

Splenectomy in Chronic Hepatic Disorders: Portal Vein Thrombosis and Improvement of Liver Function

Yuichiro Ushitora^a Hirotaka Tashiro^a Shoichi Takahashi^b Hironobu Amano^a
Akihiko Oshita^a Tsuyoshi Kobayashi^a Kazuaki Chayama^b Hideki Ohdan^a

Departments of ^aGastroenterological Surgery, and ^bGastroenterology and Hepatology, Hiroshima University Hospital, Hiroshima, Japan

Key Words

Splenectomy · Interferon · Portal vein thrombosis

Abstract

Background: Splenectomy is gaining increasing importance for cirrhotic patients with hypersplenism. However, its safety and efficacy for patients with chronic liver disease remain unclear. **Methods:** We retrospectively examined the medical records of 38 consecutive cirrhotic patients who underwent splenectomy or simultaneous hepatectomy and splenectomy for hepatocellular carcinoma. **Results:** White blood cell and platelet counts significantly increased 3 months after splenectomy. Serum levels of total bilirubin and prothrombin time significantly improved 1 year after splenectomy. Interferon therapy was administered to 25 patients after splenectomy. A sustained viral response was achieved in 8 patients (42%). The total incidence of portal or splenic vein thrombosis (PSVT) detected by postoperative dynamic computed tomography was 13/38 (34.2%). Multivariate analysis revealed preoperative spleen volume (SV) to be the sole independent predictor of postoperative PSVT. Receiver-operator characteristic curve analysis showed that a cut-off SV of 450 ml corresponded to a sensitivity of 85% and a specificity

of 56%. **Conclusions:** Splenectomy improved the liver function and facilitated effective interferon therapy in cirrhotic patients with hypersplenism, although preoperative SV was frequently associated with postoperative PSVT.

Copyright © 2011 S. Karger AG, Basel

Splenectomy has been performed as a part of Hassab's operation or esophageal transection for control of variceal hemorrhage [1]. Moreover, splenectomy evidently decreases portal pressure [2] and reverses hypersplenism [3], and it has been concurrently performed in patients with small-for-size liver grafts in the setting of living donor liver transplantation [4–6]. Splenectomy is gaining increasing importance for cirrhotic patients with hypersplenism. Thrombocytopenia is a common complication of liver cirrhosis; because of this complication, initiation of antiviral treatment with interferon (IFN) becomes difficult in cirrhotic patients. Patients with thrombocytopenia often receive inadequate or incomplete IFN therapy because of exacerbation of thrombocytopenia. Recently, splenectomy has been indicated and performed in cirrhotic patients undergoing treatment for hepatocellular carcinoma (HCC) to improve thrombocytopenia and

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2011 S. Karger AG, Basel
0253-4886/11/0281-0009\$38.00/0

Accessible online at:
www.karger.com/dsu

Hirotaka Tashiro
Department of Gastroenterological Surgery
Hiroshima University Hospital
Hiroshima 734-8551 (Japan)
E-Mail htashiro@hiroshima-u.ac.jp

prior to induction of IFN therapy for patients with hepatitis C (HCV) [7, 8]. However, the safety and efficacy of IFN therapy after splenectomy remain unclear.

Liver function parameters have been reported to improve after splenectomy [3, 9, 10]. Chen et al. [10] reported that bilirubin levels were significantly lower on postoperative day 7 in patients who underwent simultaneous hepatectomy and splenectomy than in those who underwent only hepatectomy. Sugawara et al. [3] reported a significant reduction in the serum bilirubin levels 1 week after splenectomy. However, the long-term effects on liver function parameters after splenectomy remain unclear.

Portal vein thrombosis (PVT), which was considered as an uncommon complication, has been reported to be a possible cause of death after splenectomy [11]. However, with the advancements in diagnostic modalities, the incidence of portal or splenic vein thrombosis (PSVT) may apparently be greater than clinically appreciated, and this condition may be asymptomatic [12–15]. Winslow et al. reported that PSVT occurred in 8 (8%) of 101 patients who underwent splenectomy [12]. Sugawara et al. [3] reported that PVT occurred in 3 (7%) of 48 patients who underwent splenectomy. However, recent studies reported the incidence of PSVT to be between 14 and 55% [16–18]. Ikeda et al. [18] reported that PSVT occurred in 12 (55%) patients who underwent laparoscopic splenectomy. Several risk factors contribute to the onset of PSVT. A large spleen is associated with PSVT development after splenectomy [17, 18]. However, the weight of the spleen cannot be precisely measured because of the loss of blood from the spleen during the operation.

The aim of the current study is to determine the long-term effects of splenectomy on hepatic functions, the efficacy of IFN treatment after splenectomy, and the risk factors, including the volume of the spleen for PSVT.

Patients and Methods

Patients

Between January 2003 and December 2008, 38 consecutive patients underwent splenectomy or simultaneous hepatectomy and splenectomy for HCC at Hiroshima University Hospital. In this study, patients who underwent splenectomy for idiopathic thrombocytopenic purpura or after living donor liver transplantation were excluded. Thirteen patients underwent only open splenectomy, and 2 patients underwent laparoscopic splenectomy. Seven patients underwent Hassab's operation. Sixteen patients underwent simultaneous hepatectomy and splenectomy. Splenectomy was performed by laparotomy or hand-assisted laparoscopic splenectomy (HALS). In open surgery, a midline incision was used, and the vessels at the splenic hilum were ligated and divided at the

tail of the pancreas. For HALS, patients were placed in the right semilateral position, and a 7-cm skin incision for the hand insertion was made in the left subcostal area. The first trocar (12 mm) was inserted on the left of the umbilicus, where the laparoscope was placed. A CO₂ pneumoperitoneum was then created. Three other trocars were inserted under visual control into the epigastric area, the midclavicular line of the subcostal margin, and the left flank. Splenic attachments were divided using electrocautery, or an ultrasonic dissector. The splenic hilar pedicle was transected with an endoscopic linear vascular stapler. In open surgery, an antithrombotic catheter was inserted via the jejunal vein immediately after laparotomy. The top of the catheter was positioned in the recipient portal vein. A transducer was used to measure the portal vein pressure (PVP) during surgery, and the catheter was removed before closing the abdominal operative wound. Computed tomography (CT) was performed preoperatively and at one week, 1 and 6 months after surgery, or when indicated clinically. Spleen volume (SV) was measured from CT images obtained with a workstation (Virtual Place Advance 300, AZE, Ltd.) [19]. The diagnosis of PSVT was made by a radiologist, and PSVT was classified according to the location of the thrombus. When the thrombus was located solely in the splenic vein, the patient was diagnosed with splenic vein thrombosis (SVT). On the other hand, when the thrombus extended into the trunk of the portal vein, the patient was diagnosed with PVT. PVTs were further classified as intrahepatic and extrahepatic thrombosis. Upon the detection of PSVT with the CT scan, we started anticoagulation therapy that consisted of heparin (10,000 U/day, intravenously) followed by warfarin. The dosages of warfarin were adjusted to achieve an international normalized ratio from 1.5 to 2.0. Until PSVT disappearance was confirmed by CT, the administration of warfarin was continued. Antiviral treatment including pegylated IFN α -2b plus ribavirin was started when platelet counts were increased, and no severe surgical complications developed after splenectomy.

Statistical Analysis

Continuous data are shown as mean \pm SEM. The clinical data were compared using the Mann-Whitney U test or Fisher's exact test. The Cox proportional hazards model was used for multivariate analysis. The paired t test was used for comparison of perioperative laboratory data. Receiver-operator characteristic (ROC) curve analysis was performed to determine the optimal cut-off values. Each cut-off value was determined by seeking the most optimal combination of high sensitivity and specificity values, while maintaining the lowest likelihood ratio of a negative test and the highest likelihood ratio of a positive test. p value of less than 0.05 was considered significant. Statistical analysis was performed using the SPSS 16.0 software (SPSS, Chicago, Ill., USA).

Results

Table 1 presents the clinical characteristics of the patients. The patients were 20 men and 18 women (median age, 60.0 years; range, 40–77 years). Thirty-six (94.7%) patients were positive for HCV. The severity of liver disease was evaluated on the basis of the Child-Pugh classification. Of the 38 patients, 27 (71.1%) were classified as Child-

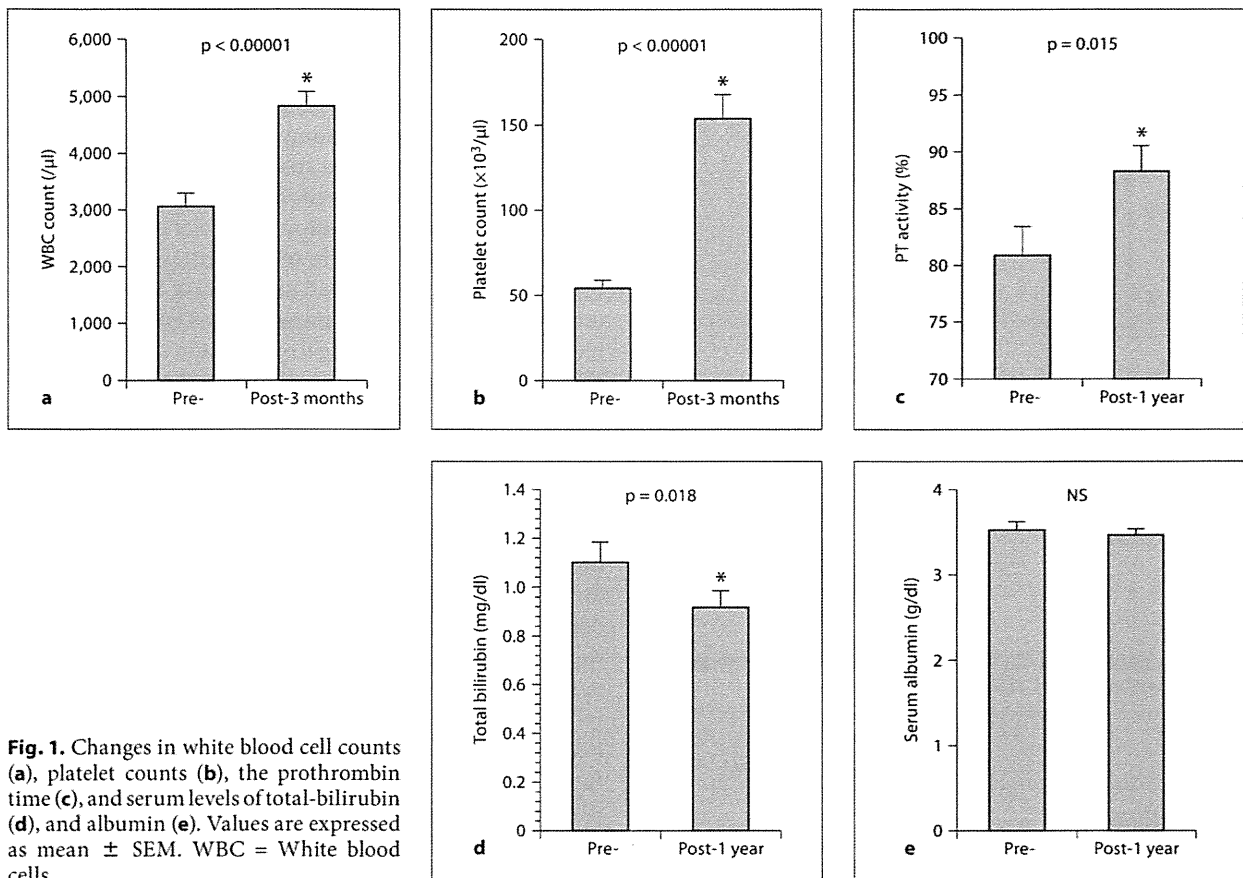


Fig. 1. Changes in white blood cell counts (a), platelet counts (b), the prothrombin time (c), and serum levels of total-bilirubin (d), and albumin (e). Values are expressed as mean \pm SEM. WBC = White blood cells.

Pugh A, and the remaining 11 (28.9%) were classified as Child-Pugh B. Twenty-six (68.4%) patients had previously received IFN therapy, and 19 (50.0%) patients had HCC. Indications for splenectomy included bleeding tendency due to thrombocytopenia (n = 1), difficulties in the induction or continuation of IFN therapy due to thrombocytopenia (n = 31), difficulties in therapies for HCC due to thrombocytopenia (n = 2), endoscopic treatment-resistant esophagogastric varices (n = 8). The changes in the laboratory data are shown in figure 1. Hematological tests conducted before and 3 months after the operation revealed a significant increase in the white blood cell count from $3,055 \pm 224$ to $4,821 \pm 252/\mu\text{l}$ ($p < 0.00001$), and in the platelet count, from 54.2 ± 4.3 to $153.5 \pm 13.5 \times 10^3/\mu\text{l}$ ($p < 0.00001$). Moreover, biochemical tests performed before and 1 year after the operation revealed a significant decrease in the serum levels of total bilirubin from 1.10 ± 0.08 to 0.91 ± 0.07 mg/dl ($p = 0.018$), and prothrombin

Table 1. Clinical characteristics of the patients (n = 38)

Age, years	60 (40–77)
Male, n	20
Female, n	18
Hepatitis HBV, n	1
Hepatitis HCV, n	36
Hepatitis NBNC, n	1
Child-Pugh A, n	27
Child-Pugh B, n	11
White blood cell count, /μl	$3,019 \pm 224$
Hemoglobin, g/dl	12.2 ± 0.29
Platelets, $\times 10^3/\mu\text{l}$	52.4 ± 4.3
Spleen volumetry, ml	493 ± 45.0
Anamnesis of IFN therapy, n	26
Complicating HCC, n	19

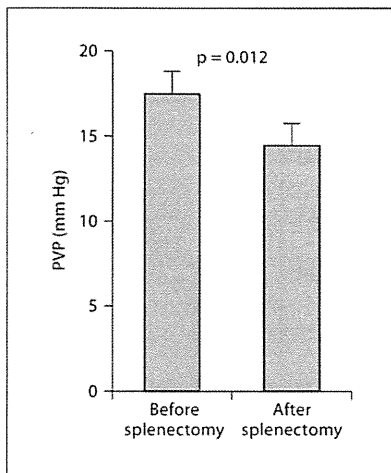


Fig. 2. Changes in portal vein pressure. Values are expressed as mean \pm SEM.

Table 2. Comparison of clinical characteristics between patients with or without PSVT after splenectomy

	No PSVT (n = 25)	PSVT (n = 13)	P value
Sex, male/female	16/9	4/9	0.054
HCC, -/+	13/12	6/7	0.73
Preoperative hemoglobin, mg/dl	12.6 \pm 0.38	11.5 \pm 0.32	0.026
Preoperative platelets, $10^4/\text{mm}^3$	5.24 \pm 0.37	5.25 \pm 0.95	0.414
Preoperative PT, %	81.7 \pm 2.7	74.0 \pm 2.5	0.031
Antithrombin III	60.0 \pm 3.4	57.5 \pm 4.8	0.410
Child-Pugh, A/B	20/5	7/6	0.096
Splenic volumetry, ml	422 \pm 47.7	621 \pm 82.9	0.011
Operating time, min	263 \pm 15.7	253 \pm 24.2	0.678
Blood loss, ml	508 \pm 62.9	433 \pm 73.8	0.399
Transfusion, -/+	22/3	9/4	0.164

Table 3. Multivariate logistic regression analysis of predictors of PSVT

Variable	p value	OR	95% CI
Sex, female	0.15	3.927	0.609–25.325
Preoperative hemoglobin	0.436	0.773	0.407–1.467
Preoperative PT	0.422	0.96	0.868–1.061
Splenic volumetry	0.036	9.582	1.156–79.415

time (PT) improved significantly from 81 ± 2.5 to $88.8 \pm 2.2\%$ ($p = 0.015$); however, the serum levels of total albumin did not improve significantly. PVP was significantly decreased from 17.5 ± 1.33 to 14.4 ± 1.38 mm Hg immediately after splenectomy ($p = 0.012$) (fig. 2). Of the 38 patients who underwent splenectomy, 25 received IFN plus ribavirin therapy. The median time between the start of IFN plus ribavirin therapy and splenectomy was 48 days (range 13–977 days). In total, a sustained virological response (SVR) was achieved in 8 (42%) of 19 patients who received IFN therapy. At present, 6 patients continue to receive IFN therapy. Owing to depression and severe diarrhea, IFN therapy was discontinued in 3 patients. No patients who underwent splenectomy discontinued IFN therapy due to thrombocytopenia.

The total incidence of PSVT detected by postoperative dynamic CT (asymptomatic or symptomatic) was 13/38 (34.2%). Specifically, 8 patients exhibited SVT, and 5 exhibited PVT. In 11 of these patients, PSVT was detected for 7–10 days after the operation. In 1 patient, PSVT was detected by dynamic CT approximately 6 months after the operation. In addition, it was detected in another patient approximately 3 months after the operation, and total bilirubin levels in this patient temporarily reached 8 mg/dl. Antiplatelet and anticoagulant drugs were successful in all patients, but PSVT recurred in 2 patients. Univariate analysis (table 2) revealed that the preoperative hemoglobin (Hgb) levels and the preoperative PT were significantly lower in patients with PSVT than in those without PSVT. Moreover, the preoperative SV measured by CT volumetry was significantly higher in patients with PSVT than in those without PSVT. However, multivariate analysis (table 3) revealed SV to be the sole predictor of PSVT in patients who had undergone splenectomy ($p = 0.036$). The optimal cut-off value was determined by ROC curve analysis (fig. 3). A cut-off SV of 450 ml corresponded to a sensitivity of 85% and specificity of 56%.

Postoperative complications associated with splenectomy developed in 7 patients; superficial surgical site infection ($n = 3$) and intra-abdominal abscess ($n = 2$) were treated by percutaneous drainage, and ileus ($n = 2$) that occurred about 1 year after splenectomy were treated by open surgery.

Discussion

In our study, platelet counts significantly increased after splenectomy, and this result is consistent with those obtained in other studies [3, 7]. Improvements in the liver

function parameters after splenectomy have been reported in the literature. Shimada et al. [9] showed that the Child-Pugh class and ammonia levels of cirrhotic patients improved after splenectomy. Chen et al. [10] reported a significant decrease in the serum bilirubin levels after splenectomy in hepatectomized patients. Sugawara et al. [3] also reported that serum bilirubin levels significantly decreased after splenectomy. However, these assessments were performed in the range from 7 to 40 days after splenectomy. A few studies have reported the mid-term (less than 6 months) effects of splenectomy. Ikegami et al. [20] reported that in 2 cirrhotic patients, liver function parameters, including total bilirubin, serum albumin levels, and PT, improved 3 months after splenectomy. Morihara et al. [8] reported that total bilirubin levels improved 6 months after splenectomy. However, few studies have reported the long-term (more than 6 months) effects of splenectomy. In our study, the serum level of total bilirubin and PT improved significantly 1 year after splenectomy, although the serum levels of albumin did not significantly improve. These results suggest that splenectomy results in long-term improvement in liver function for more than 6 months after surgery. The hemodynamic status of patients with liver cirrhosis is characterized by a systemic hyperdynamic status. Portal hypertension with hyperdynamic splanchnic inflow increases mesenteric permeability, causing leakage of a large amount of fluid into the abdominal cavity. Splenectomy can decrease the inflow into the portal system, resulting in decreased portal pressure [2, 4]. In the current study, PVP was decreased after splenectomy, and this result is consistent with the previous reports. Although the mechanism by which splenectomy improves the liver function is difficult to explain, the improvement in liver function might be associated with a decrease in PVP after splenectomy. Moreover, splenectomy may modify inflammatory mediator generation. In the endotoxin-induced liver injury model, splenectomy prevented liver injury by suppressing Kupffer cell function and tumor necrosis factor [21]. In the warm ischemia-reperfusion model in rats, splenectomy protected against acute multiple organ damage by inhibiting leukocyte infiltration in livers, release of TNF- α , and expression of caspase-3 [22]. Splenectomy promoted the regeneration and healing of livers in cirrhotic rats via inhibition of the production of TGF- β [23]. Splenectomy may improve the liver function through the modification of hemodynamic status and inflammatory mediators. Furthermore, an SVR was achieved in 8 patients (42%) in this study. The effectiveness of the IFN therapy might indirectly contribute to the improvement in liver function.

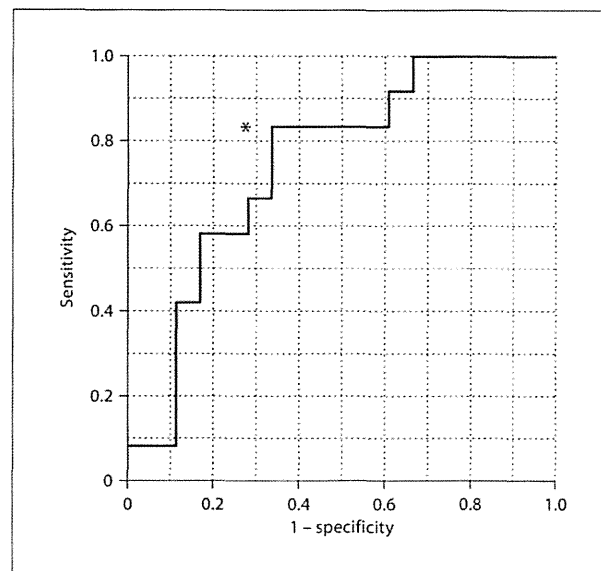


Fig. 3. Receiver-operator characteristics curve of SV for predicting postoperative PSVT in 38 patients who underwent splenectomy: * 450 ml; sensitivity, 85%; specificity, 56%.

Initiation of antiviral therapy with IFN is difficult in patients with thrombocytopenia. Patients with low platelet counts often receive inadequate or incomplete IFN therapy because of the necessary dose reduction or the discontinuation of treatment. Splenectomy is known to improve platelet counts in cirrhotic patients with thrombocytopenia. Recently, some studies have reported that IFN therapy can be safely administered to HCV-positive patients with thrombocytopenia after they have undergone splenectomy. Furthermore, these studies showed that none of the patients required a dose reduction or discontinuation of treatment because of thrombocytopenia, and that the treatment outcomes were similar to those for patients without thrombocytopenia [7, 8]. In the current study, an SVR was achieved in 42% of patients with thrombocytopenia, and IFN therapy did not have to be discontinued because of thrombocytopenia in any of the patients.

In the current study, multivariate analysis revealed that an increase in SV is associated with PSVT after splenectomy. ROC curve analysis revealed that an SV greater than 450 ml is associated with PSVT development after splenectomy. Recent reports have shown that the spleen was significantly heavier in patients with PSVT than in those that did not have PSVT; this finding suggests that

the spleen weight is a possible predictor of postoperative PSVT [17, 18]. However, because of blood loss from the spleen during splenectomy, the weight of the resected spleen is not identical to that of the spleen before the operation. This is the first study to show that the SV recorded before splenectomy is associated with PSVT development after splenectomy; in addition, postoperative PSVT can be predicted by performing preoperative CT volumetry of the spleen. In 11 (84%) of 13 patients who developed PSVT, PSVT was detected 7–10 days after the operation. Postoperative dynamic CT should be planned approximately 1 week after splenectomy for early detection of PSVT. Kawanaka et al. [24] reported that preoperative antithrombin (AT)-III activity was the most important predictive factor for PVT, and that treatment

with AT-III concentrates is likely to prevent the development of PSVT. In this study, we have shown that preoperative AT-III activity was not associated with postoperative PSVT. However, as shown in Kawanaka's report, prophylactic administration of AT-III concentrate in the early postoperative phase may prevent postoperative PSVT in case of SV greater than 450 ml, and it might be better to start anticoagulation therapy with a low dose of warfarin 3 or 4 days after the operation, after confirming that postoperative bleeding did not occur.

In conclusion, splenectomy exhibited a long-term positive effect on liver function; furthermore, preoperative SV was associated with postoperative PSVT. IFN therapy did not have to be discontinued because of thrombocytopenia in any of the patients.

References

- Hassab MA: Gastroesophageal decongestion and splenectomy in the treatment of esophageal varices in bilharzial cirrhosis: further studies with a report on 355 operations. *Surgery* 1967;61:169–176.
- Shimada M, Ijichi H, Yonemura Y, et al: The impact of splenectomy or splenic artery ligation on the outcome of a living donor adult liver transplantation using a left lobe graft. *Hepatogastroenterology* 2004;51:625–629.
- Sugawara Y, Yamamoto J, Shimada K, et al: Splenectomy in patients with hepatocellular carcinoma and hypersplenism. *J Am Coll Surg* 2000;190:446–450.
- Sato Y, Yamamoto S, Oya H, et al: Splenectomy for reduction of excessive portal hypertension after adult living-related donor liver transplantation. *Hepatogastroenterology* 2002;49:1652–1655.
- Ikegami T, Shimada M, Imura S, Arakawa Y, Nii A, Morine Y: Current concept of small-for-size grafts in living donor liver transplantation. *Surg Today* 2008;38:971–982.
- Tashiro H, Itamoto T, Ohdan H, et al: Should splenectomy be performed for hepatitis C patients undergoing living-donor liver transplantation? *J Gastroenterol Hepatol* 2007;22:959–960.
- Hayashi PH, Mehia C, Reimers HJ, Solomon HS, Bacon BR: Splenectomy for thrombocytopenia in patients with hepatitis C cirrhosis. *J Clin Gastroenterol* 2006;40:740–744.
- Morihara D, Kobayashi M, Ikeda K, et al: Effectiveness of combination therapy of splenectomy and long-term interferon in patients with hepatitis C virus-related cirrhosis and thrombocytopenia. *Hepatol Res* 2009;39:439–447.
- Shimada M, Hashizume M, Shirabe K, Takenaka K, Sugimachi K: A new surgical strategy for cirrhotic patients with hepatocellular carcinoma and hypersplenism: performing a hepatectomy after a laparoscopic splenectomy. *Surg Endosc* 2000;14:127–130.
- Chen XP, Wu ZD, Huang ZY, Qiu FZ: Use of hepatectomy and splenectomy to treat hepatocellular carcinoma with cirrhotic hypersplenism. *Br J Surg* 2005;92:334–339.
- Hanazaki K, Kajikawa S, Adachi W, Amano J: Portal vein thrombosis may be a fatal complication after synchronous splenectomy in patients with hepatocellular carcinoma and hypersplenism. *J Am Coll Surg* 2000;191:341–342.
- Winslow ER, Brunt LM, Drebin JA, Soper NJ, Klingensmith ME: Portal vein thrombosis after splenectomy. *Am J Surg* 2002;184:631–636.
- Rattner DW, Ellman L, Warshaw AL: Portal vein thrombosis after elective splenectomy: an underappreciated, potentially lethal syndrome. *Arch Surg* 1993;128:565–570.
- Chaffanjon PC, Brichon PY, Ranchoup Y, Gressin R, Sotto JJ: Portal vein thrombosis following splenectomy for hematologic disease: prospective study with Doppler color flow imaging. *World J Surg* 1998;22:1082–1086.
- van't Riet M, Burger JW, van Muiswinkel JM, Kaze-mier G, Schipperus MR, Bonjer HJ: Diagnosis and treatment of portal vein thrombosis following splenectomy. *Br J Surg* 2000;87:1229–1233.
- Romano F, Caprotti R, Scaini A, Conti M, Scotti M, Colombo G: Elective laparoscopic splenectomy and thrombosis of the splenoportal axis: a prospective study with ecocolordoppler ultrasound. *Surg Laparosc Endosc Percutan Tech* 2006;16:4–7.
- Svensson M, Wiren M, Kimby E, Hagglund H: Portal vein thrombosis is a common complication following splenectomy in patients with malignant haematological diseases. *Rur J Haematol* 2006;77:203–209.
- Ikeda M, Sekimoto M, Takiguchi S, et al: High incidence of thrombosis of the portal venous system after laparoscopic splenectomy: a prospective study with contrast-enhanced CT scan. *Ann Surg* 2005;241:208–216.
- Ohira M, Ishifuro M, Ide K, et al: Significant correlation between spleen volume and thrombocytopenia in liver transplant patients: a concept for predicting persistent thrombocytopenia. *Liver Transplant* 2009;15:208–215.
- Ikegami T, Shimada M, Imura S: Recent role of splenectomy in cirrhotic hepatic disorders. *Hepatol Res* 2008;38:1159–1171.
- Suzuki S, Nakamura S, Serizawa A, et al: Role of Kupffer cells and the spleen in modulation of endotoxin-induced liver injury after partial hepatectomy. *Hepatology* 1996;24:219–225.
- Jiang H, Meng F, Li W, et al: Splenectomy ameliorates acute multiple organ damage induced by liver warm ischemia reperfusion in rats. *Surgery* 2007;141:32–40.
- Akahoshi T, Hashizume M, Tanoue K, et al: Role of the spleen in liver fibrosis in rats may be mediated by transforming growth factor β -1. *J Gastroenterol Hepatol* 2002;17:59–65.
- Kawanaka H, Akahoshi T, Kinjo N, Konishi K, Yoshida D, Anegawa G, et al: Impact of antithrombin III concentrates on portal vein thrombosis after splenectomy in patients with liver cirrhosis and hypersplenism. *Ann Surg* 2010;251:76–83.

高力価HBs抗体含有免疫グロブリン大量投与の アロ免疫応答に対する影響

田中友加・大段秀樹*

特集 B型肝炎・肝移植後の再発予防法の現状

Immunological effects of high-dose hepatitis B immunoglobulin on allo-immune responses after liver transplantation

成人生体肝移植後にリンパ球混合試験を応用した免疫モニタリングを用い、レシピエントの抗ドナー低応答性を確認しつつ免疫抑制療法の最適化を行ったところ、免疫抑制剤の完全離脱 (operational tolerance) を4例経験した。これらの寛容症例には、共通してB型肝炎感染症患者で、肝移植後早期にHBIG大量投与療法がなされていた。最近、免疫グロブリン由来のregulatory T epitopeに制御性T細胞を増加させる効果があることが報告された。本稿では、HBIGによる制御性T細胞誘導とアロ免疫応答に対する影響の可能性を考察する。

Tanaka Yuka · Ohdan Hideki*

key words : 免疫グロブリン製剤, B型肝炎, 制御性T細胞

B型肝炎肝移植患者における operational tolerance

臓器移植後の感染症や悪性腫瘍の発生に関する問題を考慮すると、個々の免疫状態を適切に監視し、至適免疫抑制療法の実践を行うことが望まれる。筆者らの施設では、肝移植後の免疫学的指標として、Carboxyfluorescein diacetate succinidyl ester (CFSE) 蛍光色素染色法を用いたリンパ球混合試験 (CFSE-MLR) による抗ドナー細胞免疫応答モニタリングに基づく必要最小限の免疫抑制療法を、肝移植後のレシピエントに対して施行している¹⁻³⁾。

その方法は、レシピエント、ドナー、サードパーティ健常人ボランティアの末梢血単核球には放射線を照射し stimulator とする。肝移植後のレシピエント単核球はCFSE色素でラベリングし、両者を数日間混合培養し、フローサイトメトリーを用いてリンパ球の分裂回数をCD4⁺T細胞、CD8⁺T細胞別に解析し、抗ドナー応答の定量化を行う。

同時に、拒絶反応の有用な指標であるCD25の表出を伴いながら分裂増殖するCD8⁺T細胞の細胞集団の存在比率を評価している。得られたデータを用いて免疫抑制剤の最適化を図るためのアルゴリズムによって、レシピエントの免疫状態を4段階に分類し、それに即した免疫抑制剤の増減を行っている (図1)³⁾。

アルゴリズムによってCD4、CD8T細胞応答のstimulation index および抗ドナー反応性CD8T細胞のCD25表出率の解析結果による免疫状態のカテゴリー分類に基づき免疫抑制剤の減量を試みたところ、現在までに4例において免疫抑制剤完全離脱 (operational tolerance) に成功した。これらの患者は、一貫して抗ドナー低応答状態を維持しており、共通して肝移植後早期に高力価HBs抗体含有免疫グロブリン (hepatitis B immunoglobulin : HBIG) 療法がなされていた。さらに、末梢血中には制御性T細胞 (regulatory T cell : Treg) の有意な増加を認めた (図2)。また、non-operational tolerance 症例でも、原疾患別に肝移植後抗ドナーstimulation index を比較すると、HBIG療法を受けたB型肝炎症例で抗ドナー応答の低下傾向を認めた (図3)。

*Department of Surgery, Division of Frontier Medical Science, Graduate School of Biomedical Sciences, Hiroshima University 広島大学大学院医歯薬学総合研究科先進医療開発科学講座外科

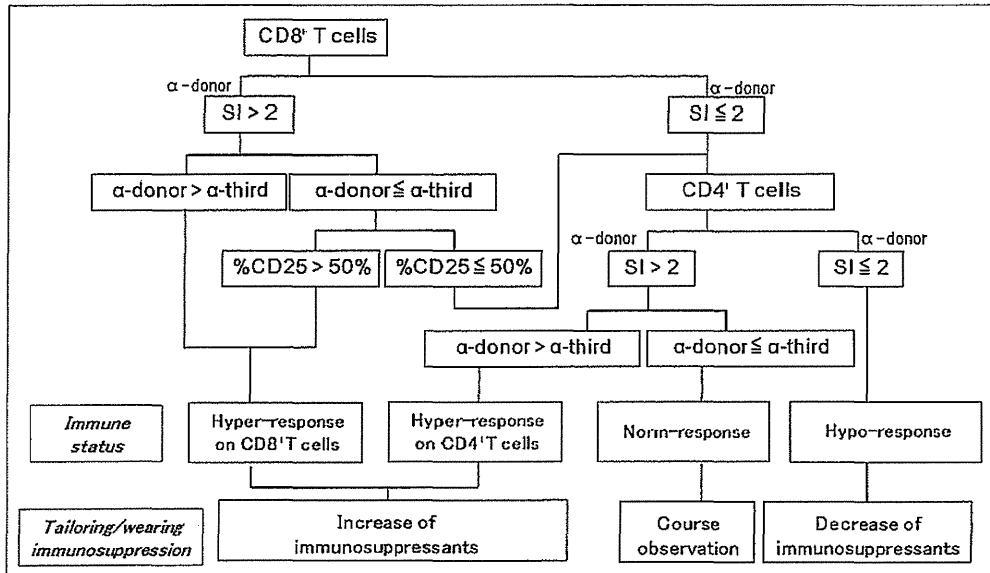


図1 CFSE-MLRに基づくアルゴリズムによる免疫抑制剤増減の指標

感作のない同種異系の組み合わせにおけるSIは平均2前後であるため、これを基準とし、抗ドナーおよび抗サードパーティのCD4⁺、CD8⁺T細胞の各SI、さらに、拒絶反応の有効な指標である反応性CD8⁺T細胞のCD25の表出率を用いて、四つのレシピエント免疫抑制状態に分類した。

細胞傷害性であるCD8⁺T細胞の抗ドナーSIが2以上で、サードパーティより有意にCD25の表出率が高い場合は、強い抗ドナー免疫応答が進行していることが予測される。すなわち、拒絶反応の存在や今後起こりうる可能性が示唆される。しかし、抗サードパーティ応答より抑制され、CD25の表出率も低いか、あるいは、SIが2以下である場合は、解析時での抗ドナー細胞傷害性は乏しいと考え、ヘルパーCD4⁺T細胞の応答を重視する。CD4⁺T細胞のSIが2を超え、抗サードパーティ応答よりも強い場合、潜在的感作状態が示唆される。しかし、抗サードパーティ応答よりも抑制されている場合、normal responseと考える。さらに、CD4⁺、CD8⁺T細胞ともにSIが低い場合は、非特異的な免疫過剰抑制状態であり、免疫抑制剤の減量が可能であるとした。

SI: stimulation index. α-donor: anti-donor response. α-third: anti-third party response. % CD25: % of CD25 expressing cells among proliferating CD8⁺T cells

この特異的抗ドナー低応答性は、B型肝炎そのものが影響するのか、あるいは投与されたHBIGが関連するのか興味を持たれる。

興味深いことには、HBIGを投与されたB型肝炎肝移植患者の急性拒絶発症率が有意に低いことが、すでに報告されている⁴⁻⁶⁾。この報告⁴⁾ではさらに、アロリンパ球混合培養系にHBIGを添加すると、抗原提示能が抑制され(共刺激分子の表出が低下)、T細胞応答が抑制されたことも示され、HBIGそのものの免疫制御機構の存在を示唆して

いる。

免疫グロブリン療法は、感染症にとどまらず自己免疫などの免疫関連疾患や炎症に対しても良好な臨床効果が得られるが、メカニズムは不明な点が多い。最近、静脈内免疫グロブリン投与(intravenous immunoglobulin: IVIG)によってTregの増加を認める報告や⁷⁻⁹⁾、IgG-Fc領域由来の合成蛋白(regulatory T-eitope: Tregitope)によるTreg誘導の報告¹⁰⁾がなされた。免疫グロブリンが投与され抗原と結合すると、それを取り込んだ抗原提示細

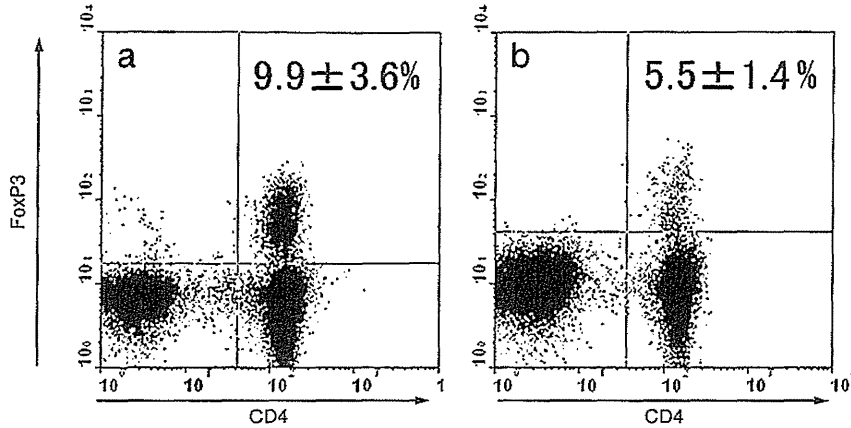


図2 Operational tolerance 症例の制御性T細胞の存在比率
Operational tolerance 症例 (a: n=3, 術後5~7年)の末梢血中 Treg 存在が高率であった。
bは Non-operational tolerant 症例

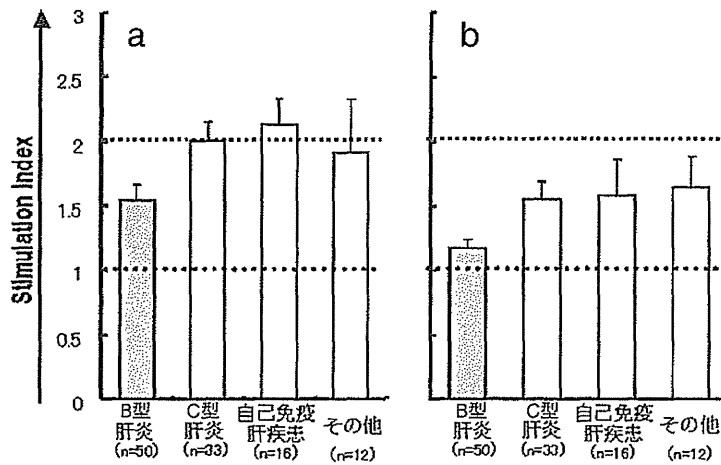


図3 肝移植後長期における原疾患別での抗ドナー免疫応答性
a: CD4T細胞, b: CD8T細胞
CFSE-MLR assay, Stimulation index: 1 = same as anti-self response

胞は、その主要組織適合複合体 (major histocompatibility complex: MHC) 上に標的抗原と regulatory T-eitope を表出し、抗原特異的な Treg が誘導されるものと考えられる (図4)。

このような報告から推測するに、B型肝炎肝移植患者に投与された HBIG によって Treg が誘導され、患者によっては免疫寛容が誘導された可能性が考えられる。

IgG-Fc 領域由来の regulatory T-eitope による免疫制御機構

免疫グロブリン可変領域 (V 領域) と結合した抗原に対する T細胞応答の抑制は、過剰な免疫応答を制御する機構として知られる。その機序は不明であったが、最近、Treg を誘導しうるエピトープ "Tregitopes" が、IgG の Fc 領域に存在することが

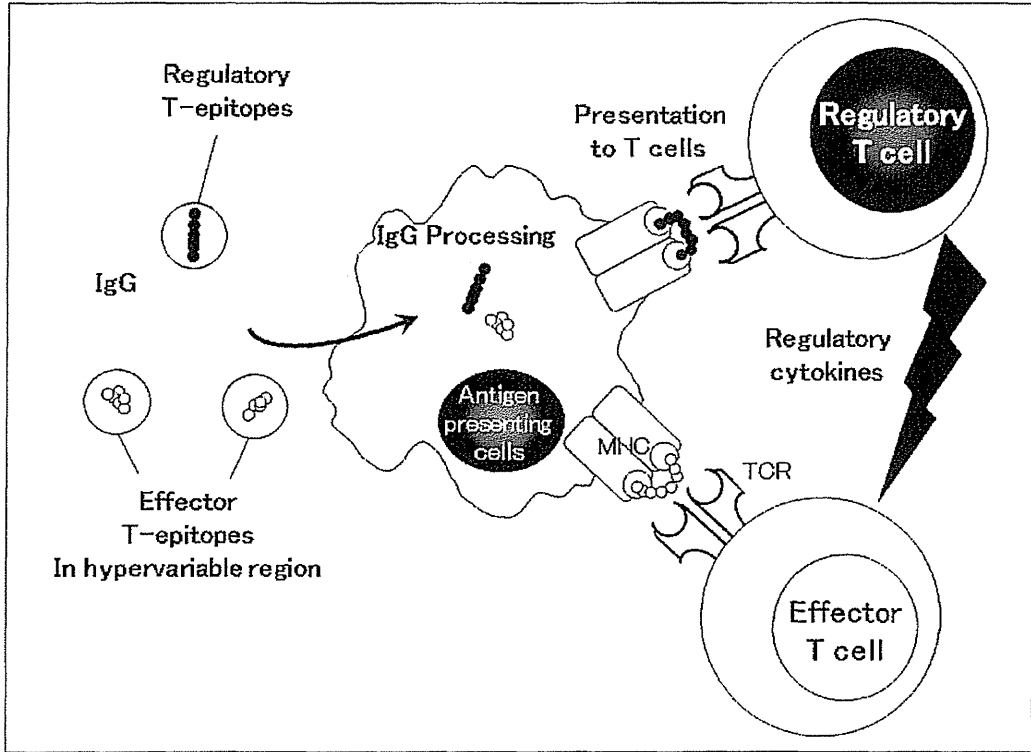


図4 IgG-Fc 領域由来の合成蛋白 (regulatory T-epitope) による 制御性 T 細胞誘導機構

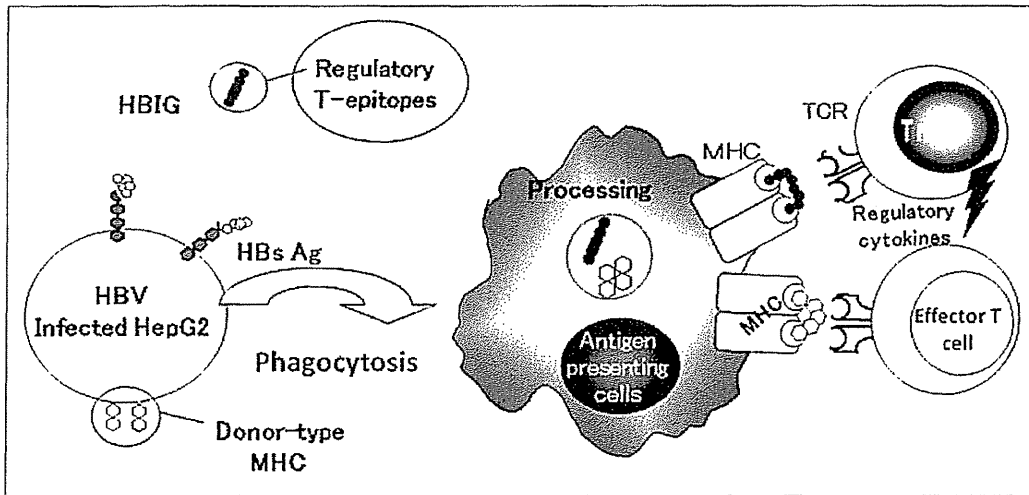


図5 抗原提示細胞への作用機序および制御性 T 細胞誘導能の仮説
TCR : T cell receptor

報告された。すなわち、IgGのC末端H鎖Peptide 289とN末端H鎖Peptide167にT細胞に対する免疫制御能があることが示された(図5)。

上述のTregitopes理論に基づいて、B型肝炎肝移植患者のアロ免疫応答を考察してみた。

投与されたHBIGがB型肝炎ウイルス(HBV)感染肝細胞に結合し傷害されると、その断片を抗原提示細胞が取り込み、IgG-Fc領域由来のTregitopeがclass II分子に表出し、それを認識したT細胞がTregへと分化する可能性が考えられる。同時にドナーHBV感染肝細胞に由来したHLA抗原もまた、抗原提示細胞のclass II上に表出され、T細胞にシグナルが伝えられる。しかし、近傍にTregが存在するため、アロ応答は特異的に抑制されている可能性があると考えられる。

現段階では、きわめて推論的ではあるが、この仮説が証明されれば、特異的免疫寛容を誘導する新たな方法へと展開される可能性が期待できる。筆者らは現在、研究を継続している。

文 献

- 1) Tanaka Y, Ohdan H, Onoc T et al. : Low incidence of acute rejection after living-donor liver transplantation : immunologic analyses by mixed lymphocyte reaction using a carboxyfluorescein diacetate succinimidyl ester labeling technique. *Transplantation* 79 : 1262-1267, 2005.
- 2) Tahara H, Tanaka Y, Ohdan H et al. : Successful hepatitis B vaccination in liver transplant recipients with donor-specific hyporesponsiveness. *Transpl Int* 22 : 805-813, 2009.
- 3) 田中友加, 大段秀樹, 田代裕尊, 浅原利正 : 細胞質染色法を応用したリンパ球混合試験に基づくアルゴリズムを用いたシクロスポリン投与量の最適化. *今日の移植* 20 : 572-575, 2007.
- 4) Kwekkeboom J, Tha-In T, Tra WM et al. : Hepatitis B immunoglobulins inhibit dendritic cells and T cells and protect against acute rejection after liver transplantation. *Am J Transplant* 5 : 2393-2402, 2005.
- 5) Farges O et al. : Incidence of rejection and infection after liver transplantation as a function of the primary disease : possible influence of alcohol and polyclonal immunoglobulins. *Hepatology* 23 : 240-248, 1996.
- 6) Couto CA, Bittencourt PL, Farias AQ et al. : Human polyclonal anti-hepatitis B surface antigen immunoglobulin reduces the frequency of acute rejection after liver transplantation for chronic hepatitis B. *Rev Inst Med Trop Sao Paulo* 43 : 335-337, 2001.
- 7) Amal E, Souleima C et al. : Expansion of CD4+CD25+ regulatory T cells by intravenous immunoglobulin : a critical factor in controlling experimental autoimmune encephalomyelitis. *Blood* 11 : 715-722, 2008.
- 8) Furuno K et al. : CD25+CD4+ regulatory T cells in patients with Kawasaki disease. *J Pediatr* 145 : 385-390, 2004.
- 9) Kessel A et al. : Intravenous immunoglobulin therapy affects T regulatory cells by increasing their suppressive function. *J Immunol* 179 : 5571-5575, 2007.
- 10) Groot AS, Moise L et al. : Activation of natural regulatory T cells by IgG Fc-derived peptide "Tregitopes". *Blood* 112 : 3303-3311, 2008.

Immunological Property of Antibodies against *N*-Glycolylneuraminic Acid Epitopes in Cytidine Monophospho-*N*-Acetylneuraminic Acid Hydroxylase-Deficient Mice

Hiroyuki Tahara,* Kentaro Ide,* Nabin Bahadur Basnet,* Yuka Tanaka,* Haruo Matsuda,[†] Hiromu Takematsu,[‡] Yasunori Kozutsumi,[‡] and Hideki Ohdan*

The generation of pigs devoid of Gal α 1,3Gal β 1,4GlcNAc (Gal) residues has stimulated interest in non-Gal Ags as potentially important targets for Ab binding leading to rejection of pig organ xenografts in humans. Although *N*-glycolylneuraminic acid (NeuGc) epitopes, which are widely expressed on the endothelial cells of all mammals except humans, are likely targets of anti-non-Gal Abs, this aspect has not been investigated intensively owing to the absence of an appropriate animal model. In this study, we used *CMAH*^{-/-} mice, which are completely deficient in NeuGc and thus produce anti-NeuGc Abs. Sera obtained from *CMAH*^{-/-} mice and healthy human volunteers having anti-NeuGc Abs initiated complement-mediated lysis against *CMAH*^{+/+} cells in vitro. The cytotoxic activity of anti-NeuGc Abs was also determined in vivo (i.e., NeuGc-expressing *CMAH*^{+/+} mouse splenocytes that had been i.v. injected were completely eliminated in syngeneic *CMAH*^{-/-} mice). *CMAH*^{-/-} mice rejected the islets transplanted from syngeneic *CMAH*^{+/+} mice. Thus, the anti-NeuGc Ab-mediated response may be crucially involved in xenograft loss. This is the first direct demonstration of the immunogenic property of NeuGc determinants as targets of the corresponding Abs in *CMAH*^{+/+}-to-*CMAH*^{-/-} transplantation setting. *The Journal of Immunology*, 2010, 184: 3269–3275.

Xenotransplantation of pig organs into humans is a potential solution to the shortage of donor organs for transplantation (1). Humans lack a functional *GalT* gene; thus, they do not express Gal α 1,3Gal β 1,4GlcNAc (Gal) carbohydrate residues and produce abundant natural Abs to the Gal epitope (2, 3). These anti-Gal Abs are a major barrier to the xenotransplantation of pig organs into humans because hyperacute rejection (HAR), which occurs in a few minutes or hours, is initiated by the binding of these Abs to Gal determinants that are ubiquitously present on porcine cells (4, 5). Recently, pigs knocked out for the gene encoding *GalT* (*GalT*^{-/-} pigs), which is responsible for the generation of the Gal epitope, have been cloned (6, 7). The availability of *GalT*^{-/-} pigs

has facilitated longer survival of nonhuman primate recipients with less immune modulation (8, 9). Thus, the absence of Gal expression has led to a substantial advance in addressing HAR; however, another study has shown that acute vascular rejection is caused by induced Abs to Ags other than the Gal epitope (non-Gal Ags) in a *GalT*^{-/-} pig-to-baboon kidney transplantation model (10). Therefore, the identification and characterization of non-Gal Ags have become a major focus of interest following the generation of *GalT*^{-/-} pigs (11, 12).

In addition to the non-Gal Ags that might be defined in the *GalT*^{-/-} pig-to-nonhuman primate model, *N*-glycolylneuraminic acid (NeuGc) epitopes, the so-called Hanganutziu-Deicher Ag (13–15), might act as an additional barrier, resulting in either HAR or acute vascular rejection of xenografts even from *GalT*^{-/-} pigs, to the eventual clinical application in humans. The most common mammalian sialic acids, which are components of the carbohydrate chains of glycoconjugates and are involved in cell–cell recognition and cell–pathogen interactions, are a group of carbohydrates of two main forms: *N*-acetylneuraminic acid and NeuGc. NeuGc is generated by the hydroxylation of CMP-Neu5Ac to CMP-Neu5Gc catalyzed by the enzyme cytidine monophospho-*N*-acetylneuraminic acid hydroxylase (*CMAH*). NeuGc epitopes are widely expressed on the endothelial cells of all mammals except humans (16) and are considered to be potential porcine targets for preformed and elicited anti-non-Gal Abs in humans (17–19), but not in baboons, that express these epitopes (16, 20). Anti-NeuGc Abs to porcine RBCs are detectable in the sera of 85% of healthy humans (17). There is a significant correlation between anti-non-Gal and anti-NeuGc Abs of both human IgM and IgG classes, and anti-NeuGc Abs constitute a significant portion of anti-non-Gal Abs (21). The presence of anti-NeuGc Abs in most normal human sera may well constitute an immunological hurdle, thereby contributing to Ab-mediated rejection; this is because the NeuGc epitope is expressed on most porcine cells, including vascular endothelial cells (22). However, this aspect has not been investigated

*Division of Frontier Medical Science, Programs for Biomedical Research, Department of Surgery, Graduate School of Biomedical Science and [†]Laboratory of Immunobiology, Department of Molecular and Applied Bioscience, Graduate School of Biosphere Science, Hiroshima University, Hiroshima; and [‡]Laboratory of Membrane Biochemistry and Biophysics, Graduate School of Biostudies, Kyoto University, Kyoto, Japan

Received for publication August 28, 2009. Accepted for publication December 31, 2009.

This work was supported by a Grand-in-Aid for Sciences Research (B) from the Japan Society for the Promotion of Science, a Grand-in-Aid for Young Scientists (Start-up) from the Japan Society for the Promotion of Science, and the Takeda Science Foundation. This work was carried out at the Analysis Center of Life Science (Hiroshima University).

Address correspondence and reprint requests to Dr. Hideki Ohdan, Division of Frontier Medical Science, Programs for Biomedical Research, Department of Surgery, Graduate School of Biomedical Science, Hiroshima University 1-2-3 Kasumi, Minami-Ku, Hiroshima 734-8551, Japan. E-mail address: ohdan@hiroshima-u.ac.jp

The online version of this article contains supplemental material.

Abbreviations used in this paper: B6, C57BL/6J; *CMAH*, cytidine monophospho-*N*-acetylneuraminic acid hydroxylase; FCM, flow cytometry; Gal, Gal α 1,3-Gal β 1,4GlcNAc; *GalT*, α 1,3-galactosyltransferase; HAR, hyper acute rejection; iEQ, islet equivalent; NAbs, natural Abs; NeuGc, *N*-glycolylneuraminic acid; WT, wild-type.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10/\$16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0902857

intensively owing to the absence of an appropriate animal model. Recently, we generated mice homozygous for a null *CMAH* allele by targeted disruption of the murine *CMAH* gene (23). In this study, we present *in vitro* and *in vivo* evidence for the cytotoxicity of non-Gal Abs against NeuGc epitopes using *CMAH*^{-/-} mice, which are completely deficient in NeuGc.

Materials and Methods

Animals, cells, and immunization

C57BL/6J (B6) (H-2^b) (syngeneic *CMAH*^{+/+}) mice, BALB/c (H-2^d) mice, and F344 rats were purchased from CLEA Japan (Tokyo, Japan) and housed in the animal facility of Hiroshima University in a specific pathogen-free microisolator environment. These mice and rats were used at an age of 8 to 16 wk. *CMAH*^{-/-} mice (23) in a B6 background (backcrossed to B6 for >10 generations), which are completely deficient in NeuGc, and *GalT*^{-/-} mice (24) in a B6 background (backcrossed seven times to B6), in which Gal expression is completely eliminated and in which naturally occurring anti-Gal Abs are present in the sera, were kindly provided by Dr. M. Sykes, Massachusetts General Hospital, Boston, MA. The porcine endothelial cell line MYP30 (25) was kindly provided by Dr. S. Miyagawa, Osaka University, Osaka, Japan. To elicit anti-NeuGc Ab production, *CMAH*^{-/-} mice were *i.p.* immunized twice with NeuGc-expressing cells (*i.e.*, thymocytes) obtained from syngeneic *CMAH*^{+/+} B6 mice, allogeneic BALB/c mice, or xenogeneic F344 rats, or with xenogeneic porcine MYP30 cells, at an interval of 1 wk (10⁷ cells/mouse at each immunization). The thymocytes or MYP30 cells were washed twice and resuspended at 10⁷/ml in medium 199 (Sigma-Aldrich, St. Louis, MO) containing 1% HEPES buffer (Life Technologies, Carlsbad, CA) and 0.08 μg/ml gentamycin (Sigma-Aldrich) before injection. All experiments were approved by the Institutional Review Board of Hiroshima University and conducted in accordance with the guidelines of the National Institutes of Health.

Immunohistochemistry

Multiple tissues from wild-type (WT) *CMAH*^{+/+} B6 mice, *GalT*^{-/-} mice, and *CMAH*^{-/-} mice were analyzed for the expression of NeuGc epitopes by immunohistochemistry using a standard immunoperoxidase technique. The tissues were fixed with formaldehyde. Prior to staining, endogenous peroxidase activity was quenched, and nonspecific binding sites were blocked with normal goat serum in PBS for 30 min. Sections were then incubated with a chicken monoclonal anti-NeuGc Ab at 4°C overnight (chickens are *CMAH*-deficient). After washing the sections, biotin-conjugated goat anti-chicken IgY (H&L) polyclonal Ab (Abcam, Cambridge, MA) in PBS at a 1:100 dilution was applied for 30 min. Sections of islet grafts from *CMAH*^{+/+} or *CMAH*^{-/-} mice were incubated with guinea pig polyclonal Ab to insulin (Abcam). Biotin-conjugated goat polyclonal Ab to guinea pig IgG (Abcam) was used to detect insulin. After washing the sections, HRP-conjugated streptavidin (Histofine SAB-PO Kit; Nichirei, Tokyo, Japan) was applied for 30 min. Peroxidase activity was visualized by staining with 3,3'-diaminobenzidine (Muto Pure Chemicals, Tokyo, Japan) for 10 min in combination with H&E counterstaining.

Pools of isolated islets from *CMAH*^{+/+} or *CMAH*^{-/-} mice were fixed with cold acetone and frozen for immunofluorescence staining. The cryosections were incubated with *CMAH*^{-/-} mouse sera containing anti-NeuGc Abs, and then FITC-conjugated goat anti-mouse IgM (Vector Laboratories, Burlingame, CA) and IgG (Zymed, Invitrogen, Carlsbad, CA) were applied.

Flow cytometry analysis for detecting NeuGc Ags and anti-NeuGc Abs

All flow cytometry (FCM) analyses were performed using a FACSCalibur dual-laser flow cytometer (BD Biosciences, San Jose, CA). The expression of NeuGc epitopes on the surface of hematopoietic cells (RBCs, bone marrow cells, splenocytes, and thymocytes) in WT, *GalT*^{-/-}, and *CMAH*^{-/-} mice was analyzed. These cells were incubated with a chicken mAb specific for NeuGc epitopes. The cells were then washed, incubated with FITC-conjugated goat anti-chicken IgG (Bethyl Laboratories, Montgomery, TX), and subjected to FCM analysis. Indirect immunofluorescence staining of thymocytes from syngeneic *CMAH*^{+/+} B6 mice was used to detect anti-NeuGc Abs. To elicit the production of anti-NeuGc Abs, *CMAH*^{-/-} mice were sensitized with NeuGc-expressing thymocytes obtained from *CMAH*^{+/+} syngeneic B6 mice, allogeneic BALB/c mice, xenogeneic F344 rats, or porcine MYP30 cells. NeuGc-expressing thymocytes (1.0 × 10⁶ cells) were incubated with 10 μl mouse serum, washed, incubated with PE-conjugated rat anti-mouse IgM, IgG1, IgG2a/2b, and IgG3 monoclonal Abs (BD Biosciences), and subjected to FCM analysis.

In vitro Ab-dependent cytotoxicity assay

Serum samples from *CMAH*^{-/-} mice and healthy human volunteers were incubated for 30 min at 56°C to inactivate complement. The samples were serially diluted with RPMI 1640 medium (plus 10% FBS; Biological Industries, Beit Haemek, Israel). Thymocytes of *CMAH*^{+/+} or *CMAH*^{-/-} B6 mice used as target cells were labeled with [⁵¹Cr] (4 μCi/well) for 60 min at 37°C. Labeled cells were washed three times and loaded onto round-bottomed 96-well microtiter plates (BD Biosciences) at a density of 1.0 × 10⁶ cells (80 μl per well) (suspended in RPMI 1640 medium plus 10% FBS). The labeled cells were incubated with 80 μl diluted *CMAH*^{-/-} mouse serum for 30 min at 4°C. Postincubation, the cells were washed and further incubated with 40 μl of 20% rabbit complement (Cedarlane Laboratories, Burlington, Ontario, Canada) for 45 min at 37°C. Negative controls used as target cells were incubated with medium and complement alone in the absence of serum, whereas positive controls cells lysed with 2% Nonidet P-40 (Nacalai Tesque, Kyoto, Japan) to determine the maximum [⁵¹Cr] release. Postincubation, the plates were centrifuged, and the cell-free supernatants were carefully harvested. The release of radioactive [⁵¹Cr] was analyzed using a γ counter (ARC-370M; Aloka, Wallingford, CT), and the percentage of specific lysis was calculated as follows: % specific lysis = [(A - B)/(C - B)] × 100, where A represents the experimental release (cpm in the supernatant from target cells incubated with serum and complement), B is the spontaneous release (cpm in the supernatant from target cells incubated with medium and complement alone), and C is the maximum release (cpm released from target cells as positive controls). The spontaneous release was <10% of the maximum release. Each experiment was performed in triplicate.

In vivo cytotoxicity assay

Splenocytes were prepared from WT *CMAH*^{+/+} B6 mice and resuspended in PBS. CFSE (Molecular Probes, Eugene, OR) was added to give a final concentration of 5 μM, and the cells were gently mixed and incubated for 5 min at 37°C in a CO₂ incubator protected from light. Labeling of cells was terminated by adding cold PBS containing 2% FBS, and the cells were then washed and resuspended in medium 199 containing 1% HEPES buffer and 0.08 μg/ml gentamycin. NeuGc-expressing splenocytes labeled with CFSE (2.0 × 10⁷ cells in 0.5 ml) were injected *i.v.* into *CMAH*^{+/+} or *CMAH*^{-/-} B6 mice in which anti-NeuGc Abs had been induced by immunization with rat thymocytes. The prospective recipient mice were irradiated with 6 Gy/whole body to facilitate the efficient engraftment of target cells. Splenocytes were collected from each recipient mouse 7 d postinoculation, and single-cell suspensions were prepared and analyzed by FCM. The percentage of surviving CFSE-labeled splenocytes from each recipient mouse was calculated.

Islet transplantation

CMAH^{+/+} or *CMAH*^{-/-} B6 mice immunized with rat thymocytes were rendered diabetic through a single *i.p.* administration of 170 mg/kg streptozotocin (Sigma-Aldrich) at 6 d before islet transplantation. The diabetic mice that had nonfasting blood glucose levels of >400 mg/dl on the day of transplantation were used as the recipients. The blood glucose levels were monitored intermittently with a blood glucose test meter (Medisafemini GR-102; Terumo, Somerset, NJ). When no islet transplantation was performed, the diabetes persisted in all the diabetic mice (blood glucose level, >350 mg/dl), and no spontaneous reversal of diabetes was observed for at least the next 3 mo. The donors were WT *CMAH*^{+/+} B6 females aged 10–12 wk. The pancreas was isolated after common bile duct perfusion of 4°C collagenase P (1 mg/ml; Roche, Somerville, NJ) in HBSS (Life Technologies), which was followed by incubation of the distended pancreas in a 37°C water bath for 20 min to achieve mechanical disruption. The pancreatic islets were purified on a Percoll (GE Healthcare, Piscataway, NJ) density gradient. Postisolation, 200, 350, or 500 *CMAH*^{+/+} islet equivalents (iEQs; diameter >150 μm) were handpicked and immediately transplanted into the recipient left renal subcapsular space.

Heterotopic heart transplantation

Neonatal F344 rat (10–14 d old) heart xenografts or *CMAH*^{+/+} syngeneic B6 mouse (14–16 wk old) heart grafts were transplanted. Cervical heterotopic heart transplantation was performed using the cuff technique modified from a previously described method (26). Briefly, the right external jugular vein and right common carotid artery of recipients were dissected free, mobilized as far as possible, and fixed to the appropriate cuffs. The cuffs were composed of polyethylene tubes (2.5 Fr; Portex, Smiths Medical, Watford, U.K.), the diameters of which were adjusted by physical extension. For anastomoses, the aorta and the main pulmonary artery of the harvested donor hearts were drawn over the end of the common carotid artery and the external jugular vein, respectively. To enhance anti-NeuGc Ab production,

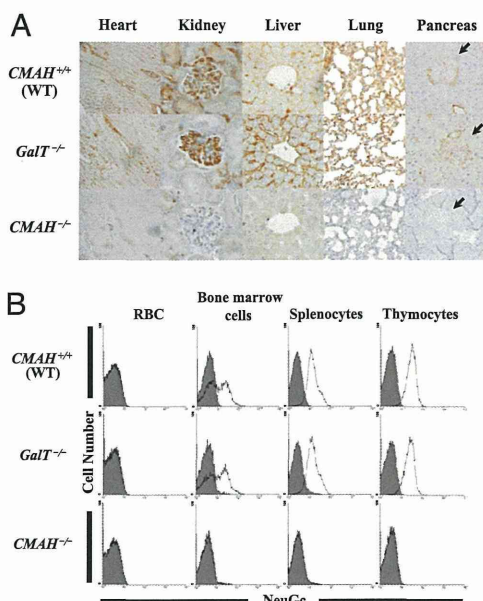


FIGURE 1. The detection of NeuGc expression in multiple tissues and on the surface of hematopoietic cells. *A*, NeuGc expression was studied by immunohistochemistry in multiple tissues (heart, kidney, liver, lung, and pancreas [islets are indicated by arrows]) from WT *CMAH*^{+/+}, *GalT*^{-/-}, and *CMAH*^{-/-} mice using a chicken anti-NeuGc mAb, and examples are shown. *CMAH*^{-/-} mice are completely deficient in NeuGc. Original magnification ×200. *B*, The expression of NeuGc epitopes on the surface of hematopoietic cells (RBCs, bone marrow cells, splenocytes, and thymocytes) was analyzed using FCM. These cells were incubated with a sensitive chicken polyclonal Ab specific for NeuGc epitopes followed by incubation with anti-chicken IgG. Expression of NeuGc was also completely absent in *CMAH*^{-/-} mice. The filled histogram represents a negative control stained with secondary Ab alone.

CMAH^{-/-} recipients were immunized by injection with 10⁷ porcine MYP30 cells at 8 wk before heart transplantation. To increase lymphocytotoxic activity against recipient peripheral lymphocytes in the presence of anti-NeuGc Abs, 0.5 ml rabbit complement (Cedarlane Laboratories) was injected to the recipient jugular vein just before anastomosis of the graft. The graft ischemic time for the transplanted hearts was <30 min. The function of the grafts was monitored by daily inspection and palpation. Rejection was determined by the cessation of beating of the graft and was confirmed by histology.

Statistical analysis

The results were statistically analyzed using unpaired or paired Student *t* tests of means or the log-rank test where appropriate. A *p* value of < 0.05 was considered to be statistically significant.

Results

NeuGc epitopes were expressed on vascular endothelial cells, thymocytes, and splenocytes in wild-type CMAH^{+/+} and *GalT*^{-/-} mice but were not detected in *CMAH*^{-/-} mice

In pigs, NeuGc epitopes are ubiquitously expressed on the vascular endothelial cells of the kidney, heart, liver, pancreas, lung, etc. (16). NeuGc epitopes are expressed on porcine aortic endothelial cells at a similar level to Gal epitopes, the number of which was previously reported as 2.0 × 10⁷/cell (27). It has been reported that the extent of glycoconjugate sialylation with NeuGc is very variable and dependent on the species, tissue, and developmental stage (28). Although NeuGc was easily detectable on the vascular endothelial cells of all analyzed tissues in WT *CMAH*^{+/+} mice (i.e., lung, heart, liver, kidney, and pancreas), we were unable to detect this epitope in any tissues of the *CMAH*^{-/-} mice (Fig. 1*A*). The expression of NeuGc was analyzed on the surface of hematopoietic cells by FCM. The

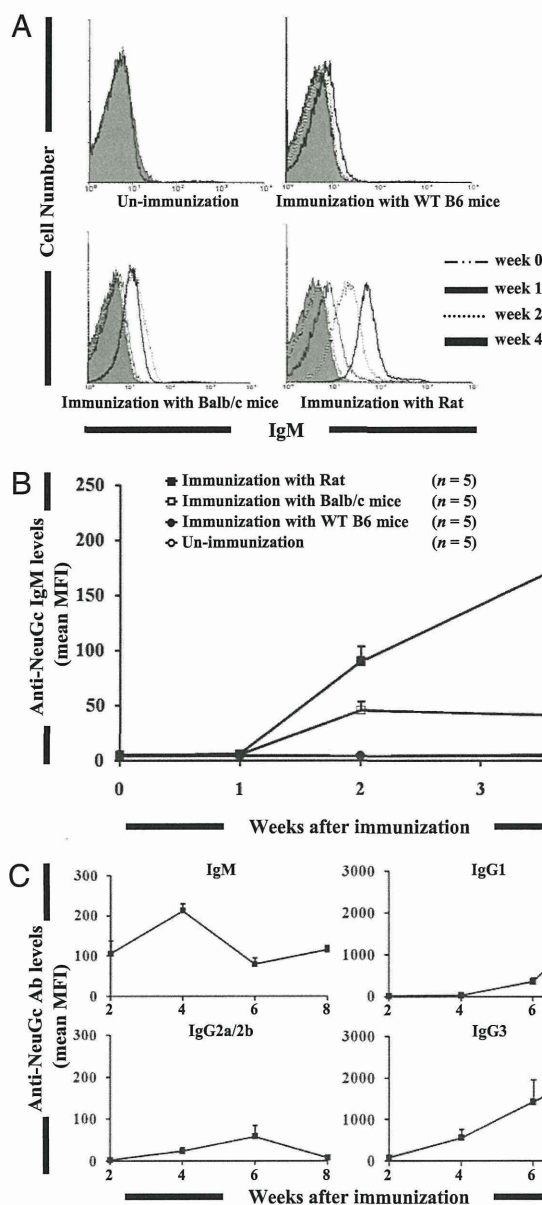


FIGURE 2. The detection of anti-NeuGc Abs. *A*, Anti-NeuGc NAb were not detected in *CMAH*^{-/-} mice. The FCM profiles shown are from representative experiments examining anti-NeuGc IgM titers at 0, 1, 2, and 4 wk postimmunization with thymocytes. The filled histogram represents a negative control stained with PE-conjugated rat anti-mouse mAb alone. *B*, The kinetics of the means of anti-NeuGc IgM titers (*n* = 5 in each group) is shown for *CMAH*^{-/-} mice at 0, 1, 2, and 4 wk after their sensitization with syngeneic, allogeneic, or xenogeneic NeuGc-expressing thymocytes. The mean ± SEM of five experiments are shown. *C*, *CMAH*^{-/-} mice can produce anti-NeuGc IgM and IgG after their sensitization with xenogeneic NeuGc-expressing rat thymocytes. A few months later, various isotypes of anti-NeuGc Ab in the sera were quantified by FCM (*n* = 5). The mean ± SEM of five independent experiments is shown.

RBCs of WT *CMAH*^{+/+} mice did not express NeuGc. However, their splenocytes and thymocytes did exhibit remarkable NeuGc expression. In contrast, we were unable to detect any NeuGc expression on these cells in *CMAH*^{-/-} mice (Fig. 1*B*). NeuGc expression was also confirmed on various tissues and cells in *GalT*^{-/-} mice at a level similar to that in *CMAH*^{+/+} mice [consistent with observations in *GalT*^{-/-} pigs (29)], which rules out the possibility that the genetic disruption of *GalT* interferes with NeuGc expression.

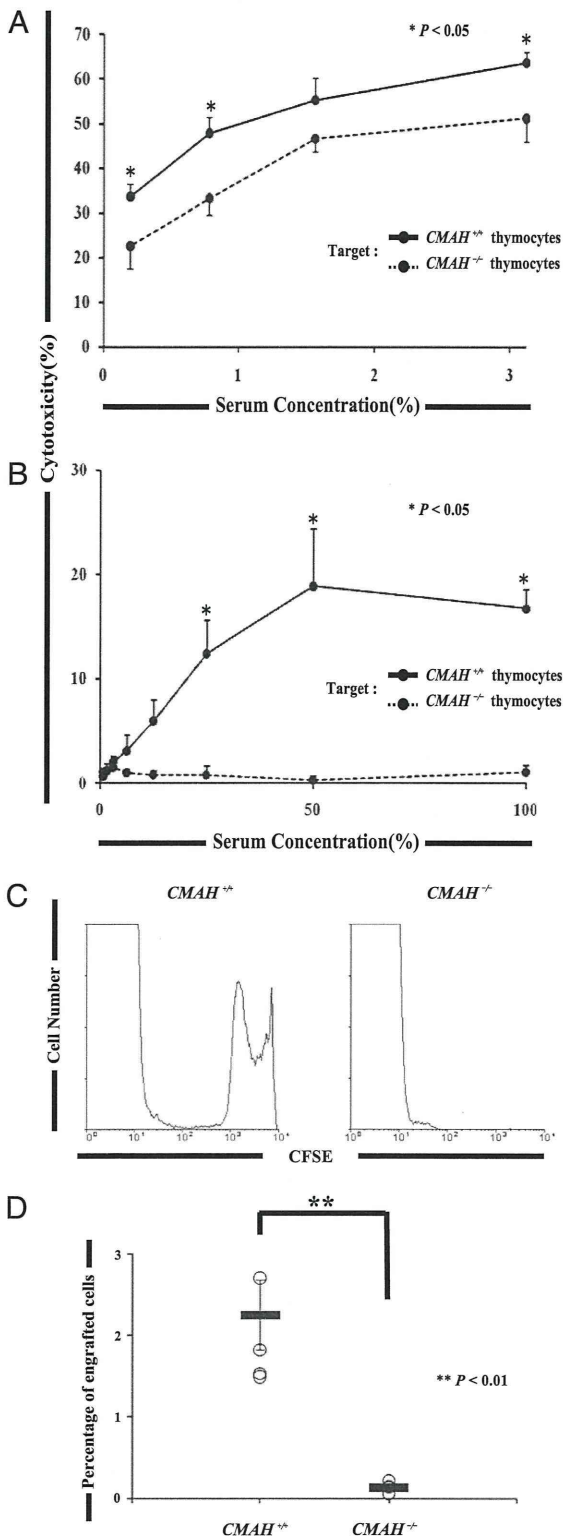


FIGURE 3. In vitro and in vivo Ab-mediated cytotoxicity assays. The results of an in vitro Ab-dependent cytotoxicity assay of sera obtained from healthy human volunteers ($n = 7$) (A) or $CMAH^{-/-}$ mice having anti-NeuGc Abs (B) against thymocytes obtained from $CMAH^{+/+}$ or $CMAH^{-/-}$ B6 mice are shown. Significantly higher lysis of NeuGc-expressing thymocytes was observed when compared with that of thymocytes lacking NeuGc. The mean \pm SEM of five independent experiments is shown. $*p < 0.05$. C, In vivo cytotoxicity assay in $CMAH^{+/+}$ or $CMAH^{-/-}$ B6 mice, which were immunized with NeuGc-expressing rat thymocytes, against CFSE-labeled splenocytes from $CMAH^{+/+}$ syngeneic B6 mice. Representative FCM results are shown. D, The percentage of engrafted cells labeled with CFSE in the recipient splenocytes ($n = 5$, respectively). Each open circle represents an individual mouse. The mean \pm SEM of five experiments is shown. $**p < 0.01$.

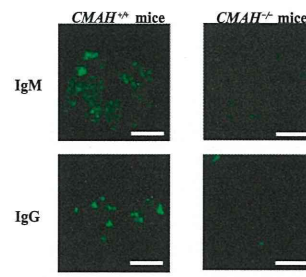


FIGURE 4. Deposition of anti-NeuGc IgM and IgG Ab in the islets isolated from $CMAH^{+/+}$ or $CMAH^{-/-}$ mice. Pictures shown are representative of the single islet from each mouse. Original magnification $\times 200$. Scale bars, 50 μ m.

CMAH^{-/-} mice produced anti-NeuGc Abs postimmunization with NeuGc-expressing cells

Naturally occurring Abs against NeuGc epitopes were undetectable in the sera of $CMAH^{-/-}$ mice. Furthermore, the elicited anti-NeuGc IgM was barely detectable several weeks postimmunization with NeuGc-expressing thymocytes obtained from $CMAH^{+/+}$ syngeneic B6 mice. The production of anti-NeuGc IgM was significantly more pronounced when $CMAH^{-/-}$ mice were immunized with thymocytes obtained from either $CMAH^{+/+}$ allogenic BALB/c mice or xenogeneic F344 rats (Fig. 2A, 2B). The production of anti-NeuGc IgG was observed a few months postimmunization with xenogeneic rat thymocytes (predominant subclasses were IgG1 and IgG3) (Fig. 2C), indicating that class switching of Ig occurs with anti-NeuGc specificity.

Anti-NeuGc Abs exhibited cytotoxicity against NeuGc-expressing cells

We examined the Ab-dependent cytotoxicity of sera obtained from $CMAH^{-/-}$ mice or healthy human volunteers having anti-NeuGc Abs against thymocytes obtained from $CMAH^{+/+}$ or $CMAH^{-/-}$ mice. Lysis of NeuGc-expressing thymocytes was significantly higher than that of thymocytes lacking NeuGc in both experiments (Fig. 3A, 3B), indicating that anti-NeuGc Abs show obvious cytotoxicity against NeuGc-expressing cells in vitro. The cytotoxic activity of anti-NeuGc Abs was also determined in vivo. In the FCM analysis, the surviving splenocyte inocula were clearly detected as CFSE-labeled cells in the spleen of control $CMAH^{+/+}$ recipients. In contrast, injected splenocytes were completely absent in the spleen of $CMAH^{-/-}$ recipients at this time point (Fig. 3C, 3D), indicating that anti-NeuGc Abs eliminated NeuGc-expressing cells in vivo.

CMAH^{-/-} mice rejected the NeuGc-expressing islets transplanted from syngeneic CMAH^{+/+} mice

Immunohistochemical analysis revealed NeuGc expression on the islets of $CMAH^{+/+}$ and even $GalT^{-/-}$ mice (Fig. 1A), but did not reveal any Gal epitopes on the islets of either $CMAH^{+/+}$ or $CMAH^{-/-}$ mice (data not shown). When the islets of $CMAH^{+/+}$ or $CMAH^{-/-}$ mice were incubated with $CMAH^{-/-}$ sera containing anti-NeuGc Abs, the deposition of both IgM and IgG Abs was observed only in the islets obtained from $CMAH^{+/+}$ mice (Fig. 4). Islets obtained from WT $CMAH^{+/+}$ or $GalT^{+/+}$ mice

representative FCM results are shown. D, The percentage of engrafted cells labeled with CFSE in the recipient splenocytes ($n = 5$, respectively). Each open circle represents an individual mouse. The mean \pm SEM of five experiments is shown. $**p < 0.01$.

were transplanted under the kidney capsules of streptozotocin-induced diabetic *CMAH*^{+/+} mice, *CMAH*^{-/-} mice having anti-NeuGc Abs, and *GalT*^{-/-} mice having anti-Gal Abs. Almost all of the *CMAH*^{+/+} and *GalT*^{-/-} mice that received 200 iEQs became normoglycemic 1 d after the transplantation (Fig. 5A). In contrast, all the *CMAH*^{-/-} mice that received the same number of syngeneic islets failed to achieve normoglycemia, thus indicating that anti-NeuGc Abs have an inhibitory effect on the engraftment of *CMAH*^{+/+} islets (Fig. 5A, 5B). In an attempt to overcome this inhibitory effect, a larger number of islets was transplanted into diabetic *CMAH*^{-/-} mice. Normoglycemia was achieved in the *CMAH*^{-/-} mice that received 500 iEQs (Fig. 5C), indicating that a high dose of islets can overcome the anti-NeuGc Ab-mediated resistance to islet engraftment.

In addition, when compared with the control *CMAH*^{+/+} recipient mice 3 d after islet transplantation, there was a significant decrease in insulin-producing viable β cells in the islet grafts of *CMAH*^{-/-} recipient mice (Fig. 6). Thus, NeuGc epitopes on the islets were targeted by the corresponding Abs in *CMAH*^{-/-} recipients (i.e., *CMAH*^{-/-} mice rejected the NeuGc-expressing islets transplanted from *CMAH*^{+/+} mice).

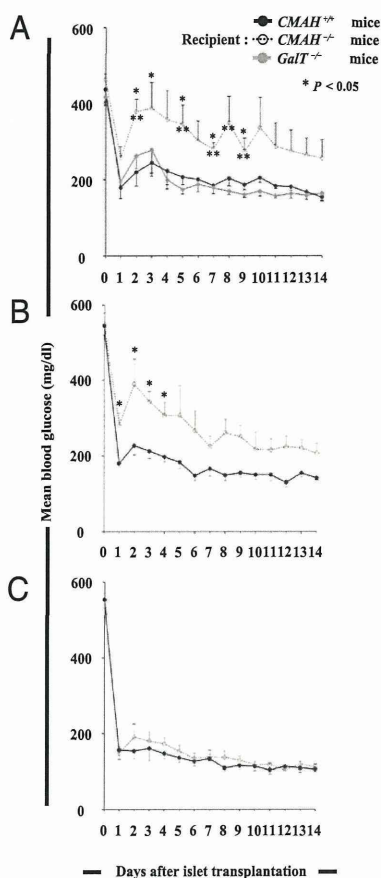


FIGURE 5. Glycemic responses to islet transplantation. Streptozotocin-induced diabetic *CMAH*^{+/+} mice, *CMAH*^{-/-} mice having anti-NeuGc Abs, or *GalT*^{-/-} mice having anti-Gal Abs received kidney capsule transplants of 200 (A), 350 (B), or 500 iEQs (C) obtained from *CMAH*^{+/+} mice [*n* = 5 for each mouse strain receiving 200 or 350 iEQs (A, B), and *n* = 4 for each mouse strain receiving 500 iEQs (C)]. The blood glucose (mg/dl) measurements of mice are expressed as the mean \pm SEM. **p* < 0.05 (compared with *CMAH*^{+/+} recipients); ***p* < 0.05 (compared with *GalT*^{-/-} recipients).

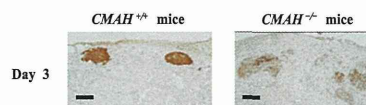


FIGURE 6. Immunohistochemical staining for insulin in the islet grafts. The islet grafts were harvested at day 3 posttransplantation from control *CMAH*^{+/+} or *CMAH*^{-/-} recipient mice receiving 200 iEQs from *CMAH*^{+/+} mice (*n* = 3, each mouse strain). When compared with the control *CMAH*^{+/+} recipient mice, the representative histological findings show a significant decrease in insulin-producing viable β cells in the islet grafts of *CMAH*^{-/-} recipient mice. Original magnification \times 200. Scale bars, 100 μ m.

Binding of NeuGc determinants on the endothelium of vascularized xenograft hearts to the corresponding Abs did not deteriorate their rejection

To examine the immunogenicity of NeuGc epitopes on the endothelium of vascularized organ grafts, we transplanted NeuGc-expressing heart grafts from either syngeneic *CMAH*^{+/+} B6 mice or xenogeneic neonatal F344 rats into either *CMAH*^{-/-} mice having anti-NeuGc Abs or control *CMAH*^{+/+} mice lacking anti-NeuGc Abs. The survival curves of the grafted hearts are shown in Fig. 7. WT B6 hearts survived indefinitely in *CMAH*^{-/-} mouse recipients (>100 d). In contrast, rat heart xenografts were rejected in *CMAH*^{-/-} recipients and control *CMAH*^{+/+} recipients with equal tempo. Even when rabbit complement was injected into these recipient animals to promote complement-dependent cytotoxicity, the tempo of rat heart xenograft rejection in *CMAH*^{+/+} and *CMAH*^{-/-} recipients did not differ. Thus, binding of the NeuGc determinants on the endothelium of vascularized xenografts to the corresponding Abs did not accelerate their rejection.

Discussion

In this study, we demonstrated that NeuGc epitopes were absent from the vascular endothelium and other tissues of *CMAH*^{-/-} mice. These animals, like humans, developed anti-NeuGc Abs, which are normally absent in WT *CMAH*^{+/+} mice, as a consequence of immunization with NeuGc-expressing cells. They thus provide a model for evaluating the immunogenic property of NeuGc determinants as targets of the corresponding Abs in *CMAH*^{+/+}-to-*CMAH*^{-/-} transplantation. Previous studies on the transplantation of *GalT*^{-/-} pig hearts into baboons have revealed that following transplantation, acute humoral xenograft rejection develops, and that this rejection is associated with the presence of

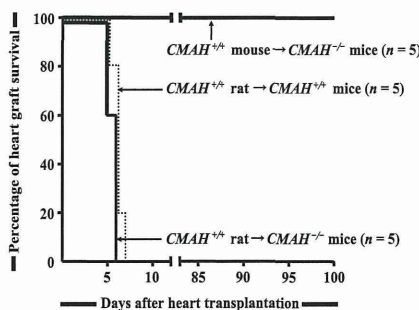


FIGURE 7. The survival curves of grafted hearts. To examine the immunogenicity of NeuGc epitopes in the endothelium of vascularized organ grafts, we transplanted NeuGc-expressing heart grafts from neonatal xenogeneic rats to either *CMAH*^{+/+} or *CMAH*^{-/-} mice. Eight weeks before heart transplantation, the recipient mice were i.p. immunized with cells of the NeuGc-expressing porcine endothelial cell line MYP30 to induce anti-NeuGc Abs. We also transplanted syngeneic heart grafts from *CMAH*^{+/+} to *CMAH*^{-/-} B6 mice that were immunized with the porcine MYP30 cells. The survival curves of the grafted hearts are shown (each *n* = 5).

performed or elicited Abs to non-Gal epitopes on *GalT*^{-/-} pigs (30, 31). However, the non-Gal Abs observed in the *GalT*^{-/-} pig-to-baboon transplantation model cannot be associated with anti-NeuGc Abs. It is very likely that anti-NeuGc Abs will prove to be important in the clinical application of xenotransplantation, but only after the acute humoral xenograft rejection in pig-to-baboon xenotransplantation is overcome.

To evaluate anti-Gal Abs, *GalT*^{-/-} mice have been used as a small animal model (24, 26, 32, 33). These mice produce anti-Gal natural Abs (NAbs), similar to humans (24, 32) and *GalT*^{-/-} pigs (34), although the baseline level of production of anti-Gal Abs in *GalT*^{-/-} mice is lower than that in humans (35). Because NAbs against Gal are thought to develop as a result of exposure to environmental bacteria that express the Gal determinant (36), the lower levels of anti-Gal NAbs in *GalT*^{-/-} mice may be due to lower levels of environmental stimulation by the gastrointestinal bacterial flora within the experimental animal facilities (35). In contrast, anti-NeuGc NAbs were undetectable in *CMAH*^{-/-} mice without stimulation and even upon similar environmental stimulation, suggesting the lower antigenicity of NeuGc epitopes compared with Gal epitopes. Anti-NeuGc Abs were, however, elicited to a certain degree by immunization with NeuGc-expressing syngeneic cells. The production of anti-NeuGc Abs was more pronounced when *CMAH*^{-/-} mice were immunized with NeuGc-expressing xenogeneic cells (Fig. 2). A similar trend of Ab production against Gal in *GalT*^{-/-} mice has been observed in previous studies (37, 38). These findings suggest that the production of anti-NeuGc and anti-Gal Abs might be facilitated by the activation of T cells responding to immunogenic xenopeptides on glycoproteins containing NeuGc and Gal epitopes. Such a distinctive contribution of T cells might be relevant to the biased class switching of anti-NeuGc Abs [i.e., IgG1 and IgG3 isotypes were dominantly detected among anti-NeuGc IgGs, resembling the anti-Gal IgG subclasses produced in *GalT*^{-/-} mice (35)]. Considering the fact that IgM and IgG3 are potent activators of complement (39), this complement-fixing isotype of anti-NeuGc Abs is likely to contribute to humoral rejection. Consistently, we have demonstrated that anti-NeuGc Abs obtained from *CMAH*^{-/-} mice or healthy human volunteers initiate complement-mediated lysis against *CMAH*^{+/+} cells in vitro (Fig. 3A, 3B). In addition, *CMAH*^{-/-} mice completely rejected NeuGc-expressing splenocytes transplanted from syngeneic *CMAH*^{+/+} mice (Fig. 3C, 3D). In that in vivo model, the other dominant isotype of anti-NeuGc IgG in the sera of *CMAH*^{-/-} mice (i.e., noncomplement-fixing IgG1) might also contribute to the rejection of NeuGc-expressing cells, probably through Ab-dependent cell-mediated cytotoxicity. This assumption is supported by the fact that noncomplement-fixing isotypes of IgG Abs in human sera have the ability to lyse porcine endothelium via Ab-dependent cell-mediated cytotoxicity (40). Further studies are needed to clarify this issue. Nevertheless, the immunogenic property of NeuGc determinants is defined in this study as targets of the corresponding Abs in *CMAH*^{+/+}-to-*CMAH*^{-/-} cell transplantation.

Islet transplantation is thought to be the most feasible clinical application of pig-to-human xenotransplantation. In a previous study, the expression of NeuGc epitopes on islets from adult or neonatal pigs was clearly detected, and the antigenicity of adult pig islets or neonatal porcine islet-like cell clusters was found to be mainly associated with N-linked sugars, including NeuGc epitopes, but not Gal Ags (41–44). These findings led us to investigate the immunogenicity of NeuGc epitopes located on the islets by using a *CMAH*^{+/+}-to-*CMAH*^{-/-} mouse islet transplantation model. As shown in Figs. 4–6, *CMAH*^{-/-} mice rejected the NeuGc-expressing islets transplanted from *CMAH*^{+/+} mice. This result provides further insight into the non-Gal Ab-mediated rejection of porcine islet xenografts and suggests that the genetic

manipulation of porcine cells for NeuGc expression could be a novel approach for attenuating non-Gal Ab-mediated islet xenograft rejection. As a further model to investigate xenogeneic setting, we carried out rat-to-*CMAH*^{-/-} mouse islet transplantation in a separate experiment. However, the rat islet xenografts were rejected in *CMAH*^{-/-} recipients and control *CMAH*^{+/+} recipients with equal tempo (Supplemental Fig. 1A). This result could be due to the much lower expression of NeuGc epitopes in rat islets than in porcine and mouse islets (Supplemental Fig. 1B, 1C), indicating that rat-to-*CMAH*^{-/-} islet transplantation could not be a relevant model to investigate anti-NeuGc Ab-mediated rejection.

In contrast, *CMAH*^{-/-} mice did not reject vascularized NeuGc-expressing heart grafts, even if exogenous rabbit complement was added (Fig. 7). We have previously described the rejection of *GalT*^{+/+} mouse or rat hearts by *GalT*^{-/-} mice and demonstrated this to be a model of HAR, which would occur in pig-to-human/ primate xenotransplantation, where anti-Gal Abs and complement mediate HAR (26, 35). The different outcome of *CMAH*^{+/+} heart grafts in *CMAH*^{-/-} and *GalT*^{-/-} mice indicates that the anti-NeuGc Ab-mediated immune response may not be crucially involved in the graft loss in xenogeneic organ transplantation. However, further investigations using organ xenotransplantation models other than the heart should be undertaken to clearly establish whether it is necessary to eliminate NeuGc epitopes because the expression of NeuGc is thought to be dependent on the tissue and developmental stage (28).

Acknowledgments

We thank Dr. Megan Sykes for helpful review of the manuscript, Drs. Kohei Ishiyama, Masayuki Shishida, Toshimitsu Irei, Masahiro Ohira, and Masataka Banshodani for advice and encouragement, Yuka Igarashi, Midori Kiyokawa, and Yuko Ishida for technical assistance, and Dr. Syuji Miyagawa for providing porcine MYP30 cells.

Disclosures

The authors have no financial conflicts of interest.

References

- Sachs, D. H. 1994. The pig as a xenograft donor. *Pathol. Biol. (Paris)* 42: 217–219.
- Galili, U., E. A. Rachmilewitz, A. Peleg, and I. Flechner. 1984. A unique natural human IgG antibody with anti- α -galactosyl specificity. *J. Exp. Med.* 160: 1519–1531.
- Galili, U., B. A. Macher, J. Buehler, and S. B. Shohet. 1985. Human natural anti- α -galactosyl IgG. II. The specific recognition of $\alpha(1 \rightarrow 3)$ -linked galactose residues. *J. Exp. Med.* 162: 573–582.
- Ye, Y., F. A. Neethling, M. Niekraz, E. Koren, S. V. Richards, M. Martin, S. Kosanke, R. Oriol, and D. K. Cooper. 1994. Evidence that intravenously administered α -galactosyl carbohydrates reduce baboon serum cytotoxicity to pig kidney cells (PK15) and transplanted pig hearts. *Transplantation* 58: 330–337.
- Xu, Y., T. Lorf, T. Sablinski, P. Gianello, M. Bailin, R. Monroy, T. Kozlowski, M. Awwad, D. K. Cooper, and D. H. Sachs. 1998. Removal of anti-porcine natural antibodies from human and nonhuman primate plasma in vitro and in vivo by a $\alpha(1-3)\text{Gal}\beta(1-4)\text{Glc-X}$ immunoaffinity column. *Transplantation* 65: 172–179.
- Phelps, C. J., C. Koike, T. D. Vaught, J. Boone, K. D. Wells, S. H. Chen, S. Ball, S. M. Specht, I. A. Polejaeva, J. A. Monahan, et al. 2003. Production of $\alpha(1,3)$ -galactosyltransferase-deficient pigs. *Science* 299: 411–414.
- Lai, L., D. Kolber-Simonds, K. W. Park, H. T. Cheong, J. L. Greenstein, G. S. Im, M. Samuel, A. Bonk, A. Rieke, B. N. Day, et al. 2002. Production of $\alpha(1,3)$ -galactosyltransferase knockout pigs by nuclear transfer cloning. *Science* 295: 1089–1092.
- Kuwaki, K., Y. L. Tseng, F. J. Dor, A. Shimizu, S. L. Houser, T. M. Sanderson, C. J. Lancos, D. D. Prabharasuth, J. Cheng, K. Moran, et al. 2005. Heart transplantation in baboons using $\alpha(1,3)$ -galactosyltransferase gene-knockout pigs as donors: initial experience. *Nat. Med.* 11: 29–31.
- Yamada, K., K. Yazawa, A. Shimizu, T. Iwanaga, Y. Hisashi, M. Nuhn, P. O'Malley, S. Nobori, P. A. Vagefi, C. Patience, et al. 2005. Marked prolongation of porcine renal xenograft survival in baboons through the use of $\alpha(1,3)$ -galactosyltransferase

- gene-knockout donors and the cotransplantation of vascularized thymic tissue. *Nat. Med.* 11: 32–34.
10. Chen, G., H. Qian, T. Starzl, H. Sun, B. Garcia, X. Wang, Y. Wise, Y. Liu, Y. Xiang, L. Copeman, et al. 2005. Acute rejection is associated with antibodies to non-Gal antigens in baboons using Gal-knockout pig kidneys. *Nat. Med.* 11: 1295–1298.
 11. Cooper, D. K. 1998. Xenoantigens and xenoantibodies. *Xenotransplantation* 5: 6–17.
 12. Ezzelarab, M., D. Ayares, and D. K. Cooper. 2005. Carbohydrates in xenotransplantation. *Immunol. Cell Biol.* 83: 396–404.
 13. Higashi, H., M. Naiki, S. Matuo, and K. Okouchi. 1977. Antigen of “serum sickness” type of heterophile antibodies in human sera: identification as gangliosides with N-glycolylneuraminic acid. *Biochem. Biophys. Res. Commun.* 79: 388–395.
 14. Merrick, J. M., R. Schifferle, K. Zadarlik, K. Kano, and F. Milgrom. 1977. Isolation and partial characterization of the heterophile antigen of infectious mononucleosis from bovine erythrocytes. *J. Supramol. Struct.* 6: 275–290.
 15. Merrick, J. M., K. Zadarlik, and F. Milgrom. 1978. Characterization of the Hanganutziu-Deicher (serum-sickness) antigen as gangliosides containing n-glycolylneuraminic acid. *Int. Arch. Allergy Appl. Immunol.* 57: 477–480.
 16. Morozumi, K., T. Kobayashi, T. Usami, T. Oikawa, Y. Ohtsuka, M. Kato, O. Takeuchi, K. Koyama, H. Matsuda, I. Yokoyama, and H. Takagi. 1999. Significance of histochemical expression of Hanganutziu-Deicher antigens in pig, baboon and human tissues. *Transplant. Proc.* 31: 942–944.
 17. Zhu, A., and R. Hurst. 2002. Anti-N-glycolylneuraminic acid antibodies identified in healthy human serum. *Xenotransplantation* 9: 376–381.
 18. Miwa, Y., T. Kobayashi, T. Nagasaka, D. Liu, M. Yu, I. Yokoyama, A. Suzuki, and A. Nakao. 2004. Are N-glycolylneuraminic acid (Hanganutziu-Deicher) antigens important in pig-to-human xenotransplantation? *Xenotransplantation* 11: 247–253.
 19. Tangvoranuntakul, P., P. Gagneux, S. Diaz, M. Bardor, N. Varki, A. Varki, and E. Muchmore. 2003. Human uptake and incorporation of an immunogenic nonhuman dietary sialic acid. *Proc. Natl. Acad. Sci. USA* 100: 12045–12050.
 20. Varki, A. 2001. Loss of N-glycolylneuraminic acid in humans: Mechanisms, consequences, and implications for hominid evolution. *Am. J. Phys. Anthropol. (Suppl 33)*: 54–69.
 21. Saethre, M., B. C. Baumann, M. Fung, J. D. Seebach, and T. E. Mollnes. 2007. Characterization of natural human anti-non-gal antibodies and their effect on activation of porcine gal-deficient endothelial cells. *Transplantation* 84: 244–250.
 22. Bouhours, D., C. Pourcel, and J. E. Bouhours. 1996. Simultaneous expression by porcine aorta endothelial cells of glycosphingolipids bearing the major epitope for human xenoreactive antibodies (Gal α 1-3Gal), blood group H determinant and N-glycolylneuraminic acid. *Glycoconj. J.* 13: 947–953.
 23. Naito, Y., H. Takematsu, S. Koyama, S. Miyake, H. Yamamoto, R. Fujinawa, M. Sugai, Y. Okuno, G. Tsujimoto, T. Yamaji, et al. 2007. Germinal center marker GL7 probes activation-dependent repression of N-glycolylneuraminic acid, a sialic acid species involved in the negative modulation of B-cell activation. *Mol. Cell. Biol.* 27: 3008–3022.
 24. Thall, A. D., P. Malý, and J. B. Lowe. 1995. Oocyte Gal α 1,3Gal epitopes implicated in sperm adhesion to the zona pellucida glycoprotein ZP3 are not required for fertilization in the mouse. *J. Biol. Chem.* 270: 21437–21440.
 25. Miyagawa, S., R. Shirakura, K. Iwata, S. Nakata, G. Matsumiya, H. Izutani, H. Matsuda, A. Terado, M. Matsumoto, S. Nagasawa, et al. 1994. Effects of transfected complement regulatory proteins, MCP, DAF, and MCP/DAE hybrid, on complement-mediated swine endothelial cell lysis. *Transplantation* 58: 834–840.
 26. Ohdan, H., Y. G. Yang, A. Shimizu, K. G. Swenson, and M. Sykes. 1999. Mixed chimerism induced without lethal conditioning prevents T cell- and anti-Gal α 1,3Gal-mediated graft rejection. *J. Clin. Invest.* 104: 281–290.
 27. Kobayashi, T., I. Yokoyama, A. Suzuki, M. Abe, S. Hayashi, H. Matsuda, K. Morozumi, M. E. Breimer, L. Rydberg, C. G. Groth, et al. 2000. Lack of antibody production against Hanganutziu-Deicher (H-D) antigens with N-glycolylneuraminic acid in patients with porcine exposure history. *Xenotransplantation* 7: 177–180.
 28. Malykh, Y. N., L. Shaw, and R. Schauer. 1998. The role of CMP-N-acetylneuraminic acid hydroxylase in determining the level of N-glycolylneuraminic acid in porcine tissues. *Glycoconj. J.* 15: 885–893.
 29. Diswall, M., J. Angström, H. J. Schuurman, F. J. Dor, L. Rydberg, and M. E. Breimer. 2007. Studies on glycolipid antigens in small intestine and pancreas from α 1,3-galactosyltransferase knockout miniature swine. *Transplantation* 84: 1348–1356.
 30. Hisashi, Y., K. Yamada, K. Kuwaki, Y. L. Tseng, F. J. Dor, S. L. Houser, S. C. Robson, H. J. Schuurman, D. K. Cooper, D. H. Sachs, et al. 2008. Rejection of cardiac xenografts transplanted from α 1,3-galactosyltransferase gene-knockout (GalT-KO) pigs to baboons. *Am. J. Transplant.* 8: 2516–2526.
 31. Tseng, Y. L., K. Moran, F. J. Dor, T. M. Sanderson, W. Li, C. J. Lancos, H. J. Schuurman, D. H. Sachs, and D. K. Cooper. 2006. Elicited antibodies in baboons exposed to tissues from α 1,3-galactosyltransferase gene-knockout pigs. *Transplantation* 81: 1058–1062.
 32. Thall, A. D., H. S. Murphy, and J. B. Lowe. 1996. α 1,3-Galactosyltransferase-deficient mice produce naturally occurring cytotoxic anti-Gal antibodies. *Transplant. Proc.* 28: 556–557.
 33. Yang, Y. G., E. deGoma, H. Ohdan, J. L. Bracy, Y. Xu, J. Iacomini, A. D. Thall, and M. Sykes. 1998. Tolerization of anti-Gal α 1-3Gal natural antibody-forming B cells by induction of mixed chimerism. *J. Exp. Med.* 187: 1335–1342.
 34. Dor, F. J., Y. L. Tseng, J. Cheng, K. Moran, T. M. Sanderson, C. J. Lancos, A. Shimizu, K. Yamada, M. Awwad, D. H. Sachs, et al. 2004. α 1,3-Galactosyltransferase gene-knockout miniature swine produce natural cytotoxic anti-Gal antibodies. *Transplantation* 78: 15–20.
 35. Ohdan, H., K. G. Swenson, H. Kitamura, Y. G. Yang, and M. Sykes. 2001. Tolerization of Gal α 1,3Gal-reactive B cells in pre-sensitized α 1,3-galactosyltransferase-deficient mice by nonmyeloablative induction of mixed chimerism. *Xenotransplantation* 8: 227–238.
 36. Auchincloss, H., Jr., and D. H. Sachs. 1998. Xenogeneic transplantation. *Annu. Rev. Immunol.* 16: 433–470.
 37. Ohdan, H., K. G. Swenson, H. S. Kruger Gray, Y. G. Yang, Y. Xu, A. D. Thall, and M. Sykes. 2000. Mac-1-negative B-1b phenotype of natural antibody-producing cells, including those responding to Gal α 1,3Gal epitopes in α 1,3-galactosyltransferase-deficient mice. *J. Immunol.* 165: 5518–5529.
 38. Tanemura, M., D. Yin, A. S. Chong, and U. Gallili. 2000. Differential immune responses to α -gal epitopes on xenografts and allografts: implications for accommodation in xenotransplantation. *J. Clin. Invest.* 105: 301–310.
 39. Schreiber, J. R., L. J. Cooper, S. Diehn, P. A. Dahlhauser, M. F. Tosi, D. D. Glass, M. Patawaran, and N. S. Greenspan. 1993. Variable region-identical monoclonal antibodies of different IgG subclass directed to *Pseudomonas aeruginosa* lipopolysaccharide O-specific side chain function differently. *J. Infect. Dis.* 167: 221–226.
 40. Schaapherder, A. F., M. R. Daha, M. T. te Bulte, F. J. van der Woude, and H. G. Gooszen. 1994. Antibody-dependent cell-mediated cytotoxicity against porcine endothelium induced by a majority of human sera. *Transplantation* 57: 1376–1382.
 41. McKenzie, I. F., M. Koulmanda, T. E. Mandel, and M. S. Sandrin. 1998. Pig islet xenografts are susceptible to “anti-pig” but not Gal α (1,3)Gal antibody plus complement in Gal o/o mice. *J. Immunol.* 161: 5116–5119.
 42. Komoda, H., S. Miyagawa, T. Kubo, E. Kitano, H. Kitamura, T. Omori, T. Ito, H. Matsuda, and R. Shirakura. 2004. A study of the xenoantigenicity of adult pig islets cells. *Xenotransplantation* 11: 237–246.
 43. Komoda, H., S. Miyagawa, T. Omori, Y. Takahagi, H. Murakami, T. Shigeheisa, T. Ito, H. Matsuda, and R. Shirakura. 2005. Survival of adult islet grafts from transgenic pigs with N-acetylglucosaminyltransferase-III (GnT-III) in cynomolgus monkeys. *Xenotransplantation* 12: 209–216.
 44. Omori, T., T. Nishida, H. Komoda, Y. Fumimoto, T. Ito, Y. Sawa, C. Gao, S. Nakatsu, R. Shirakura, and S. Miyagawa. 2006. A study of the xenoantigenicity of neonatal porcine islet-like cell clusters (NPCC) and the efficiency of adenovirus-mediated DAF (CD55) expression. *Xenotransplantation* 13: 455–464.

Alterations in portal vein blood pH, hepatic functions, and hepatic histology in a porcine carbon dioxide pneumoperitoneum model

Makoto Yoshida · Satoshi Ikeda · Daisuke Sumitani · Yuji Takakura ·
Masanori Yoshimitsu · Manabu Shimomura · Midori Noma ·
Masakazu Tokunaga · Masazumi Okajima · Hideki Ohdan

Received: 30 June 2009 / Accepted: 15 November 2009 / Published online: 7 January 2010
© Springer Science+Business Media, LLC 2010

Abstract

Background Intra-abdominal high pressure and acidosis by carbon dioxide (CO₂) pneumoperitoneum is known to affect various organ functions. In this study, changes in liver functions and liver histology were investigated during CO₂ pneumoperitoneum in a large animal model.

Methods Fourteen white pigs were anesthetized with intubation and controlled ventilation. The pigs in the pneumoperitoneum group (PG) were exposed to CO₂ pneumoperitoneum at an intra-abdominal pressure of 8 mmHg, and those in the open laparotomy group (OG) were subjected to laparotomy. Hemodynamics were measured and liver function tests were performed in the carotid artery and portal vein, and the liver tissue was histologically examined.

Results The blood pressure, PO₂, PCO₂, and pH in the carotid artery did not significantly differ between the groups. In the PG, blood pressure, PO₂, and PCO₂ in the portal vein were elevated while the pH was low. There were no significant differences in the levels of aminotransferases and lactate between the groups. In the PG, the arterial ketone body ratio (AKBR) was low at 90 min and

the ICG retention rate was high at 180 min; these values differed significantly compared to those at 0 min. Histological examination revealed liver congestion in the PG and no significant change in the OG. In the PG, the TUNEL assay revealed positive staining in the area with focal lytic changes.

Conclusions CO₂ pneumoperitoneum at an intra-abdominal pressure of 8 mmHg in a porcine model affected liver functions and caused histological changes in the liver. Although it is uncertain whether these alterations observed in the porcine liver occur in humans as well and whether the alterations are reversible after pneumoperitoneum, it may be necessary to pay attention to liver damage during laparoscopic surgery.

Keywords Carbon dioxide pneumoperitoneum · Laparoscopic surgery · Liver function · Liver histology

In the last decade there has been a considerable increase in the use of laparoscopic surgery for the treatment of a number of diseases. Laparoscopic surgery is believed to be a less invasive procedure than conventional open surgery because patients who undergo laparoscopic surgery experience less pain and have short hospital stays and superior cosmesis compared to those who undergo open surgery [1].

During laparoscopic surgery in humans, a pneumoperitoneum is generally created by the continuous insufflation of CO₂ into the peritoneal cavity at an intra-abdominal pressure of 8–12 mmHg. It is now commonly recognized that in animals and humans, a CO₂ pneumoperitoneum that increases intra-abdominal pressure and CO₂ absorption from the peritoneal cavity has several effects not only on inflammatory and immune reactions [2, 3], but also on organ function, including cardiovascular, respiratory, liver,

M. Yoshida · D. Sumitani · Y. Takakura · M. Yoshimitsu ·
M. Shimomura · M. Noma · M. Tokunaga · H. Ohdan
Department of Surgery, Division of Frontier Medical Science,
Programs for Biomedical Research, Graduate School of
Biomedical Sciences, Hiroshima University, Hiroshima, Japan

S. Ikeda (✉) · M. Okajima
Department of Endoscopic Surgery and Surgical Science,
Graduate School of Biomedical Sciences, Hiroshima University,
1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan
e-mail: sikeda@hiroshima-u.ac.jp

and renal function [4–6]. The mechanisms underlying the functional changes in the cardiovascular system during pneumoperitoneum have been studied in detail; however, the mechanisms underlying changes in liver function are still unclear. Several studies have reported that the levels of liver enzymes increase in patients undergoing laparoscopic surgery [7–9]; however, unaltered liver function has been also reported [10, 11]. Furthermore, various studies have reported different changes in the hepatic blood flow during pneumoperitoneum [12–16]. The differences in the duration of pneumoperitoneum and intra-abdominal pressure during pneumoperitoneum between the experimental conditions and clinical settings may result in these different outcomes [10].

Thus, the advantages and disadvantages of laparoscopic surgery with regard to liver function remain controversial in both experimental and clinical studies. In this study we investigated the effect of CO₂ pneumoperitoneum on the functions and histology of the liver in a porcine model.

Materials and methods

Experimental design

This study was carried out in accordance with the guidelines recommended by the Committee of Animal Experimentation, Hiroshima University, and the Committee of Research Facilities for Laboratory Animal Science, Natural Science Center for Basic Research and Development (N-BARD), Hiroshima University. For this study, we used 14 specific-pathogen-free, white, male pigs (mean weight = 35.4 kg; range = 35–36 kg). They were randomly allocated to two equivalent groups: the open laparotomy group (OG) and the pneumoperitoneum group (PG). Each pig was made to fast overnight. The following morning the pigs were premedicated with ketamine (10 mg/kg), desperado (0.1 mg/kg), and atropine (0.05 mg/kg) via the intramuscular route. The animals were then orotracheally intubated and mechanically ventilated, maintaining 100% oxygen inspiration (FiO₂) with a tidal volume of 10–15 ml/kg at the rate of 15 breaths/min. Anesthesia was maintained with isoflurane inhalation. Oxygen saturation was continuously monitored. Ringer's lactate solution was administered intravenously at 100 ml/h by varying the infusion rate, when necessary, through a femoral vein line. The animals were kept in the supine decubitus position throughout the experiment. The right carotid artery was cannulated for the collection of arterial blood samples and for recording the heart rate and arterial blood pressure. A midline abdominal incision of 15 and 25 cm was made for the PG and the OG, respectively. In both groups, a liver tissue biopsy sample was obtained from the right lobe for

histological analysis. At the same time, the portal vein was cannulated for obtaining portal vein blood samples and for recording the portal vein pressure. A 12-mm supraumbilical trocar was inserted, and a laser Doppler flowmeter probe was introduced into the peritoneal cavity through the trocar. After the preparation, the midline incision was closed in the PG and a pneumoperitoneum was created by injecting CO₂ and maintained at a pressure of 8 mmHg. In the OG, the laparotomy was kept open during the experiment. On completion of the experiment, the animals were euthanized with an intravenous injection of potassium chloride.

Blood sampling

Serial blood gas samples were obtained during the experiment from an arterial cannula placed in the carotid artery or the portal vein. In the OG, the first blood sample was obtained after the laparotomy and in the PG after the closing of the midline incision but before insufflation of CO₂. Blood sampling was carried out at 15, 30, 45, 60, 90, 120, and 180 min. Blood gas analysis was performed immediately in the operating room. Plasma was obtained by centrifuging the blood samples and was stored at –20°C until analysis. Alanine aminotransferase (ALT) levels, aspartate aminotransferase (AST) levels, and the arterial ketone body ratio (AKBR) were determined by standard methods.

Indocyanine green retention test

Indocyanine green (ICG) (1 mg/kg) was injected into the portal vein 15 min before and 180 min after laparotomy in the OG and 15 min before and 180 min after insufflation of CO₂ in the PG. Blood samples were obtained from the carotid artery before injection of ICG and at 5, 10, and 15 min after the injection. Pharmacokinetic parameters were calculated by using a computer program.

Hemodynamic measurements

The baseline hemodynamic measurements were obtained after laparotomy in the OG and after CO₂ insufflation in the PG. Next, measurements were performed at 15, 30, 45, 60, 90, 120, and 180 min. At each time point, the mean carotid arterial blood pressure and portal vein blood pressure were recorded by calibrated pressure transducers.

Liver blood flow measurement

A laser Doppler flowmeter (ALF21, Advance Company Ltd., Tokyo, Japan) was used for measuring liver tissue