloride (Calbiochem) as a substrate of APN, as described by Jung and Scholz [18]. APN activity was measured at 405 nm at 37°C by monitoring the increase of absorbance of *p*-nitroanilide liberated by APN during 30 min. The amount of APN that converted 1 μM of the substrate into *p*-nitroanilide at 37°C for 30 min was taken as a unit of enzyme activity. Each bile sample was diluted to 1:300 by 50 mM Tris-HCl (pH 7.8). Then each sample started the reaction by adding the substrate 2 mmol/L alanine-*p*-nitroanilide hydrochloride. A standard curve was obtained between 0-20 mU APN (porcine kidney) using alanine-*p*-nitroanilide. Each diluted bile sample (100 μL) and 30 μL of the substrate solution were applied into each well, and the diluted bile sample (100 μL) and 50 mM Tris-HCl (pH 7.8) buffer (30 μL) were applied in each of the other wells. After incubation at 37°C for 30 min, the optical density (OD) was measured at 405 nm by a UV/VIS spectrophotometer (Ultrospec 1100pro; GE Healthcare Biosciences, Uppsala, Sweden). Each bile sample was quantified using 'Coomassie' Bio-Rad protein Assay (Bio-Rad, Hercules, CA). The final APN activity (mU) was expressed relative to the amount of protein (mg) in the bile sample.

Immunostaining of Alanine Aminopeptidase N (APN)

Liver biopsy specimens of the ACR group ($n = 5$) and LD group ($n = 4$) were evaluated by immunostaining. In each case, we evaluated the expression of APN in graft liver biopsy specimens at three time points, (1) post-reperfusion during liver transplantation, (2) at the time of liver dysfunction, and (3) stable allograft function after treatment of allograft dysfunction.

Each liver biopsy specimen was fixed in formalin, embedded in paraffin, and cut into serial sections of 4 μm thickness. These were deparaffinized in xylene and rehydrated through a graded series of ethanol. Immunohistochemical staining was performed using a Vectastain ABC peroxidase kit (Vector Laboratories, Burlingame, CA). The sections were treated for antigen retrieval in 0.01 M sodium citrate buffer (pH 6.0) for 40 min at 95°C, followed by incubation in methanol containing 0.3% hydrogen peroxidase at room temperature for 20 min to block endogenous peroxidase. After blocking endogenous biotin, the sections were incubated with normal protein-block serum solution at room temperature for 20 min in wet condition, to block nonspecific staining. Then they were incubated overnight at 4°C with the primary antibody, anti-CD13 (3D8, mouse monoclonal IgG, diluted 1:100, Santa Cruz Biotechnology). After washing three times for 5 min in PBS, the sections were incubated with a biotin-conjugated secondary antibody (horse anti-mouse for CD13) at room temperature for 20 min and finally incubated with peroxidase-conjugated streptavidin at room temperature for 20 min. The peroxidase reaction was then developed with 3,3'-diaminobenzidine tetrachloride (Wako Pure Chemical Industries, Osaka, Japan). Finally, the sections were counterstained with Mayer's hematoxylin. For negative controls, sections were treated similarly except they were incubated with non-immunized rabbit IgG or Tris-buffered saline (TBS) instead of the primary antibody. Immunohistochemical staining was assessed by two investigators independently, without the knowledge of the corresponding clinicopathologic data.

Image Analysis for APN Immunostaining in the Liver

After immunostaining for APN in graft liver biopsy specimens, we evaluated the expression of APN using an image analysis software (Win roof ver. 5.6; Mitani Corporations, Fukui, Japan). Images were captured with a ×400 magnification under constant exposure control. Threshold tools were used to precisely define and measure the total hepatocyte area and the APN-stained area. The fields were edited manually to eliminate nonspecific artifacts. The average area of three fields was analyzed in each biopsy specimen. The percentage of APN immunostaining area per total field hepatocyte area was computed.

Statistical Analysis

Values were expressed as mean ± SD. Differences between groups were examined for statistical significance using the two-tailed *t*-test. Correlations analyses were performed using Pearson's correlation coefficient. P value <0.05 was considered statistically significant. All statistical analyses were performed using StatView software, ver. J 5.0 for Windows (SAS Institute, Cary, NC).

RESULTS

Identification of APN as a Candidate Biomarker by Proteomic Analysis

Comparative proteomic analysis using ^{18}O labeling identified 105 proteins in the bile samples collected at POD1 (pre-ACR period) and POD4 (peri-ACR period), and 115 proteins in those collected at POD4 (peri-ACR period) and POD14 (treated ACR period). Among these, 78 proteins were identified in both protein pools (Table 1).

Among the proteins identified in the bile samples collected at the three time periods, we compared their relative ratio at POD4/POD1 and POD4/POD14. The amount of a candidate protein marker for ACR should be higher at POD4 than POD1 and POD14. Alanine aminopeptidase N (APN) was one of the proteins that was significantly increased at POD4 and its level returned to baseline at POD14, which was confirmed by western blot analysis (Fig. 1A). We focused on this protein and evaluated its potential significance as a biomarker for ACR after liver transplantation.

APN Enzyme Activity Correlates with APN Concentration in Bile

The APN enzyme activity correlated with bile APN protein concentration, as determined by Western blot analysis (Fig. 1B, C). Based on this finding, we

TABLE 1

List of Proteins Detected in Bile Samples Obtained Based on Quantitative Protein Analysis Using ^{18}O labeling

Protein ID	Protein name	Protein ID	Protein name
1	α-1-acid glycoprotein1	41	Fibrinogen γ chain
2	α-1-antitrypsin	42	γ-glutamyltranspeptidase 1
3	α-2-macroglobulin	43	Probable G-protein coupled receptor 126
4	α-2-antiplasmin	44	Glypican-6
5	α-1-antichymotrypsin	45	Hemoglobin α subunit
6	Bile salt export pump	46	Hemoglobin β subunit
7	Angiotensin-converting enzyme 2	47	Hemoglobin delta subunit
8	Actin, cytoplasmic 1	48	Hemopexin
9	Alcohol dehydrogenase 4	49	Haptoglobin
10	Afamin	50	Haptoglobin-related protein
11	Serum albumin	51	Plasma protease C1 inhibitor
12	AMBP protein	52	Ig α-1chainCregion
13	Aminopeptidase N	53	Ig γ-1chain C region
14	Antithrombin-III	54	Ig γ-2chain C region
15	Apolipoprotein A-l	55	Ig γ-3chain C region
16	Apolipoprotein A-II	56	Ig γ-4chain C region
17	Apolipoprotein A-lV	57	Immunoglobulin J chain
18	Apolipoprotein B-100	58	Integrin β-1
19	Apolipoprotein D	59	Junctional adhesion molecule A
20	β-2-glycoproteinI	60	Ig kappa chain C region
21	β-2-microglobulin	61	Kininogen-1
22	Cathepsin Z	62	Ig λ chain C regions
23	Carboxypeptidase M	63	Lipopolysaccharide-binding protein
24	Monocyte differentiation antigen CD14	64	Ig μ chain C region
25	Ceruloplasmin	65	Neprilysin
26	Complement factor B	66	Protocadherin LKC
27	Complement C3	67	Polymeric-immunoglobulin receptor
28	Complement C4-A	68	Plasminogen
29	Complement component C9	69	Serum amyloid P-component
30	Cofilin-1	70	Tyrosine-protein phosphatase non-receptor type substrate 1
31	C-reactive protein	71	Transmembrane 4 L6 family member
32	Cysteine-rich secretory protein 3	72	Prothrombin
33	Cystatin C	73	Serotransfemn
34	EphrinA1	74	Trypsin-1
35	Ezrin	75	Pantetheinase
36	α-2-HS-glycoprotein	76	Vitamin D-binding protein
37	Fibrinogen-like protein 1	77	Vitronectin
38	Complement factor H-related protein 1	78	Zinc-α-2-glycoprotein
39	Fibrinogen α chain		
40	Fibrinogen β chain		

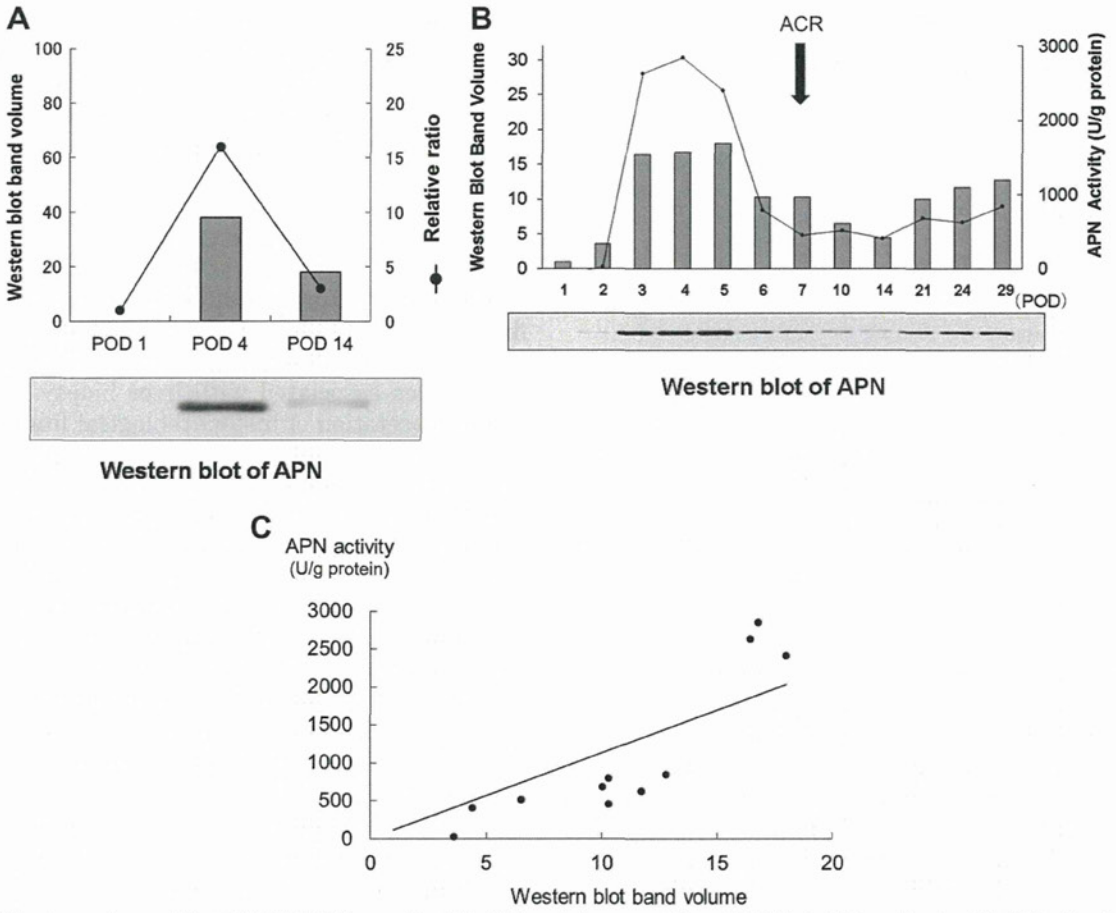

FIG. 1. Alanine aminopeptidase (APN) in bile samples. (A) APN protein expression at POD1, 4, 14 in patients with ACR. Top: Ratio of protein expression (right ordinate, ratio) based on POD1. The plot shows the relative amount of APN in bile measured by MALDI MS/MS analysis. The bar represents the Western blot band volume of APN analyzed by image software (left ordinate). Data are the amounts estimated by Western blotting and image analysis. Bottom: Western blot of APN. APN in bile increased at POD4, and then returned to the baseline. (B) Serial changes in APN activity in bile samples obtained from a single patient with ACR and measured by Western blot analysis. ACR was diagnosed at POD7. The band plot represents Western blot band volume analyzed by the image software (left ordinate) and the line plot represents APN enzyme activity (right ordinate, U/g protein). (C) Two-dimensional plot of APN enzyme activity and western blot band volume. Note the strong correlation between the two variables ($r = 0.883$, $P < 0.0001$).

determined the amount APN in the bile sample by measuring its enzyme activity, which is a simpler and easier for clinical application.

Bile APN Enzyme Activity Correlates with ACR After Liver Transplantation

Based on the inclusion criteria used in this study, recipients who were eligible for enrollment in this study were only 9 among 53 liver transplant recipients. Five of the nine recipients had biopsy-proven ACR, while the other four recipients did not have ACR (LD group). Based on the histologic diagnosis of liver biopsy, the nine recipients were classified as the ACR group ($n = 5$) and LD group ($n = 4$).

Table 2 summarizes the clinical characteristics of the nine live donors and nine liver transplant recipients. Liver biopsies at the time of donor surgery showed no fatty changes or any other histopathologic abnormalities in the nine graft livers. The cause of liver dysfunction in the LD group included small-for-size graft ($n = 1$), mild cholestasis after ABO incompatible liver transplantation ($n = 1$), and nonspecific hepatitis ($n = 2$). The bile APN enzyme activity in the nine donors was uniformly low (40.9 ± 20.1, range, 14.7–69.3 mU/mg protein).

Figure 2 shows the serial changes in APN enzyme activity in the study recipients. In the ACR group, APN activity was low after liver transplantation and, in three (60%) of five recipients of the ACR group, it gradually increased to above 500 mU/mg protein before the diagnosis of ACR, then returned to baseline after treatment of ACR with immunosuppressants and steroids. On the other hand, in two of the five recipients of the ACR group, the APN activity remained as low as that in the donor bile. In

TABLE 2

Clinical Characteristics of Recipients

	ACR cases ($n = 5$)	LD cases ($n = 4$)
Age (y) (range)	44 (19–59)	53 (40–61)
Gender (male/female)	3/2	1/3
Primary diagnosis		
HBV	1	
HBV+HCC	1	
HCV+HCC		1
Primary biliary cirrhosis	1	2
Fulminant hepatitis		1
Autoimmune hepatitis	1	
Biliary atresia	1	
Preoperative MELD score	20 (14–27)	28 (7–57)
Graft (right lobe/left lobe)	2/3	2/2
Operation time (min)	902 (642–1390)	739 (556–940)
Blood loss (mL)	3116 (1920–4400)	5800 (3350–9150)

For each variable, the mean (range) is shown.

HBV = hepatitis B virus; HCV = hepatitis C virus; HCC = hepatocellular carcinoma; ACR = acute cellular rejection; LD = liver dysfunction without ACR.

contrast, the bile APN activity remained low (<500 mU/mg protein) throughout the period in all recipients of the LD group ($n = 4$) (Fig. 2B).

Analysis of the time course of APN activity in bile of the ACR group showed that it increased 3 to 4 d before the ACR event (Fig. 2A). Therefore, APN activity within 3 d before ACR was compared with that of recipients who did not develop ACR. Available for analysis were 10 bile samples within 3 d before the ACR event and 49 bile samples outside these time periods in the ACR group ($n = 5$), while there were 47 bile samples that were not associated with ACR in the LD group ($n = 4$). APN enzyme activity in bile samples of LDLT recipients of the ACR group within 3 d before the biopsy-confirmed ACR ($n = 10$) was significantly higher (584 ± 434 U/g protein) than in bile samples of recipients free of ACR ($n = 96$, 301 ± 271 U/g protein, $P = 0.004$, Fig. 2C).

Localization of APN Along Bile Canaliculi and Its Overexpression in ACR

Immunohistochemical staining for APN in liver biopsy specimens from the donor showed APN staining in the bile canaliculi and small bile ducts. The APN expression levels in serial liver biopsy specimens from all patients of the ACR group were almost identical to that of the donor at the time of post-reperfusion, increased in the bile canaliculi and small bile ducts at ACR, then returned to the baseline after treatment of ACR and stable allograft function (Fig. 3). The lymphocyte aggregates around the portal triads did not stain for APN in the ACR group. On the other hand, the APN expression level in the LD group remained low at baseline level throughout the study period. Quantification of the immunohistochemical signal showed a significantly stronger APN staining in the ACR group at the time of ACR than all other time periods and the staining intensity in the LD group (Fig. 4).

DISCUSSION

Allograft dysfunction after liver transplantation influences post-transplant prognosis, but accurate diagnosis of this state is limited due to the risk of morbidities associated with liver biopsy and possible misinterpretation of histopathological findings. Recurrent hepatitis and ACR are often present simultaneously in clinical settings in recipients with hepatitis. Thus, it would be ideal to have an accurate, reproducible, and noninvasive method to diagnose the cause of allograft dysfunction after liver transplantation. We approached this issue previously using transcriptome analysis of liver biopsy and peripheral blood using both an animal model [19] and human samples [20, 21] and identified candidate markers associated with ACR. These studies should be continued for further validation of these candidate genes in liver and peripheral blood.

In kidney transplantation, urinary enzymes and low molecular weight proteins were reported to be useful for the diagnosis of acute rejection after the early post-transplantation phase [4–6]. The analogy of "urine" excreted from the transplanted kidney is "bile" from the allograft liver. In this study, we analyzed human bile samples using proteomic analysis to identify bile proteins that can be used as biomarkers for ACR and differentiate this condition from other causes of allograft dysfunction.

Duct-to-duct anastomosis is currently widely performed as a standard method of bile duct reconstruction in liver transplantation. Biliary drainage is quite important in order to know the amount, color, and other properties of bile output from the liver allograft as well as reducing bile duct complication [22–24]. Furthermore, it is also customary in certain cases to estimate graft function by analyzing bile bilirubin [25], bile acid [26], and other biomarkers. More importantly, bile duct reconstruction is also reported to be one of the key determinants of low morbidity in living donor liver transplantation [27, 28]. Bile is basically human waste and usually dumped without any analysis. However, it could provide a wealth of information, when another point of view is taken. The importance of biliary interleukin-6 (IL-6) in association with ACR after liver transplantation in rats [16] and deceased liver transplantation in human [17], as well as biliary ICAM-1 [8, 9] has already been reported. With this background,

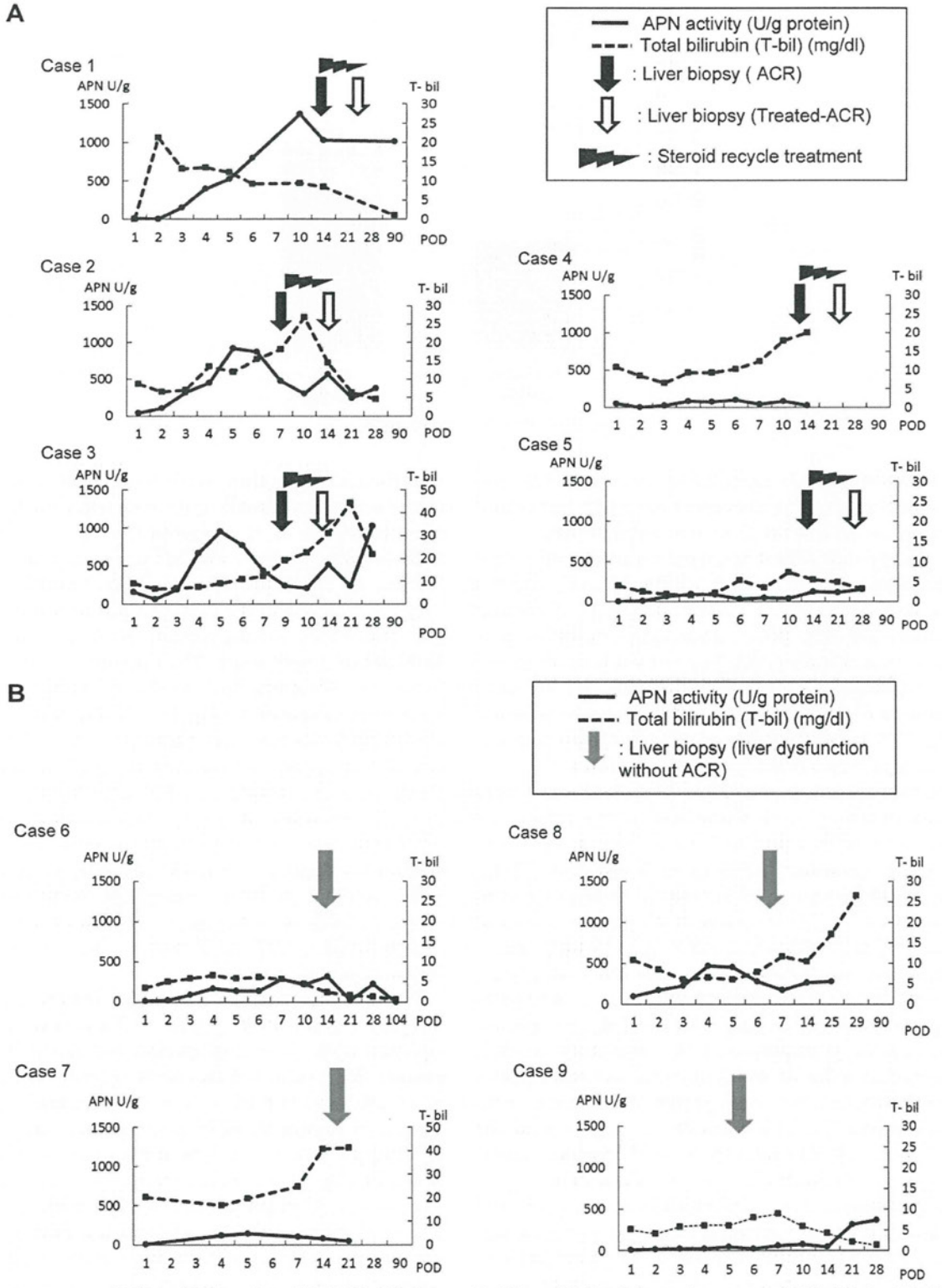

FIG. 2. Time course of biliary APN activity. (A), (B) Serial changes in biliary APN activity and serum total bilirubin level in five patients with ACR (A) and four patients with LD (B); (C) APN enzyme activity in bile samples of LDLT recipients of the ACR group within 3 d before biopsy-confirmed ACR ($n = 10$) was significantly higher than that in bile samples of patients free of ACR ($n = 96$) ($P = 0.004$). Bars indicate standard error of the mean (SEM).

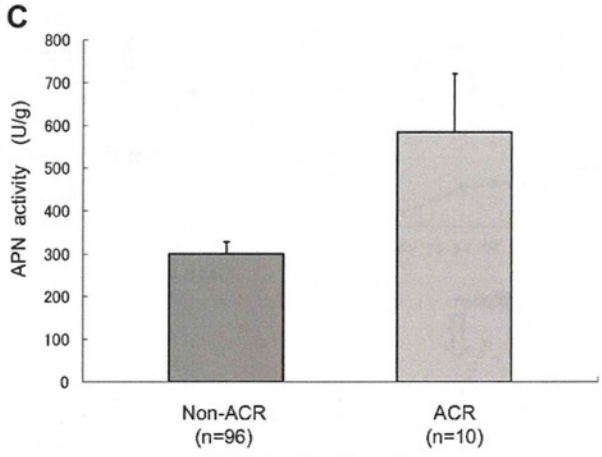

FIG. 2. (continued).

we decided to assay bile samples to determine the usefulness of bile analysis in providing clinically important information on ACR after liver transplantation.

Proteomic analysis has been used recently in the field of human clinical science such as the identification of markers for the diagnosis and/or prognosis of various malignancies [29–32]. To our knowledge, however, proteomic analysis of human bile has not yet been reported except in a limited number of studies [33, 34]. We used the technique of relative quantitative protein analysis using the ^{18}O labeling method, which allows comprehensive comparative analysis of bile proteins.

Taking this approach, we found 78 proteins that were commonly identified in all three bile samples from one recipient with ACR (obtained at POD1, 4, and 14). Among these proteins, APN (also known as CD13) was one protein whose level increased in the bile sample collected at POD4 compared with that collected at POD1 and POD14 (Fig. 1A). APN is a 150-kD transmembrane protein localized in the bile canaliculi, epithelia of the bile ducts, apical membranes of hepatocytes, mucosal cells of the gall bladder [35], peripheral blood monocytes, granulocytes [36], immature myeloid cells, epithelial cells of the intestine, synaptic membranes in the central nervous system, fibroblasts, endothelial cells, and the brush border membranes of the proximal renal tubular cells [6–8]. APN plays a pathologic role in cholelithiasis [35], biliary atresia in infants [37], and cytomegalovirus infection [38]. It was also considered as a marker of hepatocellular carcinoma, to distinguish it from metastatic tumors of the liver [39], and as a marker of cancer stem cells in hepatocellular carcinoma [40]. APN staining in the canaliculi is reported to be a highly specific marker of hepatocyte differentiation [41]. On the other hand, Jung *et al.* [4] reported that urinary APN is a significant protein associated with ACR in kidney transplantation. Surprisingly, APN was also found to correlate with liver ACR. That both bile from the liver allograft and urine from the kidney graft were linked to ACR, suggests that the mechanisms of ACR in both the liver and kidney transplants probably involve APN-related immunological and/or inflammatory processes. Further studies are necessary to establish the exact mechanism(s) of ACR, including the APN-related pathways. The amount of APN in bile detected by Western blot analysis correlated with the APN enzyme activity (Fig 1B, C). Therefore, we evaluated APN by its enzymatic activity rather than by Western blot analysis, considering its clinical applicability. Uniformly low levels of APN activity were noted in the bile samples of all donors, suggesting minimal APN activity in bile at baseline condition in the absence of liver dysfunction or ACR. Interestingly, changes in APN activity in liver transplant recipients did not correlate with other biochemical parameters such as serum bilirubin, AST, ALT, and γ-glutamyl aminotransferase (data not shown).

We classified the nine recipients into two groups; five recipients with ACR episode (ACR group) and four recipients with liver dysfunction but without ACR (LD group). We evaluated the APN activity in the patients in relation to the clinical course in both groups. As shown in Figure 2, APN activity increased above 500 mU/mg protein in a couple of days before the diagnosis of ACR in three of the five recipients of the ACR group. In contrast, APN level remained low similar to the baseline in all recipients of the LD group. Furthermore, the time course studies of APN level showed that APN increased 3–4 d before confirming the ACR by biopsy examination. Furthermore, the mean APN activity in bile samples of the LDLT recipients obtained within 3 d before ACR ($n = 10$) was significantly higher than that without ACR event ($n = 96$) ($P = 0.004$) (Fig. 2C). These results suggest that a high level of APN in the

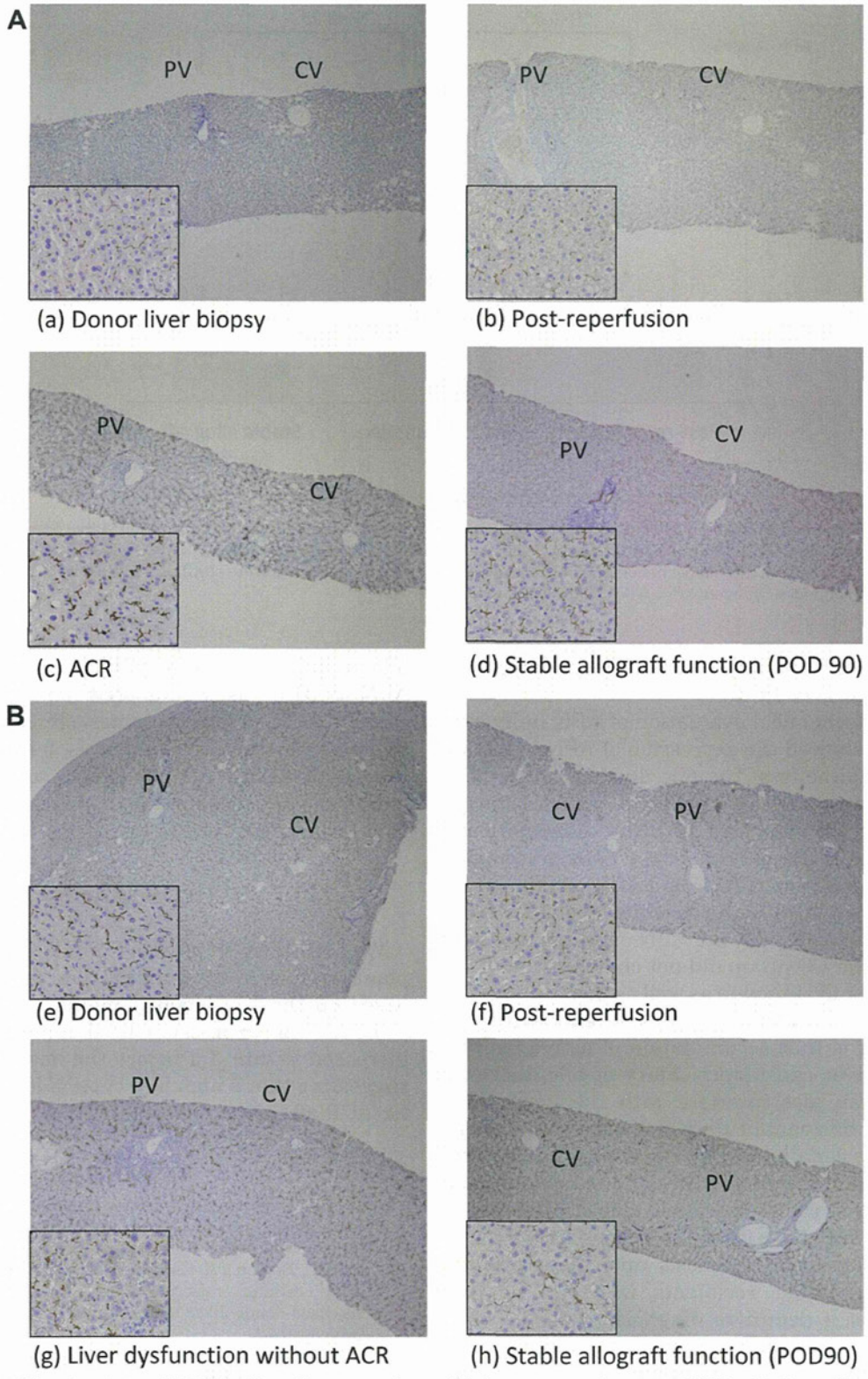

FIG. 3. Immunohistochemistry of APN in liver biopsy specimens.(A) A representative case of ACR: (a) Donor liver biopsy, (b) post-reperfusion, (c) ACR, (d) Stable allograft function (POD 90). Note the high expression of APN in patients with ACR. Note also the similarity in APN expression pattern between the donor and recipient at stable allograft function (×100, inset ×400). PV = portal vein, CV = central vein. (B) A representative case of liver dysfunction (LD): (e) Donor liver biopsy, (f) post-reperfusion, (g) liver dysfunction without ACR, (h) Stable allograft function (POD 90). Note the low APN expression compared with the patient with ACR (×400).

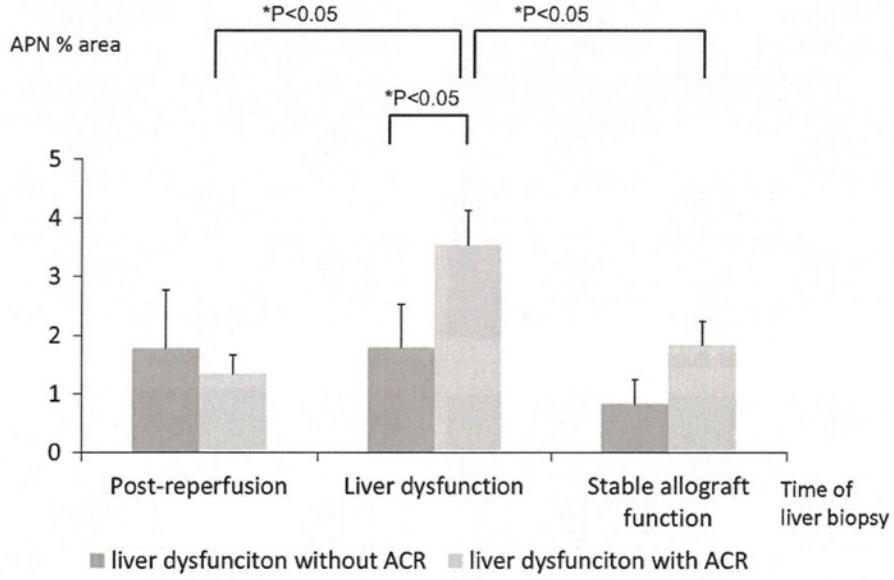

FIG. 4. Results of image analysis of APN in graft liver specimens. Data are mean ± SD of APN expression in five patients with ACR and four with LD. There was a significant difference in APN expression between the ACR and LD groups ($*P < 0.05$). The APN expression levels in liver biopsy specimens obtained 1 h after reperfusion and in the protocol liver biopsy specimens were similar, and they were significantly lower than those of the ACR group at ACR event ($P < 0.05$). Data are mean ± SD.

bile is a potentially suitable biomarker for the prediction and diagnosis of ACR.

Immunohistochemical evaluation of APN in liver biopsy samples showed the expression of APN in bile canaliculi and epithelia of the bile ducts. Furthermore, APN expression increased after liver transplantation, and such increase coincided with the confirmation of ACR by biopsy in all patients of the ACR group. Confirming the association of APN and ACR was the return of the expression level to the baseline level after treatment of ACR. In contrast, the APN expression level in recipients of the LD group did not change at all in patients with liver dysfunction as well as those with stable allograft function (Fig. 3). One possible explanation for these findings is that accumulation of active lymphocytes in the liver can induce injury of bile duct cells and, hence, can also interfere with the flow of bile stream in the bile canaliculi, which causes further injury of the bile canaliculi. This could then induce APN overexpression in the membrane of bile canaliculi cells.

The number of the recipients in this study is small, because we limited the study to recipients with confirmed histopathologic diagnosis upon liver dysfunction, excluding other recipients who had no liver biopsy, so that a definitive diagnosis could be made for liver dysfunction; ACR *versus* nonACR. Our study showed that APN level increased in the bile in association with ACR episode after liver transplantation. Furthermore, serial monitoring of APN level in the bile samples from these recipients ($n = 106$) also demonstrated increases in APN expression levels in the bile within 3 d before ACR, suggesting that biliary APN could be used as a predictor of latent and subclinical ACR, which becomes clinically apparent in the next few days. Thus, it is feasible to conclude that APN (CD13) in bile seems to be a useful and noninvasively measurable biomarker for ACR after liver transplantation.

CONCLUSION

We identified 78 proteins in bile from a liver transplant recipient by quantitative proteomic analysis based on the ^{18}O labeling method. Among these bile proteins, the expression levels of APN in bile were increased within 3 d before the development of ACR, suggesting that a high biliary APN level is a biomarker for ACR after liver transplantation.

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