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ORIGINAL ARTICLE

Salvage Cystic Duct Anastomosis Using a Magnetic Compression Technique for Incomplete Bile Duct Reconstruction in Living Donor Liver Transplantation

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In living donor liver transplantation (LDLT), bile duct reconstruction is often technically demanding due to the frequently anomalous anatomy of the bile duct, as well as the high incidence of biliary complications. A bile duct branch may also be accidentally left without anastomosis at the time of LDLT and found to be obstructed after surgery. Surgical revision for such cases is sometimes not feasible because of the invasiveness of the procedure. We report a case in which a bile duct branch was intentionally left without anastomosis and was later successfully anastomosed to the cystic duct stump using a magnetic compression anastomosis (MCA) technique. A combination of the MCA technique and cystic duct anastomosis is life-saving in certain situations and should be considered as the treatment of choice. *Liver Transpl 16:33-37, 2010.*

Reconstruction of the bile duct in living donor liver transplantation (LDLT) is often complicated because of anatomical variations, especially in right lobe liver transplantation.[1] In addition, the high incidence of biliary complications affects the postoperative course.[2]

Bile duct strictures are the most commonly encountered biliary complications after liver transplantation.[3,4] Treatment for bile duct strictures includes nonsurgical methods such as endoscopic or percutaneous bile duct balloon dilatation and stent placement, surgical revision of the bile duct anastomosis, and retransplantation in the case of allograft failure. However, endoscopic or percutaneous treatments often fail with relapse of the stricture, whereas surgical revision and retransplantation are more invasive and are associated with much morbidity.

It is possible for occlusion of the bile duct anastomosis to result as an advanced condition of a stricture[5] or to result from a bile duct branch accidentally or intentionally left without anastomosis in partial liver transplantation. In these cases, an endoscopic or percutaneous approach would not be effective, and surgical revision would be necessary for reconstruction, although it is invasive and is associated with much morbidity. The magnetic compression anastomotic technique is a simple, noninvasive, and rather effective method for recanalizing a bile duct obstruction, and we have previously reported its application in liver transplant recipients.[6]

Duct-to-duct anastomosis has now become the standard biliary reconstruction method because of its advantages over hepaticojejunostomy in both deceased

Abbreviations: B#, branch of segment #; CBD, common bile duct; ESLD, end-stage liver disease; LDLT, living donor liver transplantation; MCA, magnetic compression anastomosis; PTCD, percutaneous transhepatic cholangiodrainage.

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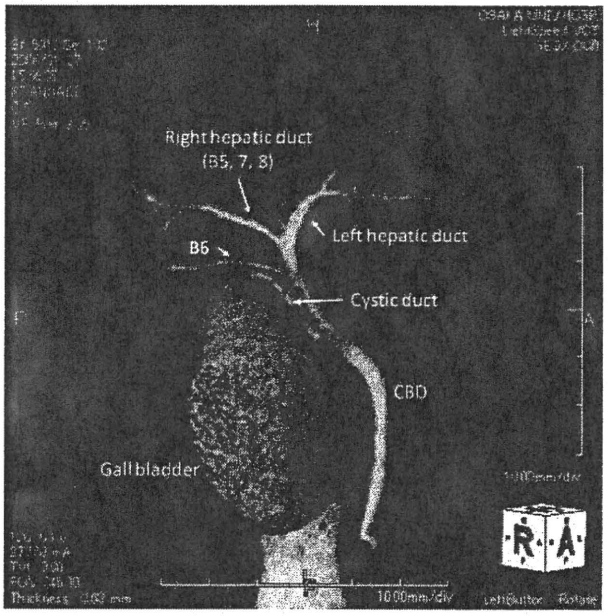

Figure 1. Three-dimensional reconstruction view of the drip infusion cholangiography computed tomography scan. The donor B6 bile duct rose from the common hepatic duct separately from the right hepatic duct (B5, B7, and B8).

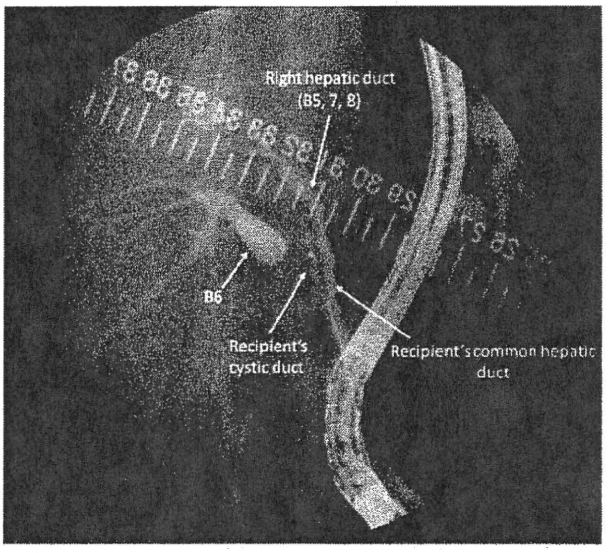

Figure 2. Cholangiography in the recipient before the magnetic compression anastomosis. Simultaneous endoscopic retrograde cholangiography combined with B6 tube cholangiography showed dilatation of the B6 bile duct and the stump of the cystic duct close to it. Duct-to-duct anastomosis of the right hepatic duct did not result in a stricture.

donor liver transplantation and LDLT.[7,8] However, duct-to-duct reconstruction to the recipient cystic duct in LDLT is a relatively new and unique concept; it was described first by Suh et al.[9] and then by Asonuma et al.[10] and has been shown to be safe and useful.

We report herein a case in which a bile duct branch was intentionally left without anastomosis and was later successfully anastomosed to the cystic duct stump with a magnetic compression technique.[6,11] The successful outcome in the present case is believed to have resulted from a combination of the magnetic compression anastomosis (MCA) technique and cystic duct anastomosis.

CASE REPORT

A 53-year-old male patient with ESLD secondary to hepatitis B virus was referred to our hospital. A preoperative evaluation revealed a small hepatocellular carcinoma (single, 10 mm) and no abnormalities of the cardiopulmonary systems. Renal function was impaired, with the serum creatinine level at 1.5 mg/dL and creatinine clearance at only 24.9 mL/minute.

His brother volunteered to donate his liver, and after extensive donor evaluations, including computed tomography volumetry of the liver, it was determined that only the right lobe could be donated, even though there was a small bile duct branch of segment 6 (B6) arising separately from the right hepatic duct (Fig. 1).

The transplant surgery was quite complicated because of the presence of portal hypertension with massive ascites, extensive coagulopathy, and severe fibrosis around the cirrhotic liver. Hepatectomy was performed as usual by the piggyback technique, and the right lobe graft was anastomosed without difficulty. Bile duct reconstruction between the donor right bile duct (B5, B7, and B8) and recipient common hepatic duct was performed with 6-0 absorbable monofilament interrupted sutures with an external tube across the anastomosis. At that time, we found that the jejunum was extensively edematous. Furthermore, the B6 bile duct was so small that we decided not to proceed with anastomosis of this bile duct, and instead we placed a 5-Fr external biliary tube. We initially planned a second-look operation of the hepaticojejunal anastomosis when the patient stabilized postoperatively.

A septic event with methicillin-resistant *Staphylococcus aureus* pneumonia necessitated reintubation and tracheotomy during the first week after LDLT. The postoperative initial graft function gradually recovered during the first 6 months without any surgical morbidity or acute cellular rejection until he suddenly developed acute heart failure of an undetermined cause with an ejection fraction of 20% at 6 months after LDLT. Subsequently, his renal function also deteriorated, and this necessitated continuous venovenous hemodialysis for 16 days. The heart failure gradually improved, with the ejection fraction recovering to 45% at post-operative month 12, whereas the renal function never recovered, with the serum creatinine level remaining around 3 mg/dL despite maintenance of normal urine output.

Surgical relaparotomy under general anesthesia for anastomosis of the B6 bile duct was considered to be contraindicated at this time point because of the multiple-organ failure. One of the solutions under these circumstances is to clamp the B6 drainage tube or inject ethanol to destroy the bile duct epithelium; how-

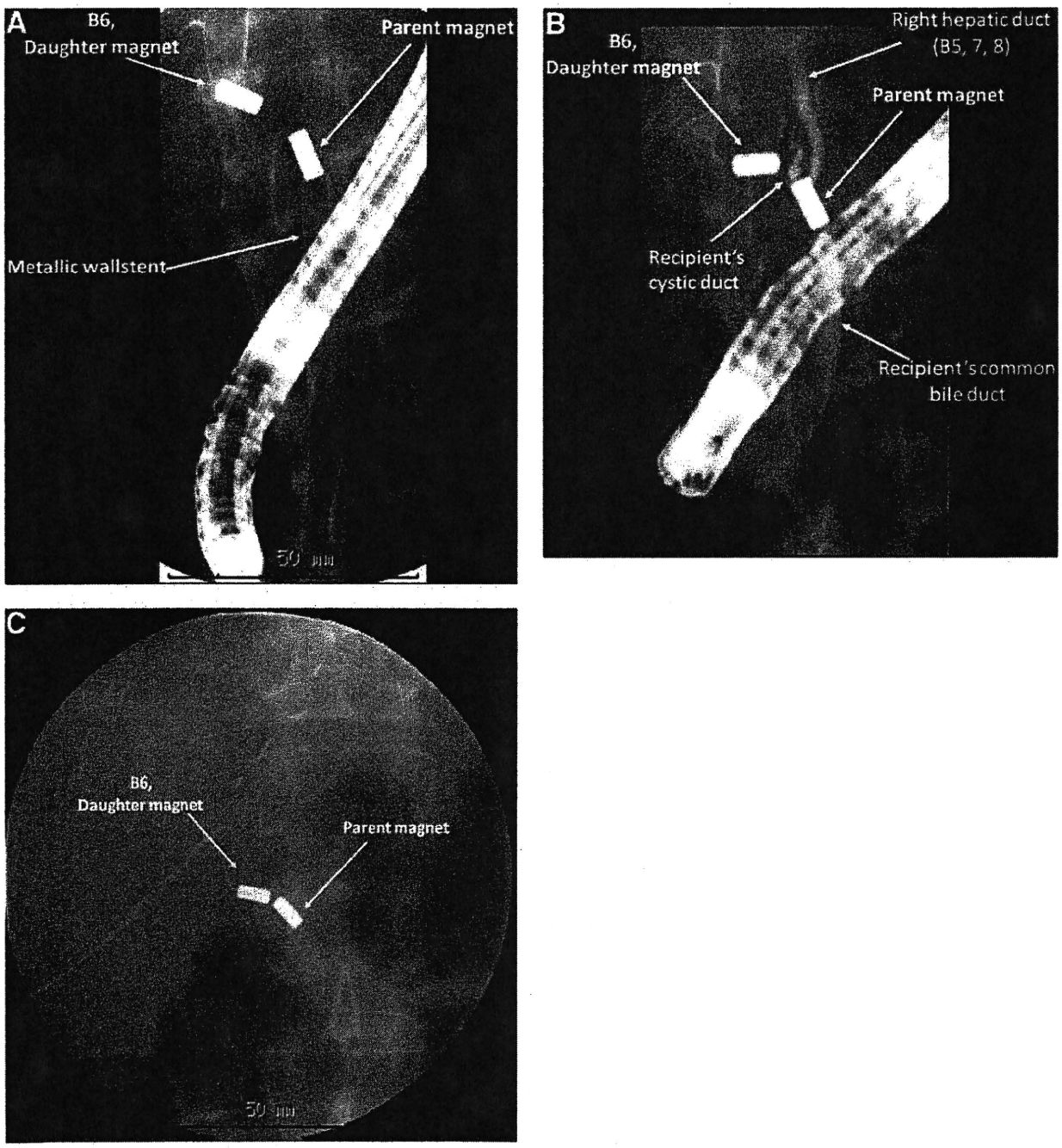

Figure 3. Magnetic compression anastomosis between the B6 bile duct and recipient's cystic duct. (A) The parent magnet was brought to the stump of the cystic duct via the common bile duct with the metallic Wallstent technique described previously. The daughter magnet was brought to the B6 bile duct via the percutaneous transhepatic cholangiodrainage route. (B) The patency of the common bile duct was confirmed by the injection of 60% urografin. (C) The parent and daughter magnets came close together by the end of this procedure.

ever, these were not recommended in this case because the area of B6 was relatively large and the bile drainage through the B6 bile duct drain was about 200 mL. Magnetic resonance imaging showed sufficient dilatation of the B6 bile duct for a percutaneous transhepatic cholangiodrainage (PTCD) tube to be inserted and placed (Fig. 2). Fortunately, the cystic duct stump was relatively close to the stump of the B6 bile duct.

The PTCD tube was inserted and sized up to 16 Fr, and MCA was performed successfully at POM 14 with a metallic Wallstent technique described previously.[6] A daughter magnet was first delivered to the end of B6.

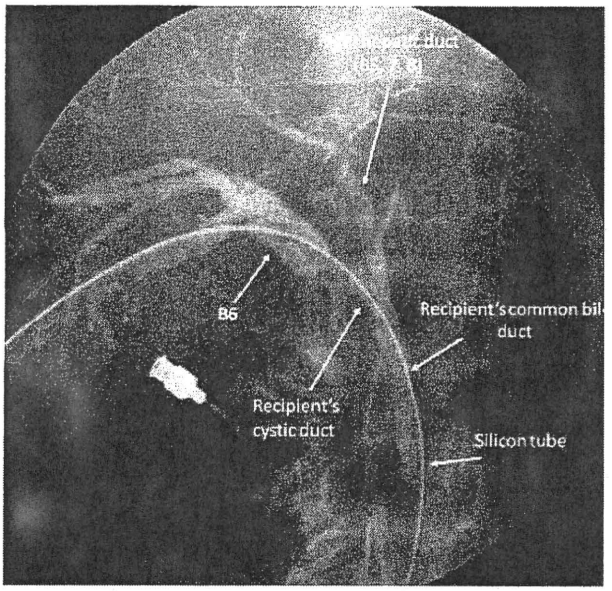

Figure 4. Cholangiography after the magnetic compression anastomosis. Both magnets were removed via the percutaneous transhepatic cholangiodrainage route. A 14-Fr silicon tube was inserted and placed.

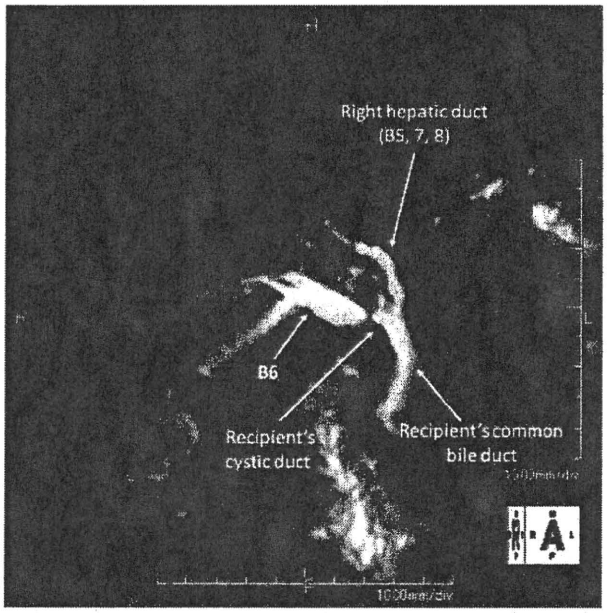

Figure 5. Magnetic resonance cholangiopancreatography image 6 months after the removal of the percutaneous transhepatic cholangiodrainage tube. There was narrowing of the magnetic compression anastomosis segment. The original anastomosis of the right hepatic duct was secure.

After endoscopic sphincterotomy, a metallic Wallstent [Wallstent RX biliary metal stent (8 mm in diameter and 60 mm long), Boston Scientific Japan K.K., Tokyo, Japan] was inserted into the cystic duct stump via the papilla of Vater and common bile duct. Then, the parent magnet was delivered safely into the cystic duct stump without interference from the common bile duct (Fig. 3A); this was confirmed by fluoroscopy (Fig. 3B). The Wallstent was removed immediately after the placement of the magnets, and the 2 magnets came close together (Fig. 3C) by the end of the procedure. There was no deterioration of the liver function after the placement of the magnets. On day 63 after the MCA procedure, both the parent and daughter magnets were removed, and a safety PTCD tube was placed across the B6 bile duct and cystic duct (Fig. 4). At 12 months after the MCA, the B6 biliary tube was removed after cholangiographic confirmation of the absence of bile leakage from the biliary system. The PTCD tube was finally removed at 18 months after the MCA procedure. Magnetic resonance imaging performed 6 months after the PTCD removal showed no bile leakage and homogeneous parenchyma in the entire graft, including segment 6, although the MCA region could not be visualized well by magnetic resonance cholangiography (Fig. 5). A hepatobiliary iminodiacetic acid scan showed normal bile drainage from B6 to the common bile duct, and this suggested normal functioning of the MCA site despite the lack of visualization of the tube between them on magnetic resonance cholangiography. The patient is now under routine follow-up at the outpatient clinic without any complaints or evidence of biliary infection and with normal serum aminotransferase, gamma glutamyl transferase, and alkaline phosphatase levels 10 months after the PTCD removal.

DISCUSSION

The clinical course of this case was uncommon because of the anomaly of the B6 bile duct and the unexpected deterioration of the heart and renal function after LDLT in the patient; however, the problem of the bile duct anastomosis of B6 was successfully resolved by a nonsurgical procedure using the MCA method. This method could be applied as one of the alternatives for performing multiple biliary reconstructions or rescuing a biliary branch that has been unexpectedly ligated during donor hepatectomy. Usage of the cystic duct has the advantage of avoidance of interference by bile flow from the rest of the grafted liver. Although it is sometimes difficult to deliver a magnet via the papilla of Vater for this MCA technique, we applied a metallic stent technique described previously[6] and successfully delivered the magnet without difficulty.

In conclusion, this case illustrates an alternative method for biliary reconstruction. A small bile duct branch in right lobe grafts can be anastomosed in a second-look fashion by the MCA technique, and this should be considered one of the options for bile duct reconstruction.

REFERENCES

1. Ohkubo M, Nagino M, Kamiya J, Yuasa N, Oda K, Arai T, et al. Surgical anatomy of the bile ducts at the hepatic

hilum as applied to living donor liver transplantation. Ann Surg 2004;239:82-86.
2. Moser MA, Wall WJ. Management of biliary problems after liver transplantation. Liver Transpl 2001;7(suppl 1):S46–S52.
3. Verdonk RC, Buis CI, Porte RJ, van der Jagt EJ, Limburg AJ, van den Berg AP, et al. Anastomotic biliary strictures after liver transplantation: causes and consequences. Liver Transpl 2006;12:726-735.
4. Pascher A, Neuhaus P. Bile duct complications after liver transplantation. Transpl Int 2005;18:627-642.
5. Matsuno N, Uchiyama M, Nakamura Y, Iwamoto H, Hama K, Ashizawa T, et al. A nonsuture anastomosis using magnetic compression for biliary stricture after living donor liver transplantation. Hepatogastroenterology 2009;56:47-49.
6. Akita H, Hikita H, Yamanouchi E, Marubashi S, Nagano H, Umeshita K, et al. Use of a metallic-wall stent in the magnet compression anastomosis technique for bile duct obstruction after liver transplantation. Liver Transpl 2008; 14:118-120.
7. Rouch DA, Emond JC, Thistlethwaite JR Jr, Mayes JT, Broelsch CE. Choledochocholedochostomy without a T tube or internal stent in transplantation of the liver. Surg Gynecol Obstet 1990;170:239-244.
8. Ishiko T, Egawa H, Kasahara M, Nakamura T, Oike F, Kaihara S, et al. Duct-to-duct biliary reconstruction in living donor liver transplantation using right lobe graft. Ann Surg 2002;236:235-240.
9. Suh KS, Choi SH, Yi NJ, Kwon CH, Lee KU. Biliary reconstruction using the cystic duct in right lobe living donor liver transplantation. J Am Coll Surg 2004;199: 661-664.
10. Asonuma K, Okajima H, Ueno M, Takeichi T, Zeledon Ramirez ME, Inomata Y. Feasibility of using the cystic duct for biliary reconstruction in right-lobe living donor liver transplantation. Liver Transpl 2005;11:1431-1434.
11. Takao S, Matsuo Y, Shinchi H, Nakajima S, Aikou T, Iseji T, et al. Magnetic compression anastomosis for benign obstruction of the common bile duct. Endoscopy 2001;33: 988-990.

Evaluation of a New Immunoassay for Therapeutic Drug Monitoring of Tacrolimus in Adult Liver Transplant Recipients

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Therapeutic drug monitoring is necessary when using tacrolimus (FK) due to the associated side effects. The aim of this study was to compare the chemiluminescent assay (CMIA) system with the previously established Abbott IMx Tacrolimus II microparticle enzyme immunoassay (MEIA) in liver transplant recipients and evaluate its accuracy. Between March and June 2008, all blood samples from the liver transplant recipients at the hospital were tested for FK trough level using 2 different methods, CMIA and MEIA. The post-transplant time, hematocrit, and other clinical parameters during the study period were recorded. FK trough level was analyzed in 398 samples from 57 liver transplant recipients by CMIA and MEIA. The correlation in FK level between the 2 methods was excellent ($r^2 = 0.941$). However, the FK level was underestimated in MEIA by more than 23% in samples with an FK level of less than 3.5 ng/mL and by 6.8% in those with an FK level between 3.5 and 5 ng/mL. CMIA is superior to MEIA in measuring low FK level, allowing the FK level to be maintained at less than 5 ng/mL in selected liver transplant recipients. The effects of maintaining low levels of FK should be evaluated in liver transplant recipients.

Keywords: *tacrolimus; liver transplantation; therapeutic drug monitoring; immunoassay*

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Tacrolimus (FK) is a potent immunosuppressant known as a calcineurin inhibitor and a key drug used in organ transplantation. FK is effective in preventing rejection and maintaining organ function in liver transplantation, but its side effects, such as nephrotoxicity,[1] diabetes mellitus,[2] hypertension, and infection, are life threatening in posttransplant management.[3] Therefore, therapeutic drug monitoring is necessary for transplant recipients to secure a better posttransplant course. Reduced exposure to elevated blood concentrations of tacrolimus provides adequate immunosuppression and improved renal function in kidney transplantation.[4] In liver transplant recipients, tacrolimus concentration should be minimized, especially in stable recipients after transplantation.

The IMx Tacrolimus II microparticle enzyme immunoassay (MEIA; Abbott Laboratories, Abbott Park, Illinois) is a standard system for monitoring tacrolimus blood concentrations.[5] However, the reported limit of quantification (LOQ) in blood concentration of tacrolimus using MEIA is 4.1 ng/mL,[6] which may be high and harmful in stable liver transplant recipients. A trough level of tacrolimus around 3 to 4 ng/mL seems adequate and could be reduced to even perhaps around 1 ng/mL.[6] A once-a-day tacrolimus dose would require a target level as low as around 1 ng/mL, although the MEIA system does not support such low levels.

The newly developed ARCHITECT tacrolimus assay using the automated chemiluminescent assay (CMIA; Abbott Laboratories) for quantitative measurement of tacrolimus on the ARCHITECT i2000SR platform is highly sensitive and provides precise

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measurement. It also allows the management of low-target concentrations in liver transplant recipients.

To our knowledge, there are no reports that compare MEIA and CMIA in the assessment of tacrolimus in liver transplant recipients. The present study was designed to compare these 2 methods in liver transplant recipients and determine the efficacy of the ARCHITECT tacrolimus assay in clinical settings.

PATIENTS AND METHODS

Abbott IMx Tacrolimus II Microparticle Enzyme Immunoassay

In this immunoassay, whole-blood sample is mixed with the microparticle reagent, followed by incubation, in which tacrolimus in the sample binds to the antibody binding sites on the microparticles. An aliquot of the mixture is then transferred to a glass-fiber filter, and tacrolimus-alkaline phosphatase conjugate is added to the filter. Conjugates that do not bind to the remaining antibody binding sites on the microparticles are removed by washing. Then, the substrate reagent is added to the filter and the rate of appearance of the fluorescence product is measured by front surface fluorescence measurements. The rate is inversely proportional to the amount of the analyte present in the patient sample or standard.

Automated CMIA for the Quantitative Determination of Tacrolimus on the ARCHITECT i2000SR Platform

The CMIA method is based on the enzyme immunoassay (EIA) principle. In contrast to the EIA, where the antigen-antibody complexes are detected by enzyme-labeled conjugate, the CMIA uses an acridinium-labeled conjugate as the detection system. The LOQ of blood concentration of tacrolimus using CMIA is as low as 0.8 ng/mL.[6]

Comparison of MEIA and CMIA

During the study period from March to June 2008, peripheral blood samples from 57 liver transplant recipients were analyzed by both techniques. Among 57 recipients, 44 patients underwent living donor liver transplantation, and 13 received deceased donor liver transplantation.

All recipients were taking tacrolimus (Prograf) every 12 hours, and blood samples were obtained at trough level. Each blood sample was divided into 2 portions and each used immediately for 1 assay. The immunosuppressive protocol consisted of tacrolimus and steroid with or without mycophenolate mofetil (MMF) or tacrolimus and anti-CD25 monoclonal antibody with MMF. Tacrolimus was started at a dose of 0.03 mg/kg and titrated 8 to 12 ng/mL as trough level during the first 3 weeks after transplant, then reduced to 6 to 8 ng/mL in the first 3 months and 4 to 6 ng/mL thereafter.

The ARCHITECT ratio (AR) was defined as [C(ARCHITECT) – C(IMx)]/C(ARCHITECT), where C(ARCHITECT) is FK trough concentration in the CMIA method, and C(IMx) is that in the MEIA method. Samples were also divided according to hematocrit into <25%, 25% to 45%, and over 45%.

Statistical Analysis

Results are expressed as mean ± standard deviation or median. Statistical examination of the correlations was based on the Pearson product-moment correlation. A P value less than .05 was considered statistically significant.

RESULTS

Samples from 57 liver transplant recipients (43 living donor liver transplantation and 14 deceased donor liver transplantation), including 3 patients who received liver transplantation during the study period, were analyzed (Table I). Time after liver transplantation ranged from 0 to 7.7 years (3.5 ± 2.2 years). The primary diagnoses of these 57 patients are listed in Table I. The total number of blood samples analyzed by the 2 methods was 398, including 35 samples within 3 weeks after liver transplantation, 57 samples between 3 weeks and 3 months, 93 samples between 3 months and 1 year, and 213 samples over 1 year. FK trough level was 11.9 ± 3.1 (MEIA), 11.5 ± 2.8 (CMIA) within 3 weeks; 11.3 ± 3.2 (MEIA), 10.8 ± 2.8 (CMIA) between 3 weeks and 3 months; 8.2 ± 4.5 (MEIA), 8.0 ± 4.1 (CMIA) between 3 months and 1 year; and 6.4 ± 3.3 (MEIA), 6.5 ± 2.9 (CMIA) over 1 year.

FK trough level ranged from 0.2 to 23.0 ng/mL in the MEIA method and from 1.4 to 21.9 in the CMIA method. The FK level in all samples was above the LOQ in the CMIA method (0.8 ng/mL), whereas 13.4% of all samples were below the LOQ (4.1 ng/mL) in the MEIA method. Samples with hematocrit less than 25% accounted for 4.0% (n = 16), whereas

Table I Patients' Characteristics

Number of patients	57
Sex (male/female)	34/23
Age, y, mean ± SD (range)	47.4 ± 14.7 (19-67)
Type of liver transplant, n	
Deceased donor	14
Living donor	43
Primary diagnosis	
Hepatitis C virus cirrhosis	22
Hepatitis B virus cirrhosis	8
Primary biliary cirrhosis	8
Primary sclerosing cholangitis	3
Alcohol cirrhosis	3
Wilson disease	2
Biliary atresia	3
Budd-Chiari syndrome	1
Citrullinemia	1
Cryptogenic	2
Fulminant hepatic failure	4
Months posttransplantation, mean ± SD (range)	41.8 ± 26.5 (0-92.4)
New transplantation, n (%)	3 (5.3)
Within 12 months posttransplantation, n (%)	9 (15.8)
12 to 36 months posttransplantation, n (%)	20 (35.1)
Over 36 months posttransplantation, n (%)	25 (43.9)

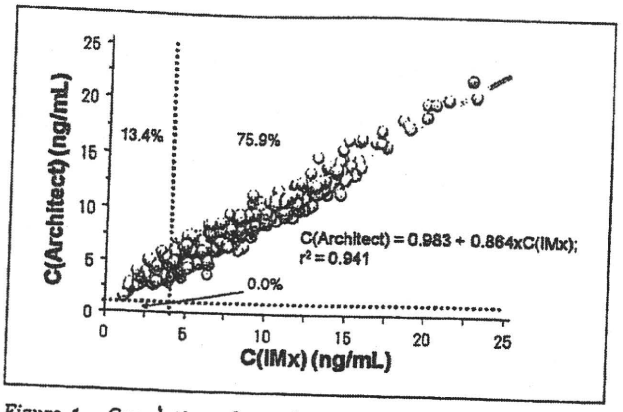

Figure 1. Correlation of tacrolimus (FK) trough concentrations measured by the microparticle enzyme immunoassay (MEIA) and chemiluminescent assay (CMIA) methods. The association between the 2 methods was excellent. C(ARCHITECT) = 0.983 + 0.864 × C(IMx); r^2 = 0.941. C(ARCHITECT) is the FK trough concentration measured by the CMIA method; C(IMx) is the FK trough concentration measured by the MEIA method. Of all samples, 13.4% were below the limit of quantification (4.1 ng/mL) in the MEIA method, whereas all samples were within the limit of qualification in the CMIA method.

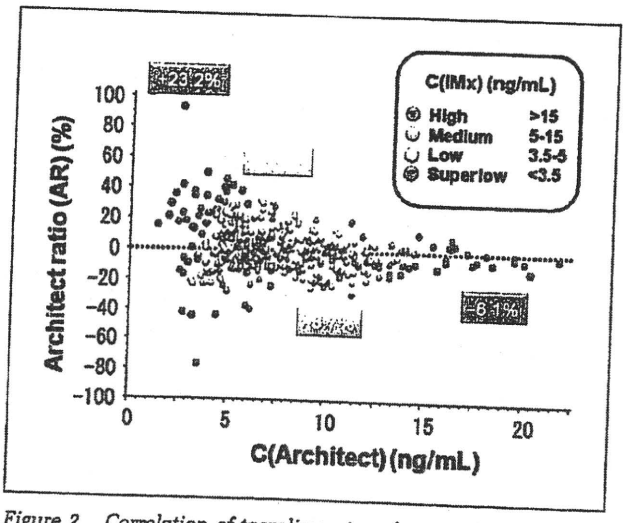

Figure 2. Correlation of tacrolimus trough concentration measured by the microparticle enzyme immunoassay (MEIA) and chemiluminescent assay (CMIA) methods using the ARCHITECT ratio (AR). The mean AR was 23.2% in samples with tacrolimus (FK) level less than 3.5 ng/mL and 6.8% in samples with FK level between 3.5 and 5 ng/mL, as measured by the MEIA. AR was defined as [C(ARCHITECT) – C(IMx)]/C(ARCHITECT).

those with hematocrit more than 45% accounted for 7.5% (n = 30).

The association between the 2 methods showed excellent correlation (Figure 1). C(ARCHITECT) = 0.983 + 0.864 × C(IMx); r^2 = 0.941. However, the discrepancy in the FK level between the 2 methods in terms of AR was large in samples with low FK level, especially less than 5 ng/mL. AR was 23.2% in samples with FK level less than 3.5 ng/mL (MEIA) and 6.8% in samples with FK level between 3.5 and 5 ng/mL (MEIA) (Figure 2). In contrast, there was excellent correlation between the 2 methods, with AR –3.7% (FK level between 5 and 15 ng/mL) and –6.1% (FK level over 15 ng/mL; Figure 2).

Analysis of the effect of hematocrit on FK level showed that the CMIA-MEIA discrepancy was larger in samples with low hematocrit (<25%), AR = –12.8%, as well as in samples with high hematocrit (>45%), AR = 11.4% (Figure 3).

In 14 of the 57 patients, the dose of FK was reduced due to renal dysfunction in 2 patients and stable graft function with more than 2 years after liver transplantation in 12 patients. The follow-up period in these 14 patients was 4.4 ± 1.8 years (range, 2.1-6.2 years). None of the patients in this study showed signs of rejection.

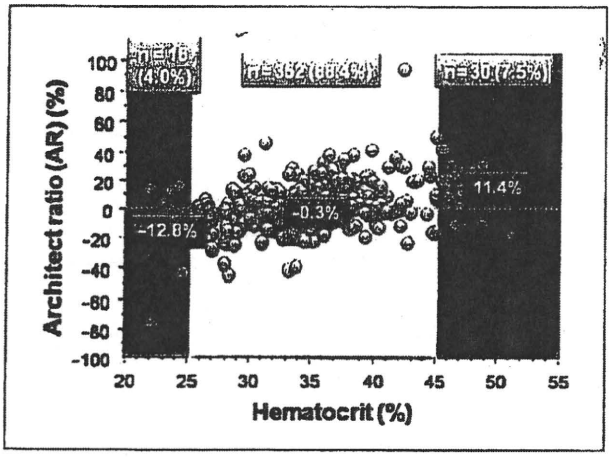

Figure 3. Correlation of ARCHITECT ratio (AR) with hematocrit (n = 398). The discrepancy in tacrolimus (FK) level measured by the chemiluminescent assay (CMIA) and microparticle enzyme immunoassay (MEIA) methods was larger in samples with low hematocrit (<25%, AR = -12.8%) and also in samples with high hematocrit (>45%, AR = 11.4%). AR was defined as [C(ARCHITECT) - C(IMx)]/C(ARCHITECT).

DISCUSSION

Therapeutic drug monitoring (TDM) is necessary when using tacrolimus due to the narrow therapeutic range and to minimize the side effects. The ideal TDM is estimated by calculating the area under the curve using several FK levels such as C0, C2, and C6. The efficacy C0 (trough) monitoring makes a suitable substitute to the AUC as it is simple and easy to measure and requires a single measure. Therefore, C0 monitoring is widely used and is an accepted measure in most transplant programs. Other methods of monitoring tacrolimus include detection of calcineurin inhibition and mixed lymphocyte reaction (MLR), which are more complex and difficult to apply in daily clinical practice. Other candidate methods for TDM include the cylex Immuknow assay, cytokines (eg, interleukin [IL]-5, IL-2, interferon [IFN]-γ, IFN-α, IL-2 receptor), neopterin, serum amyloid A, and lymphotoxin,[7,8] although their clinical application has not been established yet due to inadequate evidence of suitability for TDM.

Accurate detection of tacrolimus level in liver transplant recipients is important; however, the limitation of detecting low concentrations of tacrolimus has hampered efforts to lower the target FK level in stable liver transplant recipients. The LOQ of currently available MEIA in our hospital is 4.1 ng/mL, but we frequently encounter liver transplant recipients with normal liver function who show no sign of rejection and in whom the FK trough level is about 3.5 ng/mL. The LOQ in the CMIA method is as low as ~1 ng/mL. In our study, we maintained the FK trough level at less than 3.5 ng/mL in 13.4% liver transplant recipients.

Comparison of these 2 methods showed that the FK level varies in 2 manners. One factor relates to the hematocrit level. In the MEIA method, it has been known that there could be a substantial error in patients with low or high hematocrit.[9,10] In contrast, there is minimal error in relation to the hematocrit level in the CMIA method (Figure 3). Thus, it is likely that the discrepancy in the results of the 2 methods is due to the shortfall in the MEIA method. The more important second factor is the FK level itself. The correlation between the 2 methods was excellent at the high FK level (ie, >5 ng/mL), whereas the discrepancy between the values obtained by the 2 methods increased in samples of patients with low FK level (ie, <5 ng/mL). The FK level was underestimated, and the discrepancy ratio was more than 23% by the MEIA method at FK levels <3.5 ng/mL but was 6.8% at FK levels between 3.5 and 5 ng/mL (Figure 2). Thus, the MEIA method has a substantial error when FK level is <5 ng/mL, whereas the CMIA method is relatively accurate even in the range of 1 to 5 ng/mL.

Accurate measurement of serum FK level is necessary for TDM of FK, especially during long-term and stable periods after liver transplantation. Maintaining low FK level in stable liver transplant recipients could result in a better preservation of kidney function and less side effects such as infection, hypertension, diabetes, and hyperlipidemia. It would be useful to use once-a-day FK tablet, where lower FK trough level might be effective in avoiding rejection.

Several other methods are available for measurement of FK level in addition to CMIA and MEIA, such as the enzyme-multiplied immunoassay technique (EMIT), high-performance liquid chromatography with ultraviolet detection (HPLC-UV), and most recently mass spectrometry. Although the relationship between CMIA method and methods other than MEIA has not yet been investigated using clinical samples, to date, the CMIA method is the most accurate and clinically useful for real-time TDM of tacrolimus in liver transplant recipients.

CONCLUSIONS

We conclude based on the results of the present study that the CMIA method is superior to the

MEIA method for measurement of serum FK level, especially low FK levels (<5 ng/mL). Using this method, we can investigate the effects of low FK level in terms of reducing the chance of various short- and long-term side effects in liver transplant recipients.

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REFERENCES

1. Gonwa TA, Mai ML, Melton LB, et al. End-stage renal disease (ESRD) after orthotopic liver transplantation (OLTX) using calcineurin-based immunotherapy: risk of development and treatment. *Transplantation.* 2001;72:1934-1939.

2. Drachenberg CB, Klassen DK, Weir MR, et al. Islet cell damage associated with tacrolimus and cyclosporine: morphological features in pancreas allograft biopsies and clinical correlation. *Transplantation.* 1999;68:396-402.

3. First MR. Tacrolimus based immunosuppression. *J Nephrol.* 2004;17(suppl 8):S25-S31.

4. Ekberg H, Tedesco-Silva H, Demirbas A, et al. Reduced exposure to calcineurin inhibitors in renal transplantation. *N Engl J Med.* 2007;357:2562-2575.

5. Grenier FC, Luczkiw J, Bergmann M, et al. A whole blood FK 506 assay for the IMx analyzer. *Transplant Proc.* 1991;23:2748-2749.

6. Amann S, Parker TS, Levine DM. Evaluation of 2 immunoassays for monitoring low blood levels of tacrolimus. *Ther Drug Monit.* 2009;31:273-276.

7. Dumont RJ, Ensom MH. Methods for clinical monitoring of cyclosporin in transplant patients. *Clin Pharmacokinet.* 2000; 38:427-447.

8. Staatz CE, Tett SE. Clinical pharmacokinetics and pharmacodynamics of tacrolimus in solid organ transplantation. *Clin Pharmacokinet.* 2004;43:623-653.

9. Armendariz Y, Garcia S, Lopez RM, Pou L. Hematocrit influences immunoassay performance for the measurement of tacrolimus in whole blood. *Ther Drug Monit.* 2005;27:766-769.

10. Bouzas L, Ortega FJ, Casado P, et al. Effect of the hematocrit and its correction on the relationship between blood tacrolimus concentrations obtained using the microparticle enzyme immunoassay (MEIA) and enzyme multiplied immunoassay technique (EMIT). *Clin Lab.* 2007;53:591-596.

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ORIGINAL ARTICLE

Guanylate-binding protein 2 mRNA in peripheral blood leukocytes of liver transplant recipients as a marker for acute cellular rejection

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Keywords
acute cellular rejection, guanylate-binding protein 2 (GBP2), interferon regulatory factor 1 (IRF1), liver transplantation, peripheral blood leukocytes.

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Summary

Previously, we reported guanylate-binding protein 2 (GBP2) and interferon regulatory factor 1 (IRF1) elevated in the rat peripheral blood during acute cellular rejection (ACR), which are identified from transcriptome analysis of liver graft, as leukocyte-related gene in liver. In this study, we investigated whether these two genes could differentially diagnose ACR from other types of liver dysfunction (LD) clinically. The mRNAs from leukocytes of 19 patients with ACR and 27 with LD, as well as from liver biopsies of 12 patients with ACR and 12 with LD, were analysed by real-time PCR for GBP2 and IRF1 expression. Sensitivity and specificity were calculated using receiver operator characteristic (ROC) curves. Guanylate-binding protein 2 (GBP2) and interferon regulatory factor 1 (IRF1) gene expression levels in ACR samples were higher than that in controls, and GBP2 expression in blood was higher than that in LD (26.4 ± 3.1 and 15.6 ± 1.9, $P = 0.0203$). Multivariate analysis showed that the ratio GBP2/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was independent of ACR-related factors (OR = 0.911, $P = 0.035$). GBP2 expression levels in ACR were also higher than that in liver transplantation patients with hepatitis C or no LD. Using a cut-off value of 20, the sensitivity and specificity of GBP2/GAPDH based on ROC curve analysis were 63% and 85% respectively. GBP2 in the patients with LD may be useful for diagnosis of ACR.

Introduction

Liver transplantation with efficient immunosuppressive therapies is an established treatment for end-stage liver disease. However, acute cellular rejection (ACR) still occurs in 50–70% of transplanted patients [1–4], and can potentially lead to severe liver dysfunction (LD) and failure. The underlying genetic and molecular mechanisms of ACR remain poorly understood and liver biopsy remains the only accurate diagnostic method for ACR. However, such biopsy is invasive and moderate to severe complications needing transfusion or interventional therapies occur in up to 5% of cases [5].

We hypothesized that because ACR is a response to the transplanted tissue, and immunosuppressants work by affecting the recipient's leukocytes, changes in peripheral blood could reflect the intragraft gene expression. Using the rat allo- and iso-liver transplantation models with transcriptome analysis, we identified previously two leukocyte-associated genes, the guanylate-binding protein 2 (GBP2) and interferon regulatory factor 1 (IRF1), which reflected the state of ACR [6]. Both genes were upregulated in liver grafts and peripheral leukocytes in ACR [6]. These genes were ACR-specific and not related to other liver dysfunctions (LDs), such as bile duct ligation [6].

The present study was designed on the premise that GBP2 and IRF1 expression levels are higher in peripheral blood leukocytes of patients with ACR compared with patients with LD. Accordingly, we measured GBP2 and IRF1 mRNA expression levels in human peripheral blood

leukocytes in patients with ACR and LD, and assigned appropriate cut-off values for the diagnosis of ACR. Routine biochemical analysis was used to assess LD, and after liver biopsy, the cases were categorized as either LD or ACR with LD. Acute cellular rejection was then differentially diagnosed from the overall pool of patients with LD. Multivariate analysis was applied for comparison with LD before liver transplantation, and finally, a threshold was assigned for the diagnosis.

Materials and methods

Patients and specimens

Peripheral blood leukocytes and liver biopsy specimens were obtained from patients who received liver transplantation. From 1999 to 2007, we performed 86 liver transplantations in 84 recipients in our institution. This study used samples from patients with LD, defined as either total bilirubin >2.0 mg/dl, aspartate aminotransferase (AST) >40 U/l or alanine aminotransferase (ALT) >40 U/l. Subsequently, patients with LD were divided into those with ACR and other types of LD following liver biopsy examination (all paired blood and liver specimens were examined pathologically on the same day by two pathologists), according to the following criteria: diagnosis by either or both the pathologists as ACR with LD; or no pathologic diagnosis of ACR (LD cases). Samples obtained within 2 weeks after ACR treatment or from patients with severe complications (rejection, infection, or recurrence of primary disease) were excluded.

Guanylate-binding protein 2 and interferon regulatory factor 1 mRNA levels were evaluated using peripheral blood leukocytes from 46 patients (ACR 19, LD 27) and from 20 control blood samples obtained from donors during the last 4 years. To compare the effects of LD and liver transplantation status, we also included eight liver samples from patients with hepatitis C who received liver transplantation but did not develop LD. Furthermore, we also used eight protocol biopsy samples after liver transplantation as controls. Only fresh biopsy samples (24 in total; ACR/LD 12, LD 12) were used for this analysis.

The Institutional Review Board of Osaka University approved the study protocol and all patients provided written informed consent.

Purification of peripheral blood leukocytes

Immediately prior to liver biopsy, 8 ml of peripheral blood was collected from each patient in a Vacutainer® CPT™ cell preparation tube containing sodium citrate (Becton Dickinson, Franklin Lakes, NJ). The blood samples were centrifuged immediately at 17 000 × *g* for 20 min and the separated leukocytes were placed into a 15-ml centrifugation tube, mixed with 10 ml of phosphate-buffered saline (PBS), and then centrifuged at 800 × *g* for 10 min. After washing with 1 ml PBS, the cells were resuspended with 1 ml TRIzol Reagent (Molecular Research Center, Cincinnati, OH, USA) and stored at −80 °C until RNA isolation.

Liver biopsy and pathologic examination

Parts of the liver biopsy samples were immediately immersed in RNAlater (Qiagen, Valencia, CA, USA), before freezing in liquid nitrogen and storing at −80 °C. Hematoxylin and eosin (H&E)-stained sections of the samples were examined by two independent experienced pathologists blinded to the clinical information. Specimens diagnosed as ACR were graded according to the Banff classification [7–9]: mild (RAI: 4–5) or moderate (RAI: 6–7) ACR. After biopsy evaluation, the patients were followed to confirm that the pathologic diagnosis matched the clinical course.

Isolation of RNA

Frozen liver biopsy samples were disrupted in TRIzol reagent using Tissue Lyzer (Qiagen, Haan, Germany). Total RNA was purified from the tissue samples by TRIzol reagent according to the protocol provided by the manufacturer. Isolated RNA was quantified and assessed for purity by UV spectrophotometry. The quality of RNA was confirmed using an Agilent 2100 Bioanalyzer and RNA 6000 LabChip kit (Yokokawa Analytical Systems, Tokyo, Japan). Only high-quality RNAs with intact 18S and 28S RNA were used for subsequent experiments.

Quantitative RT-PCR

Total RNA (1 μg) was subjected to reverse transcription to generate complementary DNA (cDNA) using the Reverse Transcription System (Promega, Madison, WI, USA). The expression levels of GBP2 and IRF-1 were quantified using a real-time thermal cycler, LightCycler®, and detection system (Roche Diagnostics, Mannheim, Germany). LightCycler-DNA master SYBR green I (Boehringer, Mannheim) was used to detect the amplification products. Briefly, a 20-μl reaction volume containing 2 μl of cDNA and 0.2 μmol/l of each primer was applied to a glass capillary. In this assay, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The following primers were used: human GBP2 (forward; 5′-GGATATATTTGGCCCTTTAGAAGAA-3′, reverse; 5′-CTTTTTCCTTTTCTGAGAGTGACTG-3′), human IRF-1 (forward; 5′-AGCTCAGCTGTGCGAGTGTA-3′, reverse; 5′-TAGCTGCTGTGGTCATCAGG-3′), and human GAPDH (forward; 5′-CAACTACATGGTTTACATGTTC-3′,

reverse; and, 5′-GCCAGTGGACTCCACGAC-3′). These primers were designed using web-based software PRIMER3 (version 0.9, Whitehead Research Institute http://primer3.sourceforge.net/). The PCR for each gene was performed with cycling conditions of 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for15 s, annealing at 62 °C for10 s, and extension at 72 °C for18 s. Quantitative mRNA analysis was performed using LightCycler® analysis software (Roche Diagnostics) as recommended by the manufacturer. The relative gene expression levels were expressed as quantified gene expression divided by quantified GAPDH levels.

Statistical analysis

Data are expressed as mean ± standard error (SEM). Differences were tested by Student's *t*-test or chi-squared test and considered statistically significant at $P < 0.05$. Cut-off values for diagnosis were ascertained using the receiver operator characteristic (ROC) curve, and the sensitivity and specificity were calculated for each cut-off value. Multivariate analysis was performed by multiple logistic regression. All statistical analysis was performed using STATVIEW version 5.0 (SAS Institute, Cary, NC, USA).

Results

Clinical course and serial changes in GBP2 and IRF1 mRNA levels

Figure 1 shows serial changes in GBP2 and IRF1 mRNA expression levels in a representative patient diagnosed by liver biopsy and treated for ACR. The patient (58-year-old male) underwent transplantation using a left-lobe liver graft with middle hepatic vein necessitated because of cryptogenic liver cirrhosis. Six days after the surgery, a liver biopsy was performed to assess liver damage. Acute cellular rejection was diagnosed and the patient was placed on steroid pulse therapy (MPSL, Fig. 1). Immediately before the start of this treatment, GBP2 and IRF1 mRNA levels in the peripheral blood leukocytes showed a transient increase. However, the levels returned to pre-transplantation control levels during continued treatment.

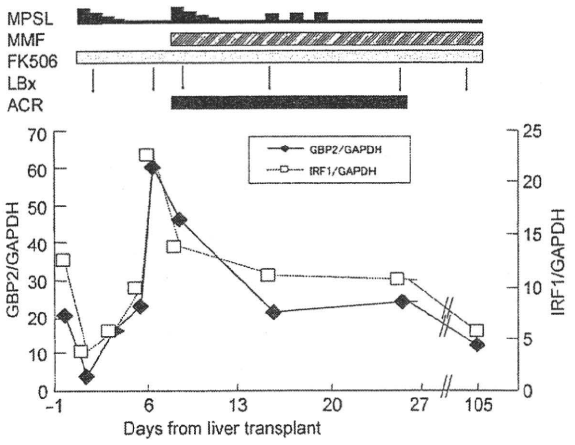

Figure 1 Serial changes in GBP2 and IRF1 mRNA expression levels during acute cellular rejection (ACR) and in response to treatment in a representative patient. The patient underwent living-relative liver transplantation of left liver grafts as a result of cryptogenic liver cirrhosis. MPSL, methylprednisolone; MMF, mycophenolate mofetil; LBx, liver biopsies.

Patients' characteristics

Table 1 summarizes the differences between patients of the ACR/LD and LD groups at liver biopsy. Assessment of type of liver damage (ACR and LD) using peripheral blood leukocyte mRNA levels required equivalent background liver damage. There were no differences in the primary disease for liver transplantation, including hepatitis C. Liver biopsy date after liver transplantation in the ACR/LD group was closer to the date of surgery than in LD patients, but the difference was not statistically significant. Biochemical analysis indicated that AST and ALT levels were >40 U/l and total bilirubin >2.0 mg/dl in both groups. However, the levels of ALT, alkaline phosphatase (ALP), and gamma glutamyl transpeptidase (γGTP) were significantly higher in the ACR/LD group than in LD patients.

Table 1. Patients' characteristics.

	ACR/LD	LD	*P*-value
n	19	27	
Age	51.7 ± 1.7	52.7 ± 1.6	0.685
Gender			
M	12	16	0.790
F	7	11	
Primary disease			
HCV	7	13	0.394
PBC/PSC/AIH	4	7	
Cryptogenic	6	3	
Others*	2	4	
Days after transplantation	104 ± 55	219 ± 70	0.193
AST	146 ± 61	67 ± 11	0.194
ALT	118 ± 16	69 ± 9	0.018
ALP	578 ± 142	220 ± 32	0.019
γGTP	272 ± 56	126 ± 23	0.019
Total bilirubin	12.9 ± 2.1	11.2 ± 2.0	0.550
PT-INR	1.30 ± 0.07	1.36 ± 0.05	0.470

Data are mean ± SD.
ACR, acute cellular rejection; LD, liver dysfunction other than ACR.
*HBV, citrullinemia.
AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γGTP, gamma glutamyl transpeptidase.

GBP2 and IRF1 mRNA levels in ACR and LD

Biochemical analysis showed no liver damage in ACR and LD patients. Donor peripheral blood was then used as a control and GBP2 and IRF1 mRNA levels were compared with those of ACR/LD and LD patients. The expression levels of GBP2 and IRF1 genes were higher in both ACR and LD patients as compared with the control group (Fig. 2). GBP2 mRNA level, but not that of IRF1 mRNA, was significantly higher in the ACR/LD group than in LD group.

In contrast, liver biopsy analysis showed higher levels of both GBP2 and IRF1 mRNAs in ACR/LD than LD, although not statistically significant (Fig. 3). We had compared previously the same two genes in the rat allotransplantation model (ACR) and iso-model (no ACR) [6]. We therefore used protocol liver biopsy samples assessed by routine biochemical analysis at 1, 2, and 5 years after liver transplantation. Both GBP2 and IRF1 mRNA levels were higher in the ACR/LD group than in protocol liver biopsy samples.

The GBP2 and IRF1 genes are mainly expressed in leukocytes [6], and leukocyte infiltration is a feature of ACR [7]; therefore, their mRNA levels in peripheral blood should correlate with those in the liver. Accordingly, we compared the mRNA expression levels in paired samples of five patients with ACR/LD and five with LD (Fig. 4), both samples were obtained on the same day in each patient. There were significant correlations in the expression levels of both GBP2 and IRF1 between peripheral blood and liver ($P < 0.05$).

GBP2 mRNA levels in the diagnosis of ACR

The above results showed higher GBP2 mRNA levels in peripheral blood of ACR/LD patients, and also high levels of ALT, ALP, and γGTP in ACR/LD patients as compared with LD. In the next step, multiple logistic regression analysis was conducted to assess whether GBP2 mRNA levels in peripheral blood is an independent diagnostic factor for ACR. The ratio of GBP2/GAPDH mRNA and ALP level were identified as independent factors, with an odds ratio for GBP2/GAPDH of 0.911 (Table 2).

Guanylate-binding protein 2 mRNA levels in peripheral blood of ACR patients were also compared with those of patients with hepatitis C (before transplantation) and in normal liver after transplantation (blood samples were obtained at the protocol liver biopsy as described above). GBP2 mRNA levels in the peripheral blood of ACR/LD patients were significantly higher than those of patients with severe liver damage and normal liver (both control and transplanted patients; Table 3).

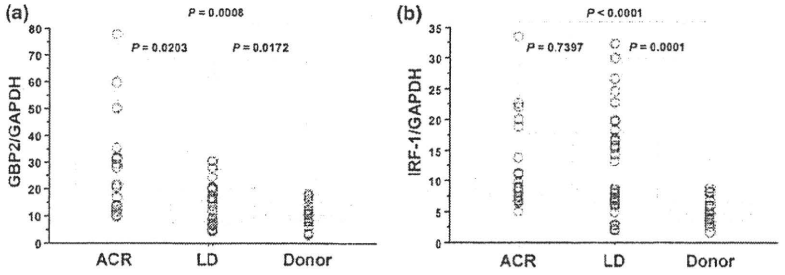

Figure 2 GBP2 (a) and IRF1 (b) mRNA expression levels in peripheral blood leukocytes from 19 ACR and 27 LD patients. Twenty donor blood leukocytes were used as controls. The pathologic diagnosis was made for each case as described in Materials and Methods. In the box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. ACR, acute cellular rejection; LD, other liver dysfunction.

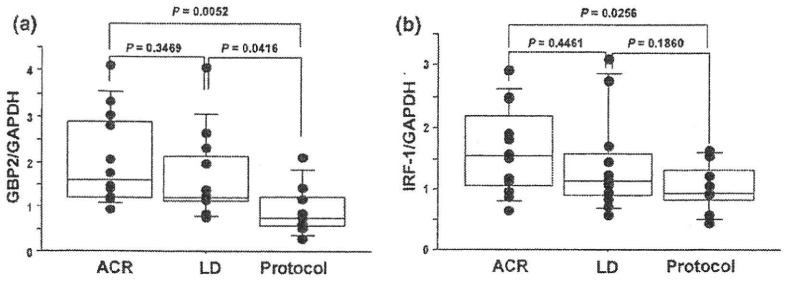

Figure 3 GBP2 (a) and IRF1 (b) mRNA expression levels in liver biopsies from 12 patients with ACR, 12 with LD, and protocol liver biopsy (no-dysfunction transplanted liver) specimens as controls. ACR, acute cellular rejection; LD, other liver dysfunction.

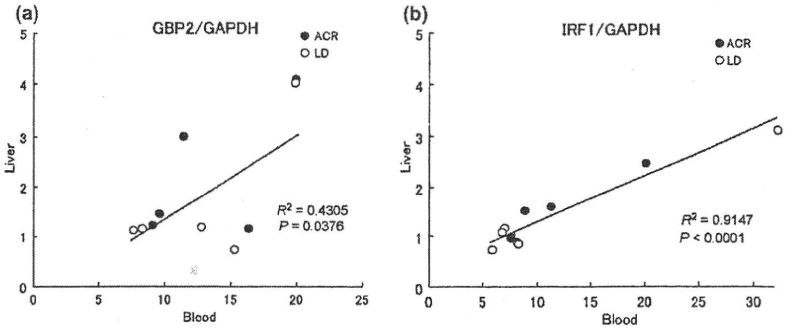

Figure 4 Correlation between GBP2 (a) and IRF1 (b) mRNA expression levels in peripheral blood leukocytes with those in liver tissues. We compared GBP2 and IRF1 expression levels of paired peripheral blood and liver biopsy samples from five ACR and five LD patients. Closed circles, ACR, acute cellular rejection; open circles, LD, other liver dysfunction.

Table 2. Results of multiple logistic regression analysis for the diagnosis of acute cellular rejection/liver dysfunction (ACR/LD).

	P	Odds ratio	95% confidence interval
GBP2/GAPDH	0.035	0.911	0.856–0.970
ALT	0.175	0.990	0.975–1.005
ALP	0.043	0.997	0.993–1.000
γGTP	0.891	1.000	0.994–1.005

GBP2, guanylate-binding protein 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γGTP, gamma glutamyl transpeptidase.

The differential diagnosis of ACR from hepatitis C recurrence is one of the most difficult issues to be resolved in respect of the damaged transplanted liver. To resolve this issue, we compared GBP2 mRNA levels in the HCV (+) recipients. Among these patients, GBP2 mRNA in ACR only were higher than those in LD (14.6 ± 2.1 vs. 11.2 ± 4.1), although the differences were not statistically significant ($P = 0.2160$).

Differential diagnosis of ACR from liver dysfunctions

To determine the cut-off value for the GBP2/GAPDH ratio for the diagnosis of ACR, a ROC curve was used to calculate the sensitivity and specificity (Fig. 5). The cut-off values ranged from 9 to 30. Using a GBP2/GAPDH cut-off value of 20, the sensitivity was 63% and specificity was 85% (Table 4).

Table 3. Guanylate-binding protein 2 (GBP2) mRNA expression levels in patients with acute cellular rejection (ACR), liver dysfunction other than ACR, donors, preoperative patients with hepatitis C, and in the protocol biopsy after liver transplantation.

	n	GBP2/GAPDH	*P*-value vs. ACR
ACR/LD	19	26.4 ± 3.1	–
LD	27	15.6 ± 2.5	0.0203
Donor	20	10.3 ± 1.0	0.0008
HCV(+) preLTx	8	16.9 ± 1.6	0.0362
Protocol	8	15.3 ± 2.6	0.0268

ACR, acute cellular rejection; LD, liver dysfunction other than ACR; HCV (+) pre LTx, preoperative patients with hepatitis C; GBP2, guanylate-binding protein 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

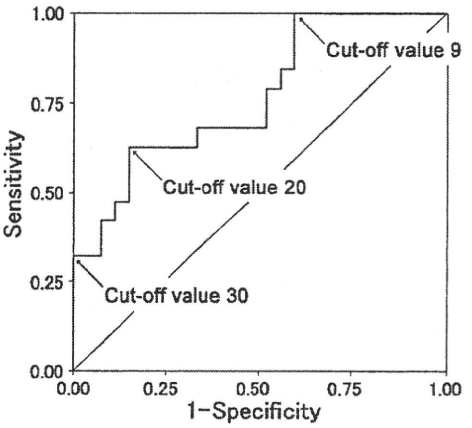

Figure 5 Receiver operator characteristic (ROC) curve for diagnosis of acute cellular rejection (ACR) using GBP2 mRNA expression levels. The GBP2/GAPDH cut-off values are indicated on the figure. Sensitivity and specificity at each cut-off values are detailed in Table 4.

Table 4. Sensitivity and specificity for acute cellular rejection (ACR) diagnosis using guanylate-binding protein 2 (GBP2) mRNA expression levels in peripheral blood leukocytes.

	GBP2/GAPDH cut-off value		
	9	20	30
Sensitivity (%)	100	63.2	31.6
Specificity (%)	40.7	85.2	100
Positive predictive value (%)	54.3	75.0	100
Negative predictive value (%)	100	76.7	67.5
Efficacy (%)	65.2	76.1	71.7

GBP2, guanylate-binding protein 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Discussion

Acute cellular rejection following transplantation is a systemic response against the grafted liver. Based on this hypothesis, we identified previously two leukocyte-related peripheral blood biomarkers (GBP2 and IRF1) from transcriptome analysis of rat liver [6]. In the present study, these same genes were investigated by mRNA expression analysis in human peripheral blood during ACR. Indeed, liver biopsy for the differential diagnosis of LD carries safety issues, and several clinicians have called for direct peripheral blood biomarkers [10–14]. However, such investigations into peripheral blood biomarker for ACR diagnosis have not so far been carried out.

The reported peripheral blood markers for ACR are related to T-cell related immune responses including regulatory T-cell levels [10–13] and apoptosis [14]. The systemic state in ACR is still unclear, although it might also involve regulatory T cells. Furthermore, IRF-1 is known to suppress regulatory T-cell function under suppression of FOXP3 [15]. On the other hand, both of our peripheral blood markers for ACR are associated with interferon (IFN)-related mechanisms [16–18]. Briefly, IRF1, which is a transcriptional factor regulated by the IFN-STAT signaling pathway [19,20], regulates GBP2 expression levels [16,17]. This system in turn elicits antiviral activity [21], macrophage activation [22], and fibroblast proliferation [23]. These results indicated that the Th1 cytokine, IFN-gamma, would stimulate IRF1, with subsequent increase in GBP2, and thus downregulate regulatory T-cell activity via suppression of FOXP3. Indeed, immunosuppressive drugs decrease the ratio of Th1/Th2 cytokines and increase regulatory T cells [24]. As ACR does not adequately respond to immunosuppressive drug, our speculation seems reasonable. In support of the hypothesis on the systemic status during ACR, one report described another peripheral blood marker for ACR [10]. AIF-1 is a promising peripheral blood marker for ACR, which increased in parallel with changes in IFN-gamma and other Th1 markers in rats [10].

Interferon regulatory factor 1 (IRF1) and guanylate-binding protein 2 (GBP2) are good peripheral blood markers for ACR detection, because unlike FOXP3 they are upregulated during ACR. Unfortunately, there was no significant difference in IRF1 expression between ACR and LD samples, as would be expected from a reported marker of ACR-related pathway. Perhaps, GBP2 would be enhanced as a downstream factor of IRF1, and might therefore be more sensitive for ACR detection, although the relationship between GBP2 and ACR remains unclear in this and previous reports [23]. In addition, GBP2 levels increased rapidly prior to detection of pathologic changes, supporting the notion that GBP2 is an enhanced factor in ACR. It is quite difficult to identify peripheral blood markers by direct 'omics' analysis, because such molecules, e.g. FOXP3, are often present at low concentrations. Thus, although GBP2 expression levels were high in ACR, the population of GBP2-related cells might be too small to show detectable changes in peripheral blood [25].

Another current topic of diagnosis in damaged liver is the challenge for the distinction of ACR from hepatitis C recurrence in HCV (+) patients. In this study, GBP2 mRNA levels in ACR were higher than those in hepatitis C recurrence; however, there were no statistically significant differences because of limited number of HCV (+) patients. To ensure the differential diagnosis of ACR from hepatitis C recurrence using GBP, further investigation would be necessary.

In summary, this study showed the potential clinical usefulness of GBP2 as a new peripheral blood marker for ACR. The GBP2 gene-related pathway and the previously reported ACR-related genes are downstream of IFN-gamma signaling. It is therefore probable that IFN-gamma-related pathways play a key role in ACR [26,27]. Prospective clinical analyses will be necessary to achieve precise diagnosis.

Authorship

HN, YD, MM 'designed research/study'. SK, HN 'performed research/study'. HN 'contributed important reagents'. HE, YT, MT 'collected data'. SK, SM, HN, TA 'analysed data'. SK 'wrote the paper'.

References

1. Wiesner RH, Ludwig J, Krom RA, Hay JE, van Hoek B. Hepatic allograft rejection: new developments in terminology, diagnosis, prevention, and treatment. *Mayo Clin Proc* 1993; **68**: 69.
2. Van Buskirk AM, Pidwell DJ, Adams PW, Orosz CG. Transplantation immunology. *JAMA* 1997; **278**: 1993.
3. Martinez OM, Rosen HR. Basic concepts in transplant immunology. *Liver Transpl* 2005; **11**: 370.
4. Liu LU, Bodian CA, Gondolesi GE, *et al.* Marked differences in acute cellular rejection rates between living-donor and deceased-donor liver transplant recipients. *Transplantation* 2005; **80**: 1072.
5. Rockey DC, Caldwell SH, Goodman ZD, Nelson RC, Smith AD. Liver biopsy. *Hepatology* 2009; **49**: 1017.
6. Hama N, Yanagisawa Y, Dono K, *et al.* Gene expression profiling of acute cellular rejection in rat liver transplantation using DNA microarrays. *Liver Transpl* 2009; **15**: 509.
7. Banff schema for grading liver allograft rejection: an international consensus document. *Hepatology* 1997; **25**: 658.
8. Ormonde DG, de Boer WB, Kierath A, *et al.* Banff schema for grading liver allograft rejection: utility in clinical practice. *Liver Transpl Surg* 1999; **5**: 261.

9. Netto GJ, Watkins DL, Williams JW, *et al.* Interobserver agreement in hepatitis C grading and staging and in the Banff grading schema for acute cellular rejection: the "hepatitis C 3" multi-institutional trial experience. *Arch Pathol Lab Med* 2006; **130**: 1157.
10. Nagakawa Y, Nomoto S, Kato Y, *et al.* Over-expression of AIF-1 in liver allografts and peripheral blood correlates with acute rejection after transplantation in rats. *Am J Transplant* 2004; **4**: 1949.
11. Sakamoto R, Asonuma K, Zeledon Ramirez ME, Yoshimoto K, Nishimori A, Inomata Y. Forkhead Box P3 (FOXP3) mRNA expression immediately after living-donor liver transplant. *Exp Clin Transplant* 2009; **7**: 8.
12. Pons JA, Revilla-Nuin B, Baroja-Mazo A, *et al.* FoxP3 in peripheral blood is associated with operational tolerance in liver transplant patients during immunosuppression withdrawal. *Transplantation* 2008; **86**: 1370.
13. Boleslawski E, BenOthman S, Grabar S, *et al.* CD25, CD28 and CD38 expression in peripheral blood lymphocytes as a tool to predict acute rejection after liver transplantation. *Clin Transplant* 2008; **22**: 494.
14. Wang YL, Zhang YY, Li G, *et al.* Correlation of CD95 and soluble CD95 expression with acute rejection status of liver transplantation. *World J Gastroenterol* 2005; **11**: 1700.
15. Fragale A, Gabriele L, Stellacci E, *et al.* IFN regulatory factor-1 negatively regulates CD4+ CD25+ regulatory T-cell differentiation by repressing Foxp3 expression. *J Immunol* 2008; **181**: 1673.
16. Briken V, Ruffner H, Schultz U, *et al.* Interferon regulatory factor 1 is required for mouse GBP gene activation by gamma interferon. *Mol Cell Biol* 1995; **15**: 975.
17. Ramsauer K, Farlik M, Zupkovitz G, *et al.* Distinct modes of action applied by transcription factors STAT1 and IRF1 to initiate transcription of the IFN-gamma-inducible gbp2 gene. *Proc Natl Acad Sci USA* 2007; **104**: 2849.
18. Lew DJ, Decker T, Strehlow I, Darnell JE. Overlapping elements in the guanylate-binding protein gene promoter mediate transcriptional induction by alpha and gamma interferons. *Mol Cell Biol* 1991; **11**: 182.
19. Lehtonen A, Matikainen S, Julkunen I. Interferons up-regulate STAT1, STAT2, and IRF family transcription factor gene expression in human peripheral blood mononuclear cells and macrophages. *J Immunol* 1997; **159**: 794.
20. Decker T, Kovarik P. Transcription factor activity of STAT proteins: structural requirements and regulation by phosphorylation and interacting proteins. *Cell Mol Life Sci* 1999; **55**: 1535. 26.
21. Carter CC, Gorbacheva VY, Vestal DJ. Inhibition of VSV and EMCV replication by the interferon-induced GTPase, mGBP-2: differential requirement for wild-type GTP binding domain. *Arch Virol* 2005; **150**: 1213.
22. Vestal DJ, Buss JE, Kelner GS, Maciejewski D, Asundi VK, Maki RA. Rat p67 GBP is induced by interferon-gamma and isoprenoid-modified in macrophages. *Biochem Biophys Res Commun* 1996; **224**: 528.
23. Gorbacheva VY, Lindner D, Sen GC, Vestal DJ. The interferon (IFN)-induced GTPase, mGBP-2. Role in IFN-gamma-induced murine fibroblast proliferation. *J Biol Chem* 2002; **277**: 6080.
24. Arias-Diaz J, Ildefonso JA, Muñoz JJ, Zapata A, Jiménez E. Both tacrolimus and sirolimus decrease Th1/Th2 ratio, and increase regulatory T-lymphocytes in the liver after ischemia/reperfusion. *Lab Invest* 2009; **89**: 433.
25. Martínez-Llordella M, Lozano JJ, Puig-Pey I, *et al.* Using transcriptional profiling to develop a diagnostic test of operational tolerance in liver transplant recipients. *J Clin Invest* 2008; **118**: 2845.
26. Nagano H, Libby P, Taylor MK, *et al.* Coronary arteriosclerosis after T-cell-mediated injury in transplanted mouse hearts: role of interferon-gamma. *Am J Pathol* 1998; **152**: 1187.
27. Nagano H, Mitchell RN, Taylor MK, Hasegawa S, Tilney NL, Libby P. Interferon-gamma deficiency prevents coronary arteriosclerosis but not myocardial rejection in transplanted mouse hearts. *J Clin Invest* 1997; **100**: 550.

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Role of the liver in determining alloimmune response *in vitro* following donor-specific spleen cell injection

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ABSTRACT

The aim of our study was to investigate the allogeneic influence inside and outside the liver *in vitro* following donor-specific cell injection (DSI). DA rats (RT1a) were used as donors and WS rats (RT1k) as recipients. WS were sensitized with DA spleen cells, followed 24 h later by total hepatectomy. The liver was transplanted into another WS (sensitized liver-grafted; SL-Grafted). The hepatectomized WS underwent liver transplantation from a naive WS (sensitized liver-removed; SL-Removed). Alloantigens accumulated in the liver in SL-Grafted and in the extrahepatic tissue/organ(s) in SL-Removed. DA hearts were transplanted 10 days after antigen administration. To analyze the immune responses, we measured Th1/Th2 cytokine profiles, and perforin mRNA in various organs, allogeneic mixed lymphocyte reaction (MLR), and donor-specific immunoglobulin. Th1 cytokine levels in the liver of SL-Grafted and in spleen of SL-Removed were highly and rapidly upregulated but decreased thereafter. IFN-γ and perforin mRNAs were significantly higher in SL-Grafted and lower in SL-Removed. MLR was significantly higher in SL-Grafted than SL-Removed and controls. There was no significant difference in the donor-specific immunoglobulin level. Our findings suggest that liver and other organs may behave differently to alloantigen, suggesting the importance of an early Th1 reaction in the liver and spleen.

1. Introduction

Despite the difficulty in inducing tolerance, antigen-specific unresponsiveness of mature lymphocytes can be induced *in vivo* through various techniques. For example, donor-specific transfusion prior to transplantation induces permanent acceptance of a subsequent allograft [1–4]. The dosage and interval between antigen administration and transplantation are two important factors as they determine whether active immunity or tolerance can be induced by antigen exposure [5,6]. In addition, intraportal administration of donor antigens could induce unresponsiveness, whereas intravenous administration may cause host sensitization, similar to hyper-acute rejection of the allograft [7,8], suggesting that the route of antigen administration is particularly important in the recipient response [9]. Following intraportal administration, donor antigens may accumulate in the liver first, whereas intravenous administration results in the distribution of these antigens in various body organs. Therefore, the liver may be an important immunomodulatory organ and determines whether active immunity or tolerance develops after antigen administration.

To our knowledge, there is little or no information on the relationship between immunologic reactions in intrahepatic and extrahepatic tissues after administration of alloantigens. The most difficult issue in investigating this relationship is the technique used for targeting antigens to the liver only or to extrahepatic tissue/organ(s) only. We developed previously a model to define the relationship between liver and extrahepatic tissue/organ(s) in the alloimmune responses induced by antigen administration [10,11]. In this study, we investigated the allogeneic reaction *in vitro* that reflects the immune response following donor-specific cell injection (DSI) in these models.

2. Materials and methods

2.1. Animals

Male DA rats (RT1a, Shizuoka Laboratory Animal Center, Japan) and Wistar Shionogi rats (WS, RT1k, Shionogi Pharmaceutical Co., Osaka, Japan) were used as donors and recipients. The rats were maintained

Abbreviations: DSI, donor-specific cell injection; HTx, heterotopic heart transplantation; MLR, mixed lymphocyte reaction; SL-Grafted, sensitized liver-grafted; SL-Removed, sensitized liver-removed.

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under specific pathogen-free conditions at the animal facility in Graduate School of Medicine, Osaka University. All experiments were approved by the animal care committee of our university.

2.2. Administration of alloantigen

Spleens were obtained from DA rats for the preparation of allogeneic cells and minced on glass slides. They were prepared by passage through nylon mesh after lysis of erythrocytes with 0.83% Tris-ammonium chloride solution and washed three times with RPMI 1640 medium. Cell viability was always more than 90%, as assessed by the trypan blue exclusion test. The final step of this procedure included preparation of DA splenocytes at a density of 5×10^7/ml in RPMI medium, followed by intravenous systemic administration of these cells via the penile vein into the recipients (WS rats) 10 days prior to cardiac transplantation.

2.3. Experimental groups

Animals were divided into four experimental groups based on the distribution of donor antigens before allograft transplantation (Fig. 1): 1) untreated group; 2) DSI group consisting of WS rats sensitized by systemic intravenous administration of DA spleen cells without orthotopic syngeneic liver transplantation; 3) sensitized liver-grafted (SL-Grafted) group (in this group, 24 h after DA-to-WS DSI, liver grafts obtained from sensitized WS rats were transplanted into naïve WS); and 4) sensitized liver-removed (SL-Removed) group (in this group, 24 h after DA-to-WS DSI, WS rats were hepatectomized and transplanted [replaced] with liver from naïve WS rats). The animals were subsequently challenged with a DA heart allograft 10 days after administration of donor spleen cells (9 days after liver isografting in SL-Grafted and SL-Removed). In the DSI group, alloantigens were distributed in the whole body including the liver at day 1 after alloantigen administration. In the SL-Removed group, alloantigens were distributed throughout the whole body but not the liver at day 1 after alloantigen administration. In the SL-Grafted group, alloantigens were distributed solely in the liver at day 1 after alloantigen administration.

2.4. Orthotopic liver transplantation

Orthotopic liver transplantation was performed using the cuff technique reported by Kamada and Calne [12], with some modifications [13].

2.5. Heterotopic heart transplantation (HTx)

Cervical heterotopic heart transplantation was performed from DA-to-WS rats as described previously [14] on day 10 after injection of donor splenocytes on day 0. Graft survival was evaluated by daily palpation of the neck, and graft rejection was defined as the complete cessation of heartbeats, which was later confirmed by histological examination.

2.6. Semiquantitative RT-PCR of cytokine transcription

Cytokine transcription in the liver, spleen, and cardiac allograft was evaluated in rats treated by DSI and/or HTx. Tissue samples were obtained on days 1, 2, 3, 5, 7, 10 (before HTx), and 14 after DSI to determine the mRNA levels of IFN-γ, IL-2, IL-4, IL-10 and perforin by RT-PCR as described previously [15–17]. Briefly, liver, spleen, and

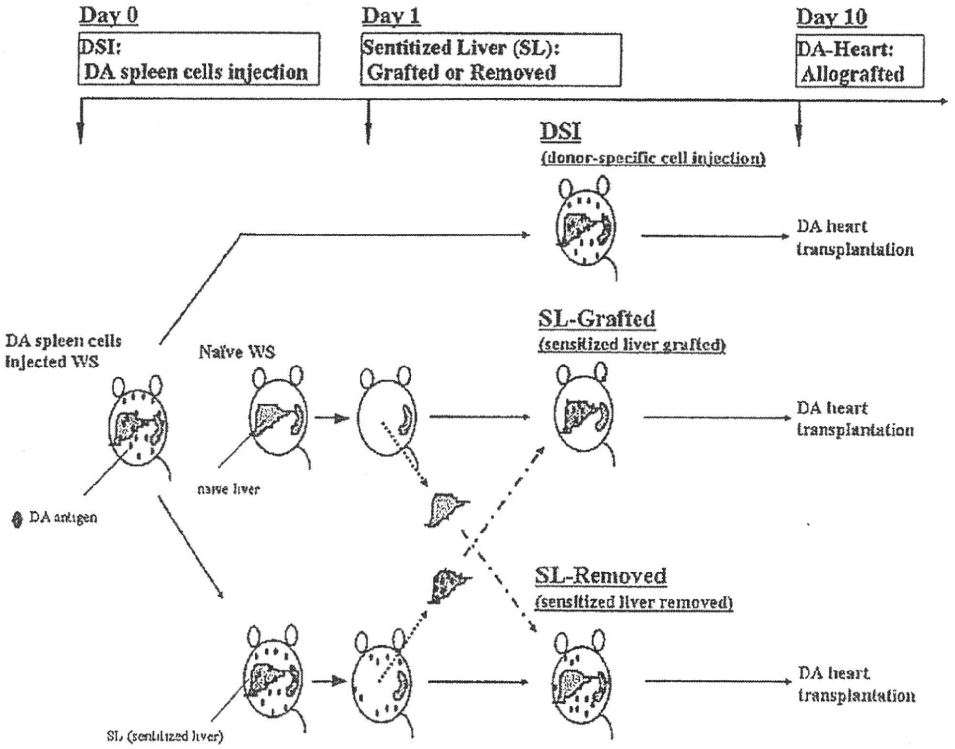

Fig. 1. Experimental groups. WS rats were divided into four groups as described in Materials and methods, based on the distribution of donor antigens prior to allograft transplantation: 1) untreated group; 2) DSI group consisting of WS rats sensitized by systemic intravenous administration of donor spleen cells without orthotopic liver transplantation; 3) sensitized liver-removed (SL-Removed) group. In this group, after DA-to-WS DSI, WS rats were hepatectomized and then underwent replacement liver transplantation from naïve WS rats; and 4) sensitized liver-grafted (SL-Grafted) group. In this group, after DA-to-WS DSI, liver grafts obtained from sensitized WS rats were transplanted into naïve WS.

cardiac allograft samples were harvested from the rats and frozen immediately in liquid nitrogen and stored at −80 °C until extraction procedure. The tissue was homogenized in liquid nitrogen and total RNA was extracted with Isogen (Gibco BRL, Grand Island, NY) before spectrophotometric adjustment of appropriate concentration for RT. One μg of total RNA was used for first stranded cDNA synthesis according to the protocol of the SuperScript reverse transcription kit (Gibco). Subsequently, 1 μl of the resulting cDNA solution was used for PCR. The thermal cycler (Gene Amp PCR System 9600, Perkin Elmer, Foster City, CA) was set for initial denaturation at 94 °C for 3 min, and followed by a single 7-min extension at 75 °C. Then, 10 μl of PCR products was electrophoresed onto 2% agarose gel and stained with ethidium bromide. The filters on which Southern blot was performed were hybridized with appropriate internal oligo end-labeled with (^{32}P) ATP. The position of internal oligo sequences in cytokine cDNAs is internal to that of the original two primers. RT-PCR of all samples was performed simultaneously in a single comparative study using the stock reagents to ensure the same biochemical conditions. Semiquantitative analysis was conducted using a densitometer (BAS 2000, Fujix, Tokyo). To normalize the cytokine mRNA levels of comparable samples, the cytokine level in each sample was expressed in densitometric units, representing the ratio between the cytokine level and β-actin level [(the density of Southern blotted band for cytokine/the density of Southern blotted band for β-actin) ×100].

2.7. Mixed lymphocyte reaction (MLR)

One-way MLR was performed as described previously [18,19]. Lymphocytes from a cervical lymph node of a WS rat were obtained at day 10 (just before HTx) and DA splenocytes were used as the responder and stimulators. The responder and stimulator cells were cultured in 96-well tissue culture plates. Triplicate cultures of 2×10^5 responder cells and 2×10^5 irradiated (2000 rad) stimulator cells were cultured in a final volume of 0.2 ml RPMI supplemented with 10 mM N-2-hydroxyetylpiperazine-N′-ethanesulfonic acid (HEPES) buffer, 5×10^{-5}M 2-mercaptoethanol, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum. The cultures were incubated in a humidified atmosphere of 5% CO_2 in air for 1 to 5 days at 37 °C. Next, 0.4 μCi of [^{3}H]thymidine was added to each well 6 h prior to the termination of culture. The cultures were harvested with a multiple sample harvester, and the [^{3}H]thymidine uptake was determined by liquid scintillation spectrometry. The results were calculated from the [^{3}H]TdR uptake and were expressed as the mean uptake in counts per min ± SEM of triplicate counts.

2.8. Flow cytometry

Sera were obtained from Groups DSI, SL-Grafted, and SL-Removed on days 1, 3, 5, 7, 10 and 14. Cervical lymph nodes from DA rats were used as the source of target cells for flow cytometry staining. Harvested lymph nodes were transferred to phosphate-buffered saline containing 0.5% bovine serum albumin (Sigma, St. Louis, MO) and 0.02% NaN_3 (PBS-BSA-AZ), then minced on glass slides. They were washed three times in PBS-BSA-AZ and resuspended at a density of 3×10^6cells/ml. Aliquots of 50 μl containing 1.5×10^5 lymph node lymphocytes were incubated in 12×75-mm plastic tubes with 50 μl of diluted sera (1:4, 1:16, and 1:64) for 45 min at 4 °C; then washed twice in 1 ml of PBS-BSA-AZ. To stain for IgM, IgG1 and IgG2a alloantibodies, the washed cells were reacted with 50 μl of PBS-BSA-AZ containing a mixture of fluorescein isothiocyanate (FITC)-conjugated goat antibodies specific for the Fc portion of rat IgG1 or IgG2a (PharMingen, San Diego, CA) and phycoerythrin-conjugated goat antibodies specific for the Mu chain of rat IgM (Jackson Immunoresearch Laboratories, West Grove, PA) for 30 min at 4 °C. After staining, the cells were washed twice, and analyzed on a FACScan (Becton-Dickinson, Mountain View, CA).

2.9. Data processing and statistical analysis

All data are expressed as mean ± SEM. One-way analysis of variance (ANOVA) was used for comparisons between groups, followed by Bonferroni/Dunn's test as the post hoc comparison. Pearson correlation analysis was used for analysis of the association of two variables. A corrected p value <0.05 was considered significant.

3. Results

3.1. Survival of cardiac allograft

SL-Grafted rats rejected cardiac allografts after a period of 4.3 ± 0.3 days ($p<0.001$, vs. other groups). In contrast, SL-Removed rats rejected donor type cardiac grafts after a significantly longer period (25.4 ± 1.9 days; vs. other groups, $p<0.001$) (Table 1).

3.2. mRNA expression of perforin, Th1 (IFN-γ, IL-2) and Th2 (IL-4, IL-10) cytokines

Fig. 2 shows the mRNA levels of cytokines in the liver and peripheral tissues on days 1–10 after alloantigen administration but before HTx. After alloantigen administration, in the liver of SL-Grafted, the highest level of IFN-γ was noted on day 2 (279 ± 96) (i.e., day 2 for SL-Grafted was one day after sensitization by administered allogeneic splenocytes, 1 day before liver transplantation) followed by day 1 (33 ± 14), day 3 (2.0 ± 2.0), day 7 (0.001 ± 0.001), and day 10 (17 ± 8.0) (Fig. 2). In the spleen of SL-Removed, IFN-γ was rapidly upregulated reaching a peak level on day 2 but decreased thereafter. The expression of IL-2 in the spleen on day 1 was significantly higher than on day 3 (66 ± 21 vs. 2.1 ± 1.3, $p<0.005$), and day 5 (66 ± 21 vs. 2.2 ± 0.8, $p<0.005$) (Fig. 2). These results suggest that the survival of SL-Grafted was associated with the upregulation of IFN-γ in the liver while that of SL-Removed was associated with the upregulation of IFN-γ and IL-2 in the spleen. On the other hand, IL-4 was not different between groups. IL-10 levels in the liver and spleen in SL-Grafted on days 2 and 3 were higher than other days in the same group. In SL-Removed, IL-10 level in the spleen on day 3 was significantly higher than on day 1 (364 ± 12 vs. 90 ± 20, $p<0.0001$), day 2 (364 ± 12 vs. 105 ± 25, $p<0.0001$), day 5 (364 ± 12 vs. 99 ± 5.7, $p<0.0001$) and day 10 (364 ± 12 vs. 136 ± 21, $p<0.0001$) (Fig. 2). In DSI, SL-Grafted, and SL-Removed rats, perforin mRNA levels in the liver and spleen were the highest on day 5 compared to other days (Fig. 2).

Fourteen days after DSI, IFN-γ mRNA levels in SL-Grafted were higher than in SL-Removed in the liver (179 ± 12 vs. 31 ± 12, $p<0.05$) and in the allograft (1385 ± 254 vs. 564 ± 82, $p<0.05$), respectively. Perforin levels in SL-Grafted were higher than in SL-Removed in the spleen (428 ± 84 vs. 34 ± 10, $p<0.005$) and in the allograft (1108 ± 184 vs. 158 ± 81, $p<0.001$), respectively. In SL-Grafted, IFN-γ (1385 ± 254 vs. 269 ± 92, $p<0.005$) and perforin (1108 ± 184 vs. 250 ± 50, $p<0.001$) were higher than in DSI in the allograft. Perforin mRNA levels in SL-Grafted were higher than in SL-Removed in liver (737 ± 46 vs. 48 ± 13, $p=0.25$) (Fig. 3). There were no significant differences between groups with regard to IL-2, IL-4, and IL-10 levels in the liver, spleen, and alloheart. Graft survival in SL-Grafted correlated with IFN-γ and perforin mRNA levels on day 14.

3.3. MLR on day 10 after alloantigen administration

The peak of MLR proliferation in lymphocytes from cervical lymph nodes of SL-Grafted, SL-Removed, and DSI rats was noted one day earlier than in naive WS rats. The MLR proliferation of WS responder cells toward donor DA stimulator cells in SL-Grafted was significantly higher than in SL-Removed (114,412 ± 2899 vs. 87,691 ± 1308, $p<0.0001$) and DSI (114,412 ± 2899 vs. 105,376 ± 1821, $p<0.005$) (Fig. 4).

3.4. Donor-specific alloantibody response

In SL-Grafted, SL-Removed, and DSI, each treatment after alloantigen administration elicited an IgM response that reached a peak level at day 7 and switched to an IgG1 and an IgG2a response that reached peak levels on day 10 (Fig. 5). There were no significant differences in the latency to peak switching from IgM to IgG1 and IgG2a responses among groups.

Table 1
Survival of cardiac allografts.

Experimental groups	Cardiac graft	
	Survival[a](days)	Mean ± SEM (days)
Untreated	10, 10, 10, 11, 11, 12, 13, 13, 14	11.6 ± 0.5
DSI	8, 9, 9, 10, 10, 11, 13, 14	10.5 ± 0.7[b]
SL-Grafted	3, 3, 4, 4, 4, 5, 5, 5, 6	4.3 ± 0.5[c]
SL-Removed	18, 18, 19, 22, 25, 26, 28, 31, 33, 34	25.4 ± 1.9[c]

[a] Graft survival was evaluated by daily palpation of the neck, and graft rejection was defined as the complete cessation of the palpable heart beat, which was later confirmed by histological examination.
[b] Not significant vs. untreated, Bonferroni/Dunn's test.
[c] $p<0.001$ vs. other groups, Bonferroni/Dunn/s test.