outcomes of anatomical subsegmentectomy and non-anatomical minor hepatectomy for single HCC, based on a Japanese nationwide survey, recommends anatomical resection, especially when the size of HCC ranges 2-5 cm[19]. In our study, non-anatomical resection was a significantly poor prognostic factor in disease-free survival and tended to be a poor prognostic factor in cumulative survival in patients with PVI-S. Therefore, anatomical resection is preferable in patients with HCC accompanied by PVI. Recent studies have demonstrated that HCC with PVI can be predicted by several factors[11-15]. Therefore, patients with HCC that have high risk factors for PVI preoperatively should be recommended for anatomical resection. To clarify this hypothesis, further examination is necessary.

In PVI-M patients, there was no significant difference in the outcome between anatomical and non-anatomical resection. Recently, in patients with portal vein thrombi in major portal branches, adjuvant chemotherapy has been reported to be effective following hepatectomy and thrombectomy[16,17,20,21]. Patients with HCC and PVI-M may also be good candidates for adjuvant chemotherapy.

In conclusion, in patients with HCC, accompanied by microscopic PVI, the presence of PVI-M is a poor prognostic factor. In PVI-S patients, anatomical resection is preferable to non-anatomical resection. Patients with HCC and PVI-M may be good candidates for adjuvant chemotherapy. Further studies aimed at improving the outcome of patients with PVI after hepatectomy are necessary.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is a malignant tumor with periportal venous metastasis. Vascular invasion, especially portal vein invasion (PVI), is a major determinant of outcome after hepatic resection in patients with HCC. Nevertheless, the prognostic factors of HCC with microscopic PVI have remained elusive and the operative procedures for this type of HCC have not been determined.

Research frontiers

The prognostic factors in cumulative and disease-free survival in patients with HCC, accompanied by microscopic PVI, were evaluated in the present study.

Innovations and breakthroughs

The presence of multiple PVI (PVI-M) was a poor prognostic factor in patients with HCC, accompanied by microscopic PVI. Anatomical resection is recommended in these patients with HCC. Patients with HCC with PVI-M may be good candidates for adjuvant chemotherapy.

Applications

In patients with HCC, accompanied by microscopic PVI, the presence of PVI–M is a poor prognostic factor. In solitary PVI patients, anatomical resection is preferable to non-anatomical resection. Patients with HCC and PVI-M may be good candidates for adjuvant chemotherapy.

Terminology

When clusters of cancer cells were present in the extra tumoral portal vein, accompanied with bile duct and hepatic artery, it was defined as positive for extra tumoral PVI. When more than two clusters of cancer cells (PVI) were present in different portal vein branches, it was defined as PVI-M. When only one cluster was present in a single portal vein branch, it was defined as solitary PVI.

Peer review

This paper was correctly planned to investigate the prognostic factors in patients with HCC accompanied by microscopic PVI. This research is of clinical importance in post-resection management of patients with HCC. The results provide robust clinical evidence to suggest firm scientific conclusions.

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Technical standardization of laparoscopic splenectomy harmonized with hand-assisted laparoscopic surgery for patients with liver cirrhosis and hypersplenism

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Abstract
Background/purpose The aims of this study were to standardize the techniques of laparoscopic splenectomy (LS) to improve safety in liver cirrhosis patients with portal hypertension.
Methods From 1993 to 2008, 265 cirrhotic patients underwent LS. Child-Pugh class was A in 112 patients, B in 124, and C in 29. Since January 2005, we have adopted the standardized LS including the following three points: hand-assisted laparoscopic surgery (HALS) should be performed in patients with splenomegaly (≧1,000 mL), perisplenic collateral vessels, or Child-Pugh score 9 or more; complete division and sufficient elevation of the upper pole of the spleen should be performed before the splenic hilar division; and when surgeons feel the division of the upper pole of the spleen is too difficult, conversion to HALS should be performed.
Results There were no deaths related to LS in this study. After the standardization, conversion to open surgery significantly reduced from 11 (10.3%) of 106 to 3 (1.9%) of 159 patients ($P < 0.05$). The average operation time and blood loss significantly reduced from 259 to 234 min ($P < 0.01$) and from 506 to 171 g ($P < 0.01$), respectively.
Conclusions With the technical standardization, LS becomes a feasible and safe approach in the setting of liver cirrhosis and portal hypertension.

Keywords Laparoscopic splenectomy · Liver cirrhosis · Hypersplenism · Portal hypertension

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Introduction

In the setting of liver cirrhosis and portal hypertension, splenectomy has been performed either for bleeding tendency due to thrombocytopenia or as part of an operative procedure, such as devascularization of the upper stomach and esophageal transection for the control of variceal hemorrhage [1, 2]. Recently, splenectomy has been performed to improve thrombocytopenia in cirrhotic patients undergoing treatment of hepatocellular carcinoma (HCC) [3, 4] and prior to the induction of pegylated-interferon (IFN) plus ribavirin therapy for hepatitis C infection [5]. Moreover, in living-donor liver transplantation (LDLT), splenectomy has been performed concurrently with liver transplantation to reduce graft congestion, and after liver transplantation to alleviate persistent thrombocytopenia [6–8]. Splenectomy is of increasing importance for patients with liver cirrhosis and hypersplenism. Therefore, if splenectomy could be performed safely and less invasively with a laparoscopic procedure, cirrhotic patients with portal hypertension would have an opportunity to receive a wider range of treatments for liver cirrhosis.

Laparoscopic splenectomy is widely accepted as a standard treatment for hematologic disorders such as idiopathic thrombocytopenic purpura. It has been shown that laparoscopic splenectomy is superior to open splenectomy in terms of postoperative pain, length of hospital stay, and

perioperative complication [9–11]. Laparoscopic splenectomy is occasionally converted to open splenectomy, and the conversion is often due to massive intraoperative bleeding [12, 13]. Therefore, some authors suggest that patients with liver cirrhosis and portal hypertension are contraindications for laparoscopic splenectomy because of several technical hurdles such as bleeding tendency due to low platelet counts and impaired coagulation factors, splenomegaly, and development of collateral vessels [14, 15]. Our previous study including 73 patients with liver cirrhosis and portal hypertension showed that 7 patients (9.6%) had conversion to open splenectomy, and that the estimated blood loss was 375 g [16]. We consider that although such an outcome is relatively good, the technical hurdles greatly limit the applicability of laparoscopic splenectomy in the setting of liver cirrhosis and portal hypertension to all but experienced surgeons and institution.

Splenomegaly has also been reported to be a contraindication for laparoscopic splenectomy [17–19]. Hand-assisted laparoscopic surgery (HALS), which allows surgeons to use one hand in the intraperitoneal cavity while maintaining the benefits of a minimally invasive procedure, has been applied in the setting of splenomegaly [20, 21]. In addition to recent advances in laparoscopic instrumentation, the use of HALS may overcome the technical hurdles and render laparoscopic splenectomy as a safe procedure in the setting of liver cirrhosis and portal hypertension. To universally perform laparoscopic splenectomy safely on patients with liver cirrhosis and portal hypertension, we standardized the techniques and clarified the indications of HALS for splenectomy.

Patients and methods

Patients

Two hundred and sixty-five patients (132 males and 133 females; Table 1) with liver cirrhosis and portal hypertension underwent laparoscopic splenectomy either in the Department of Surgery and Science, Kyushu University, or in the Department of Surgery, Fukuoka City Hospital, in the period from September 1993 to August 2008. The mean age was 57.7 ± 10.0 years (range, 43–73 years). The etiologies were hepatitis B virus (HBV)-related cirrhosis ($n = 29$), hepatitis C virus (HCV)-related cirrhosis ($n = 193$), HBV and HCV-related cirrhosis ($n = 3$), alcoholic cirrhosis ($n = 16$), primary biliary cirrhosis ($n = 3$), and cirrhosis of unknown origin ($n = 21$). Child-Pugh class was class A in 112 patients, class B in 124, and class C in 29. Eighty-six patients (32.5%) had concomitant HCC. Average preoperative platelet counts were 47 ± 18 ($\times 10^3/\mu L$). Indications for splenectomy included bleeding tendency due to thrombocytopenia (platelet counts $<30 \times 10^3/\mu L$) ($n = 62$), difficulties with therapies for HCC due to thrombocytopenia ($n = 66$), difficulties with induction or continuation of pegylated IFN therapy due to thrombocytopenia ($n = 92$), and severe portal hypertension such as endoscopic treatment-resistant esophagogastric varices, portal hypertensive gastropathy (PHG) bleeding, and refractory ascites ($n = 45$).

Table 1 Patient characteristics

Characteristic	Value
No. of patients (M/F)	265 (132/133)
Age (years)	57.7 ± 10.0
Etiology of cirrhosis	
Viral hepatitis (HCV/HBV/both)	225 (193/29/3)
Alcoholism	16
PBC	3
Unknown	21
Child-Pugh class (A/B/C)	112/124/29
Child-Pugh score	7.1 ± 1.7
Concomitant HCC	86 (32.5%)
Platelet counts ($\times 10^3/\mu L$)	47 ± 18
Indications	
Bleeding tendency (platelet $<30 \times 10^3/\mu L$)	62
Difficulties with therapies for HCC	66
Difficulties with induction or continuation of IFN therapy	92
Severe portal hypertension (endoscopic treatment-resistant esophagogastric varices, PHG bleeding, and refractory ascites)	45

Operative procedures for laparoscopic splenectomy

From 1993 to December 2004, we performed laparoscopic splenectomy on 106 patients with liver cirrhosis and portal hypertension. Eleven (10.3%) of 106 patients had conversion to open splenectomy. We analyzed preoperative patient characteristics, preoperative CT findings, and operative videos to evaluate factors related to conversion to open splenectomy in cirrhotic patients with portal hypertension. Based on these results, we have adopted the following standardized procedures for laparoscopic splenectomy since January 2005.

First, we decided whether the patient should be treated by purely laparoscopic surgery or by HALS. A preoperative multi-detector row CT (MD-CT) was performed for each patient to evaluate the splenic volume and to determine the location and extent of the collateral vessels (Fig. 1a). Patients with huge splenomegaly, the presence of large perisplenic collateral vessels, and/or a Child-Pugh score of 9 or more were treated by HALS (Fig. 1b).

Patients were placed in a semilateral position with the left flank elevated at a 60° angle (Fig. 1c) [16]. A 12-mm laparoscope trocar was inserted through an incision on the left side of the umbilicus, which was made by a minimal open laparotomy. A CO_2-pneumoperitoneum was created using a high-flow electric insufflator, and a laparoscope with 30° forward oblique was introduced through the trocar. Three other trocars were then inserted under visual control into the epigastric area, the midclavicular line of the subcostal line, and the left flank. Regarding the spleen extending below the left subcostal margin, two exterior trocars were placed at the level of the lower pole of the spleen. The hand port for HALS was inserted through a 7-cm midline incision in the epigastric area (Fig. 1d).

Splenic attachments were divided using electrocautery, ultrasound dissector, and/or particularly the LigaSure vessel-sealing system (Valleylab, Boulder, CO, USA) in order as shown in Fig. 1e. Before dissecting the perisplenic attachments, we usually dissected the splenic flexure of colon and mobilized it to visualize the retroperitoneal attachments well. As collateral vessels were often lying along the lower aspects of the spleen, we did not touch the spleno-colic ligaments at the start of the operation. In the majority of the patients, the retroperitoneal splenic attachments were densely covered by small collateral vessels, and they were dissected using the LigaSure vessel-sealing system without bleeding. Although in some of the patients, large collateral vessels were noted along the lower and/or posterior aspects of the spleen overlying the kidney and the tail of the pancreas as shown in Fig. 1a, the spleen could be detached from the retroperitoneum without injuring or dividing these collateral vessels. The splenogastric ligaments were divided using the LigaSure vessel-sealing system without any bleeding. After the upper pole of the spleen was entirely dissected away from the diaphragm, we transected the splenic hilar pedicles with an endoscopic linear vascular stapler (Echelon 60–2.5 mm; Ethicon Endo-Surgery, Cincinnati, OH) (Fig. 1f). Regarding the dissection of spleno-colic ligaments, in cases in which the length of the splenic hilar pedicles was too long for the first stapler, we dissected the spleno-colic ligament to reduce the length of splenic hilar pedicles just before the splenic hilar transection. In cases in which the upper pole of the spleen was not elevated because of insufficient upper division of the spleen, or in which it was difficult for an endoscopic vascular stapler to cross the thick splenic hilar pedicles, we converted from purely laparoscopic surgery to HALS without hesitation (Fig. 1g). The resected spleen was placed into a plastic bag and extracted.

Operative time was measured from initial incision to skin closure. Splenic weight was based on the weight of the morcellated spleen.

Statistical analysis

The characteristics of the patients were compared using the Student's t test for parametric data, the Mann Whitney U test for nonparametric data, and χ^2 test for categorical data. A P value of less than 0.05 was considered to be statistically significant. All values were expressed as the mean ± SD. All calculations were performed using the StatView software package for Windows (version 5.0; SAS Institute, Cary, NC, USA) (see Tables 2, 3).

Results

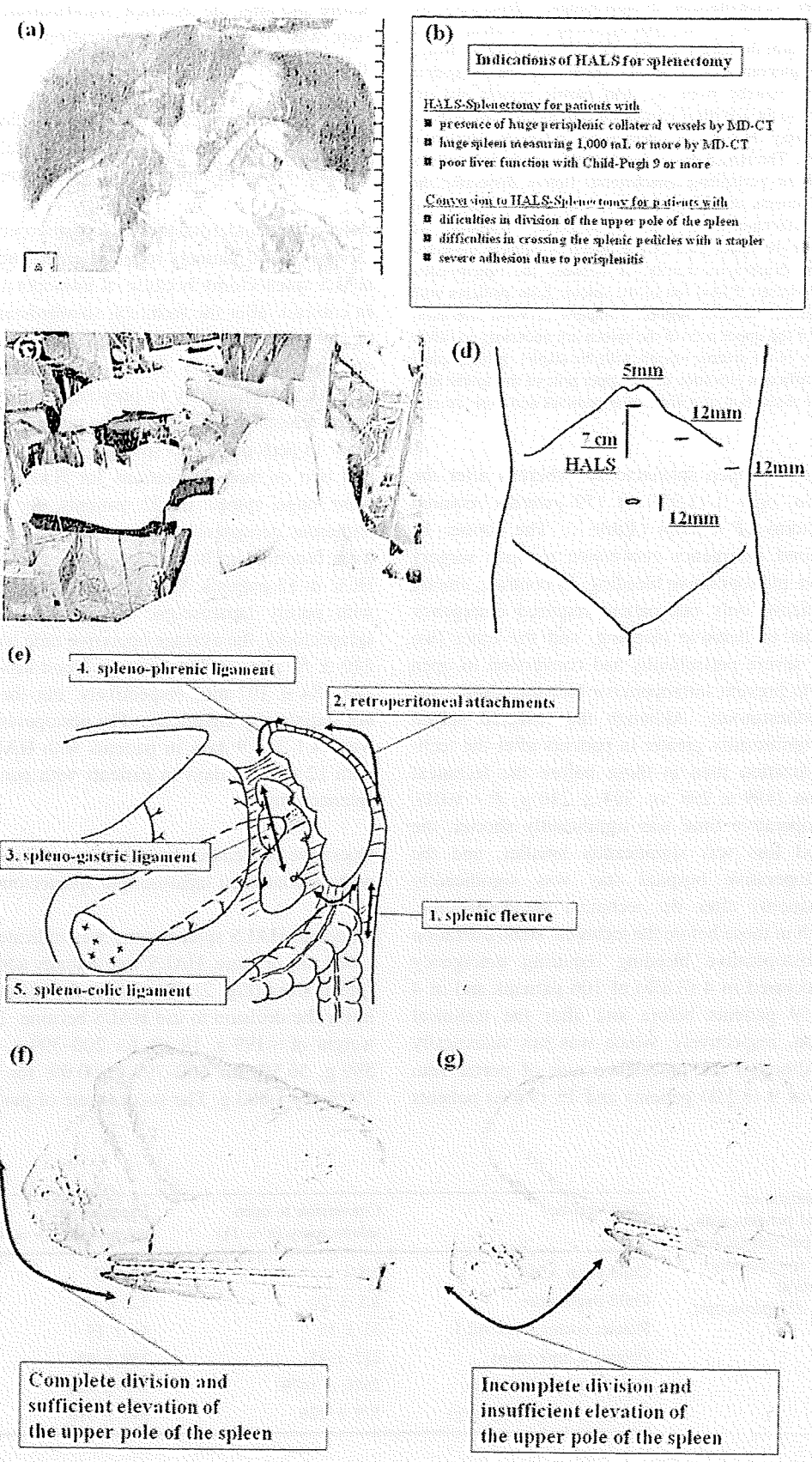
Characteristics related to conversion to open splenectomy in patients with liver cirrhosis and portal hypertension before the technical standardization of laparoscopic splenectomy

As shown in Table 2, conversion to open surgery was significantly related to an exacerbation of liver function as shown by Child-Pugh class ($P < 0.05$) or Child-Pugh score ($P < 0.01$). Resected spleen weight was significantly greater in patients with conversion to open splenectomy than in those without conversion to open surgery ($P < 0.05$).

When we retrospectively evaluated preoperative CT findings of all 11 patients with conversion to open splenectomy, all the patients had both moderate or huge splenomegaly and numerous perisplenic collateral vessels. From a technical standpoint, we analyzed the operative videos of 11 cases with conversion to open splenectomy. Massive bleeding occurred during the division of the splenic hilar pedicles with an endoscopic linear vascular stapler in seven patients and during the dissection of the upper pole of the spleen in four patients. Bleeding site was at the tip of the first staple line in the former patients, in which the first stapler could cross only a part of the splenic hilar pedicles. In the latter patients, there were densely developed collateral vessels including short gastric vessels along the upper aspects of the spleen.

Operative outcome before and after the technical standardization of laparoscopic splenectomy for patients with liver cirrhosis and portal hypertension

In the present study, there were no deaths related to laparoscopic splenectomy in patients with liver cirrhosis and portal hypertension. From 1993 to December 2004, 106 cirrhotic patients with portal hypertension underwent laparoscopic splenectomy, and from January 2005 to August 2008, 159 cirrhotic patients underwent the technically standardized laparoscopic splenectomy. Before the standardization of the procedure, 11 (10.3%) of 106 patients


(a)
(b)
Indications of HALS for splenectomy
HALS-Splenectomy for patients with
presence of huge perisplenic collateral vessels by MD-CT
huge spleen measuring 1,000 mL or more by MD-CT
poor liver function with Child-Pugh 9 or more
Conversion to HALS-Splenectomy for patients with
dificulties in division of the upper pole of the spleen
difficulties in crossing the splenic pedicles with a stapler
severe adhesion due to perisplenitis
(c)
(d)
5mm
12mm
7 cm
HALS
12mm
12mm
(e)
4. spleno-phrenic ligament
2. retroperitoneal attachments
3. spleno-gastric ligament
1. splenic flexure
5. spleno-colic ligament
(f)
(g)
Complete division and sufficient elevation of the upper pole of the spleen
Incomplete division and insufficient elevation of the upper pole of the spleen

◀ **Fig. 1** Technical standardization of laparoscopic splenectomy for patients with liver cirrhosis and portal hypertension. **a** Preoperative evaluation by multi-detector-row CT (MD-CT). MD-CT image showing a huge splenomegaly measuring 900 mL and the perisplenic collateral vessels running from the short gastric vessels and the splenic hilar vein to the left renal vein. **b** Indications for hand-assisted laparoscopic surgery (HALS) for splenectomy. **c** Patient position. **d** Trocar placement. The HALS device is oriented in the upper midline. **e** Dissection of the perisplenic attachments. Before dissecting the perisplenic attachments, the splenic flexure of colon is mobilized. The retroperitoneal attachments and the spleno-gastric ligaments are divided, and then the upper pole of the spleen is entirely dissected away from the diaphragm. Lastly, if needed, the spleno-colic ligaments are dissected. **f** Division of the splenic hilar pedicles with an endoscopic linear vascular stapler. Complete division and sufficient elevation of the upper pole of the spleen are necessary to safely cross the entirety of the splenic vessels with the stapler. **g** Incomplete division and insufficient elevation of the upper pole of the spleen lead to an incomplete dissection of splenic hilar pedicles and raise the risk of bleeding

had conversion to open splenectomy, whereas after the standardization, only 3 (1.9%) of 159 patients required open splenectomy ($P < 0.05$) (Table 3). The former 11 patients required emergency conversion to open surgery due to massive intraoperative bleeding. In contrast, among the latter patients, only one patient required emergency conversion due to massive bleeding, and the other two patients with severe perisplenitis had conversion to open splenectomy to prevent intraoperative bleeding during the perisplenic adhesiotomy. Although the resected spleen weight was significantly greater in patients after the technical standardization than in those before the technical standardization (499 ± 298 vs. 394 ± 210 g; $P < 0.01$), the average operative time was significantly shorter, the average blood loss was significantly smaller, and the average postoperative hospital stay was significantly shorter in patients after the technical standardization, compared with in those before the technical standardization (Table 3). Postoperative bleeding requiring emergency hemostasis occurred in 4 (3.8%) of 106 patients and in 4 (2.5%) of 159 patients before and after the technical standardization, respectively, which was not statistically significant. Although the prevalence rate of portal vein thrombosis was 9 (8.5%) patients and 15 (9.4%) patients before and after the technical standardization, respectively, there was no significant deterioration in the liver function in any of these patients.

Patient characteristics for the determination of operative procedures after the technical standardization of laparoscopic splenectomy

Before the standardization of laparoscopic splenectomy, 6 (5.7%) of 106 patients required emergency conversion to HALS splenectomy because of intraoperative hemorrhage. In contrast, after the technical standardization, 79 (49.7%) of 159 patients underwent HALS splenectomy according to our criteria (Table 4). Seven patients required conversion to HALS splenectomy to prevent intraoperative bleeding. In the remaining 72 patients, the preoperative decision was made to perform HALS splenectomy. Twenty-four patients had two or three indications for HALS. The indications were huge spleen in 22 patients, the development of numerous perisplenic collateral vessels in 25 patients, poor liver function in 30 patients, and concurrent therapy for HCC in 13 patients. When we compared between patients with purely laparoscopic splenectomy and with HALS splenectomy, the average operative time was 221 ± 61 and 240 ± 57 min, and the average blood loss was 135 ± 170 and 184 ± 197 mL, respectively, but the difference was not statistically significant. The postoperative hospital stay was 14.1 ± 4.9 days in patients with HALS as compared with 12.1 ± 3.3 days in patients with purely laparoscopic splenectomy.

Operative procedures by spleen size in patients with standardized laparoscopic splenectomy

Although HALS splenectomy was indicated for spleens of 1,000 mL or more, HALS was actually adopted for various spleen sizes (Fig. 2). The larger the spleen size, the more likely the decision to use HALS became: 15.4% for spleen weight of ≤199 g, 18.2% for 200–399 g, 38.3% for 400–599 g, 56.5% for 600–799 g, 90.0% for 800–999 g, and 100% for 1,000 g. The success rate of purely laparoscopic

Table 2 Comparison of characteristics of patients with conversion to open splenectomy or laparoscopic splenectomy before the technical standardization of laparoscopic splenectomy

Characteristic	Conversion to open splenectomy ($n = 11$)	Laparoscopic splenectomy ($n = 95$)	P value
Child-Pugh A/B/C	2/5/4	40/46/9	<0.05
Child-Pugh score	8.5 ± 2.4	7.2 ± 1.4	<0.01
Platelet counts ($\times 10^3/\mu L$)	35 ± 15	46 ± 19	<0.05
Operative time (min)	212 ± 45	259 ± 69	<0.01
Estimated blood loss (g)	2576 ± 1479	506 ± 723	<0.01
Resected spleen weight (g)	579 ± 226	394 ± 210	<0.05

Table 3 Comparison of operative outcome before and after the technical standardization of laparoscopic splenectomy

Characteristic	Before the standardization ($n = 106$)	After the standardization ($n = 159$)	P value
Child-Pugh A/B/C	42/51/13	70/73/16	n.s.
Child-Pugh score	7.3 ± 1.6	7.0 ± 1.8	n.s.
Conversion to open splenectomy	11 (10.3%)	3 (1.9%)	<0.05
HALS for splenectomy	6 (5.7%)	79 (49.7%)	<0.01
Operative time (min)	259 ± 69	234 ± 62	<0.01
Estimated blood loss (g)	506 ± 723	171 ± 219	<0.01
Spleen weight (g)	394 ± 210	499 ± 298	<0.01
Postoperative hospital stay (days)	17.6 ± 10.7	13.0 ± 4.1	<0.01

Table 4 Patient characteristics for the determination of operative procedures after the technical standardization of laparoscopic splenectomy

Patient characteristic	Number (%)
Purely laparoscopic splenectomy	77/159 (48.4%)
Conversion to open splenectomy	3/159 (1.9%)
Perisplenitis	2
Bleeding from the splenic pedicles	1
Conversion to HALS splenectomy	7/159 (4.4%)
Post-LDLT	1
Post-PSE	1
Difficulties in the upper splenic division	3
Perisplenitis	2
HALS splenectomy	72/159 (45.3%)
Huge splenomegaly	10
+ Collateral vessels	4
+ Poor liver function	4
+ Collateral vessels + poor liver function	3
Poor liver function	13
+ Collateral vessels	8
+ Collateral vessels + HD	1
Collateral vessels	8
Post-LDLT	4
Post-PSE + HD	2
Post-PSE + huge splenomegaly	1
Post-PSE + poor liver function + collateral vessels	1
Concurrent treatment for HCC	13

HALS Hand-assisted laparoscopic surgery, *HCC* hepatocellular carcinoma, *HD* hemodialysis, *LDLT* living-donor liver transplantation, *PSE* partial splenic arterial embolization

splenectomy was 11 (100%) of 11 patients with spleen weight of ≤199 g, 32 (88.9%) of 36 patients with 200–399 g, 26 (89.7%) of 29 patients with 400–599 g, 8 (80.0%) of 10 patients with 600–799 g, and 0 (0%) of 1 patient with 800–999 g.

Discussion

Our present study demonstrates a technically standardized laparoscopic splenectomy harmonized with HALS to be a safe and feasible procedure in the setting of liver cirrhosis and portal hypertension. Massive bleeding requiring emergency conversion to open splenectomy was related to splenomegaly, development of perisplenic collateral vessels, and poor liver function, and it occurred either during the dissection of the splenic hilar pedicles with an endoscopic linear vascular stapler or during the dissection of the upper pole of the spleen. Therefore, the technical standardization of laparoscopic splenectomy includes the following three points: the preoperative decision to use HALS should be made in high-risk patients with splenomegaly (>1,000 mL), numerous perisplenic collateral vessels, or poor liver function (Child-Pugh score 9 or more); complete division and sufficient elevation of the upper pole of the spleen can avoid uncontrollable bleeding during the splenic hilar division; and when surgeons find the division of the upper pole of the spleen to be difficult because of development of collateral vessels or insufficient manipulation, conversion to HALS should be performed without hesitation.

The advancement of laparoscopic technology has contributed to the prevention of massive bleeding during laparoscopic splenectomy. The use of the LigaSure vessel-sealing system was reported to decrease operation time and increase safety for laparoscopic splenectomy [22, 23]. This new system, which we have been using for laparoscopic splenectomy since 2002, provides excellent hemostasis in division of the gastro-splenic ligaments including the short gastric vessels and the vascularized retroperitoneal ligaments in patients with portal hypertension [5, 12, 24]. We have also adopted this vessel-sealing system at the time of concurrent splenectomy during the LDLT, and it reduced the mean blood loss to only 80 mL [8]. In our experience, we cannot count on the ultrasonic scissors to safely perform division of the gastro-splenic ligaments including the

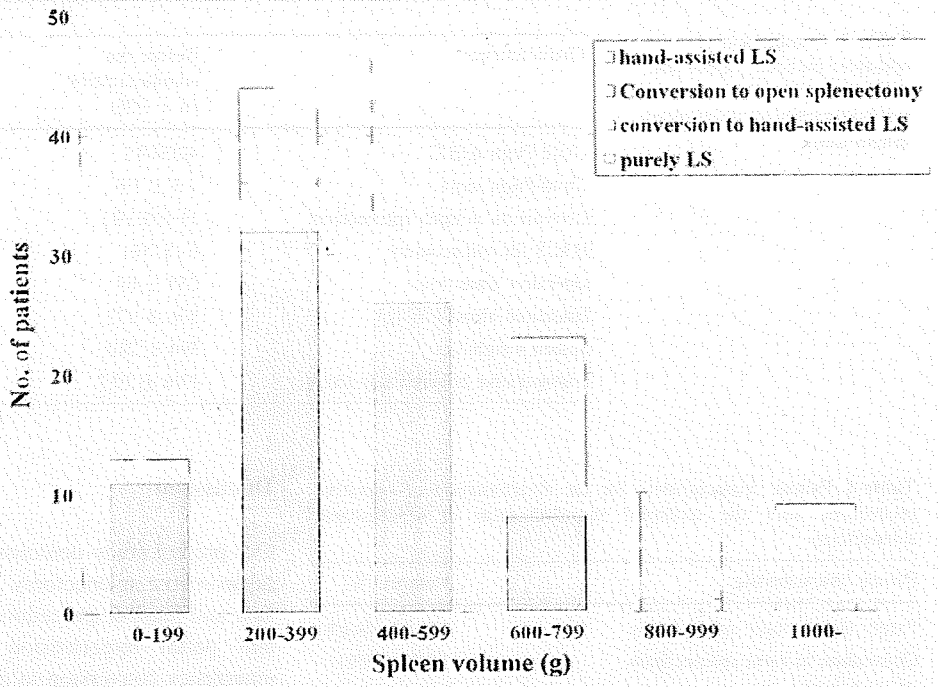


Fig. 2 Operative outcome by spleen size in patients who underwent standardized laparoscopic splenectomy. *LS* Laparoscopic splenectomy

short gastric vessels in the setting of liver cirrhosis and portal hypertension. In addition, the selection of the vascular stapling device is important. The tip of the staple line sometimes becomes broken in thick tissues. As the splenic hilar pedicles are usually extremely thick in the setting of liver cirrhosis and portal hypertension, we used Echelon 60–2.5 mm for the splenic hilar division in 130 recent patients, and it led to safe and effective hemostasis.

From a technical standpoint, we analyzed the operative videos of 11 patients who required emergency conversion to open splenectomy. Uncontrollable bleeding occurred during the division of the splenic hilar pedicles with an endoscopic linear vascular stapler in seven patients and during the dissection of the upper pole of the spleen in four patients. Regarding the splenic hilar bleeding, hemorrhage occurred from the tip of the staple line in which the first stapler could not cross the entire splenic hilar pedicles. As an incomplete dissection of splenic vessels, especially the splenic vein, may leave high splenic blood flow in the partially resected splenic vein, we consider that the first stapler should entirely cross the splenic hilar pedicles, especially the splenic vein, to intercept the splenic blood flow. Several authors reported that, in the setting of splenomegaly, the transection of splenic hilar pedicles should be made before the dissection of the upper pole of the spleen following the splenic hilar division [13, 25]. However, to cross the entirety of the splenic vessels with the first stapler, the complete division and sufficient elevation of the upper pole of the spleen are necessary before handling the splenic hilar pedicles, which is a distinctive point in our standardized laparoscopic splenectomy (Fig. 1f). In patients with huge splenomegaly and/or development of the short gastric venous collaterals, the complete division of the upper pole of the spleen is a potentially frustrating task. In fact, in our series before the technical standardization, four patients had bleeding requiring conversion to open splenectomy during the upper splenic division. Therefore, when surgeons find the division of the upper pole of spleen to be difficult because of development of collateral vessels or insufficient manipulation, conversion to HALS should be performed without hesitation. The use of HALS can allow surgeons to manipulate the spleen with one hand and secure the operative space between the stomach and the spleen widely, and deal safely with vessels, rendering dissection of the short gastric vessels and subsequent division of the upper pole of the spleen safe and definite [20, 21].

For successful achievement of laparoscopic splenectomy in patients with liver cirrhosis and portal hypertension, detailed knowledge of splenic vascular anatomy and volumetric data is essential. It has been shown that multidetector-row CT (MD-CT) provides not only reproducible splenic volumetric data but also accurate splenic vascular anatomy for laparoscopic splenectomy [26]. In our series after the technical standardization, we performed a preoperative evaluation by MD-CT in all patients as shown in Fig. 1a. This allows us to intuitively understand the splenic vascular anatomy and the relationship between the spleen and the surrounding organs such as stomach and colon by reading three-dimensional reconstructions and by scrolling

Table 5 Modified indications of HALS for splenectomy in patients with liver cirrhosis and portal hypertension

Surgical technique	Indications
HALS splenectomy	Huge spleen measuring 600 mL (previously 1,000 mL) or more by MD-CT
	The presence of huge perisplenic collateral vessels by MD-CT
	Poor liver function with Child-Pugh 9 or more
	Post-LDLT
	Post-PSE
	End stage of renal disease with HD
Conversion to HALS splenectomy	Difficulties in division of the upper pole of the spleen
	Difficulties in crossing the entire splenic hilar pedicles with an endoscopic linear vascular stapler
	Severe adhesion due to perisplenitis

HALS Hand-assisted laparoscopic surgery, *HCC* hepatocellular carcinoma, *HD* hemodialysis, *LDLT* living-donor liver transplantation, *PSE* partial splenic arterial embolization, *MD-CT* multi-detector-row CT

the transverse images, finally deciding on the surgical strategy—a purely laparoscopic approach or HALS.

The large spleen is an impediment to manipulation, which can be traumatic and result in bleeding or rupture. Several authors have described that splenomegaly, excluding massive splenomegaly, appears not to affect the conversion rate from laparoscopic splenectomy to open splenectomy [17, 18]. However, other authors have reported that the conversion rate for a spleen weighing less than 1,000 g was 0%, whereas the conversion rate for a spleen weighing more than 1,000 g was 60% [19], and that patients with splenomegaly (≧1,000 g) were 14 times more likely to experience postoperative complications than those without [27]. The limitation for laparoscopic splenectomy may be met at a spleen weighing 1,000 g. The use of HALS has been shown to be able to overcome the limitation of the purely laparoscopic approach in the setting of splenomegaly [20, 21]. In our technical standardization of laparoscopic splenectomy, we thought that patients with a spleen measuring more than 1,000 mL by preoperative evaluation of MD-CT should be treated with HALS. In fact, HALS was performed in patients with various spleen sizes because of other characteristics, such as collateral vessels and poor liver function, as shown in Fig. 2. HALS was performed in 56.5% of patients with a spleen of 600–799 g, 90.0% of patients with a spleen of 800–999 g, and 100% of patients with a spleen of ≧1,000 g. Even in the highly selected patients, the successful achievement rate of purely laparoscopic splenectomy was 8 (80.0%) of 10 patients with a spleen of 600–799 g and 0 (0%) of 1 patient with a spleen of ≧800 g. Therefore, to perform laparoscopic splenectomy as safely as possible in the setting of liver cirrhosis and portal hypertension, the limitation of purely laparoscopic splenectomy may be met at a spleen size of 600 mL by MD-CT, and we have now modified the indications for HALS as shown in Table 5.

The perisplenic collateral vessels often run from the short gastric vessels and/or the splenic hilar vein to the left renal vein through the spleno-colic ligament and/or the retroperitoneal ligaments (Fig. 1). Dissection of the spleen away from the perisplenic attachments can be performed easily and safely without injury and division of these collateral vessels in the majority of such patients. However, perisplenic collateral vessels potentially cause sudden and terrifying hemorrhage. We consider that HALS should be the exclusive choice for patients with perisplenic collateral vessels, which can facilitate easy conversion to an open incision.

Child-Pugh class has been shown to be an independent risk factor for intraoperative massive hemorrhage [12]. In our series before the standardization of laparoscopic splenectomy, conversion to open splenectomy was significantly associated with poor liver function, and the average Child-Pugh score in patients with emergency conversion was 8.5 ± 2.4. Patients with poor liver function have substantial bleeding tendency, and intraoperative massive bleeding is a potential risk for liver failure. Therefore, we considered that patients with poor liver function such as Child-Pugh score 9 or more should be operated on using HALS. Among the highly selected patients who underwent purely laparoscopic splenectomy according to our criteria for HALS, the success rate of purely laparoscopic splenectomy was 26 (89.7%) of 29 patients with score 5, 22 (91.7%) of 24 patients with score 6, 15 (100%) of 15 patients with score 7, and 9 (90.0%) of 10 patients with score 8. Regarding the Child-Pugh score, the limit for purely laparoscopic splenectomy is likely to be met at a Child-Pugh score 8.

In our first experiences with patients with partial splenic arterial embolization (PSE) [28] or in cases of LDLT, we converted to HALS. PSE leads to dense adhesion to the diaphragm and/or retroperitoneal attachments, which possibly increases the difficulties of manipulation and the risk of bleeding from capsular injury and vascularized adhesion. After LDLT, the operative space between the stomach and the upper pole of the spleen was insufficient due to the hypertrophy of graft liver. For the following three patients with PSE and four patients with LDLT, we performed

HALS. In three patients with end-stage kidney disease requiring hemodialysis, we also performed HALS because such patients may be unable to tolerate bleeding, thus requiring conversion to open splenectomy.

The goal of the operation should be safe use of a minimally invasive technique, and we should decide whether to perform a purely laparoscopic approach or a HALS approach based on insightful knowledge of our own capabilities and the limitations of laparoscopic surgery [13]. To reduce blood loss and avoid emergency conversion to open splenectomy, we standardized the laparoscopic splenectomy, and now propose modified criteria for the use of HALS in the setting of liver cirrhosis and portal hypertension (Table 5). Our results support that laparoscopic splenectomy can be a feasible and safe procedure even in the setting of liver cirrhosis and portal hypertension.

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Rituximab, IVIG, and Plasma Exchange Without Graft Local Infusion Treatment: A New Protocol in ABO Incompatible Living Donor Liver Transplantation

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Background. Although graft local infusion (GLI) treatment via the portal vein or the hepatic artery has been the pivotal strategy in ABO incompatible (ABOi) living donor liver transplantation (LDLT) in Japan, the procedure is associated with a high rate of catheter-associated complications.

Methods. A new ABOi-LDLT protocol has been implemented using rituximab, intravenous immune globulin (IVIG), plasma exchange (PE), and splenectomy, without using GLI, on four patients, since 2007. Three other patients, treated before 2007, received GLI.

Results. Three of the four patients with liver cirrhosis received rituximab over 3 weeks before LDLT, followed by PEs and post-LDLT IVIG, resulting in no rebound elevation of the isoagglutinin titers. The remaining patient, with fulminant hepatitis, received rituximab 3 days before the LDLT, resulting in antibody-mediated rejection, successfully treated by IVIG and PE. All four patients that were treated with the new protocol are alive, 26, 8, 6, and 5 months after ABOi-LDLT with normal liver function. Two of the three other patients with GLI, before 2007, had catheter-associated complications, including one graft loss.

Conclusion. The new ABOi-LDLT protocol using rituximab, IVIG, and PE, without the use of GLI, therefore seems to be a safe and an effective treatment modality.

Keywords: Living donor, Liver transplantation, ABO incompatible, Intravenous immunoglobulin, Rituximab, Humoral rejection.

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Although living donor liver transplantation (LDLT) has now become a treatment of option for patients with end-stage liver disease, its application is limited by the need for an appropriate living donor (*1*). Under these circumstances, ABO incompatible (ABOi) LDLT has been practiced in Japan (*2–8*). Despite the dismal outcomes in the initial series, the application of graft local infusion (GLI) treatment, which delivers protease inhibitors, prostaglandin and steroids through the portal vein or hepatic artery, increased the survivals of ABOi LDLT to more than 50% (*2, 7*). However, such GLIs are associated with high incidence of catheter-associated problems including vascular thrombosis, bleeding, or infections (*7*). Despite the recent invention and application of rituximab, a novel anti-CD20 antibody terminating B-lymphocytes, such a protocol using GLI is still exclusively used in ABOi LDLT (*2–7*).

We have been performed ABOi LDLT only for limited number of cases using GLI, since 2001, resulting catheter-associated vascular problems in 2 of 3 cases. Therefore, such GLI has been abandoned, and a new protocol has been established and implemented, using rituximab and intravenous immune globulin (IVIG), since 2007. This report describes the preliminary results of ABOi LDLT using rituximab and IVIG without the use of GLI at Kyushu University Hospital.

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PATIENTS AND METHODS

Patients

Between October 1996 and December 2008, 304 LDLTs were performed at Kyushu University Hospital, Japan. Among them, seven patients received ABOi LDLTs (Table 1). All the LDLTs were performed after obtaining full-informed consent from all patients and approval by the Liver Transplantation Committee of Kyushu University. The basic surgical procedures and techniques were described previously (*9–11*). All seven patients received duct-to-duct biliary reconstruction. The mean follow-up period was 22±25 months. The values are expressed as the mean±standard deviation.

Immune Modulation Protocols in ABOi LDLT

The basic immunosuppression induction regimen in ABOi LDLT involved the administration of tacrolimus with mycophenolate mofetil and steroids (Table 2). Currently, mycophenolate mofetil is started 7 days before LDLT at a dose

TABLE 1. Patient demographics and survival data

		Recipient				Donor						
Protocol	ABOi No.	Age/ gender	Primary disease	MELD score	ABO	Age/ gender	ABO	Relationship	Graft	GV (g)	GV/SLV (%)	Outcomes
I	1	63 F	FHF	15	O	32 M	A	Son	LL	650	66.1	Dead (PVT) <1 mo
	2	49 F	LC-B HCC	18	A	47 M	B	Husband	RL	670	57.1	Alive 68 mo
	3	21 F	FHF	19	B	46 M	AB	Mother	RL	520	50.3	Alive 41 mo
II	4	20 F	FHF	17	O	54 M	A	Step father	RL	600	48.7	Alive 26 mo
	5	47 F	LC-B HCC	11	A	38 M	B	Husband	LL	390	35.5	Alive 8 mo
	6	45 M	LC-C HCC	20	B	45 F	A	Wife	RL	540	42.5	Alive 6 mo
	7	55 M	LC-C HCC	11	B	24 M	A	Son	RL	530	39.8	Alive 5 mo

ABOi, ABO incompatible; FHF, fulminant hepatic failure; GV, graft volume; HCC, hepatocellular carcinoma; LC-B, liver cirrhosis due to hepatitis B; LC-C, liver cirrhosis due to hepatitis C; LL, left lobe; MELD, model for end stage liver disease; PVT, portal vein thrombosis; RL, right lobe; SLV, standard liver volume.

TABLE 2. Immunomodulation protocols in ABOi LDLT

Protocol	ABOi No.	Portal infusion	IVIG (total dose)	Rituximab (pre-LDLT day)	Splenectomy	Plasma exchange (pre-, post- LDLT)	Basic immunosuppression
I	1	Yes	—	—	Yes	x2, —	Tac, MMF, steroid
	2	Yes	—	—	Yes	x8, —	Tac, MMF, steroid
	3	Yes	—	Yes (−3)	Yes	x2, x5	Tac, MMF, steroid
II	4	—	110 g, 5x	Yes (−3)	Yes	x6, x5	Tac, MMF, steroid
	5	—	125 g, 3x	Yes (−29)	Yes	x4, —	Tac->CyA, MMF, steroid
	6	—	125 g, 2x	Yes (−36)	Yes	x5, —	Tac->CyA, MMF, steroid
	7	—	100 g, 2x	Yes (−22)	Yes	x2, —	Tac, MMF, steroid

ABOi, ABO incompatible; CyA, Cyclosporine A; IVIG, intravenous immune globulin; LDLT, living donor liver transplantation; MMF, mycophenolate mofetil; Tac, tacrolimus.

of 2 g/day, and increased to 3 g/day after LDLT, then decreased to 2 g/day once the blood calcineurin inhibitor level reaches an appropriate level. Tacrolimus is started within 3 days after LDLT, once the kidney function has recovered. The target tacrolimus level ranges between 12 and 15 ng/mL for the first post-LDLT month and is titrated down to 8 to 10 ng/mL for the next few months. When patients experience tacrolimus-associated complications, especially encephalopathy, tacrolimus is converted to cyclosporine A (cases 5 and 6). The target cyclosporine A level ranges from 200 to 250 ng/mL for the first post-LDLT month and was titrated down to 100 to 150 ng/mL for the next few months. One gram of methylprednisolone is given after reperfusion and tapered from 200 mg to 40 mg over 10 days, then switched to 20 mg of oral prednisolone and tapered off in 6 months after the LDLT.

GLI through the portal vein was exclusively used between 2001 and 2006 (Protocol I, Table 2). A 16 G double lumen catheter was introduced from the umbilical vein or the mesenteric vein and protease inhibitor (Nafamostat Mesilate, 200 mg/day), Prostaglandin E1 (500 mg/day), and methylprednisolone (50 mg/day) were given for 14 days after LDLT. Plasma exchanges (PEs) were performed to lower the isoagglutinin titer less than or equal to 64. Splenectomy was performed during LDLT. Rituximab (375 mg/m^2) was given and has been administered, since the case 3 in 2005.

GLI was abandoned in 2007, and the new ABOi-LDLT protocol using rituximab and IVIG in addition to splenectomy and PE has been started (Protocol II, Table 2). Rituximab (375 mg/m^2) was given just 3 days before LDLT in case 4 because the primary disease was fulminant hepatic failure (FHF), and the high-dose IVIG (0.6 g/kg) was used as a rescue for treating antibody-mediated rejection (AMR) with rebound elevation of the isoagglutinin titer (*8*). Rituximab (375 mg/m^2) has been administered over 3 weeks before LDLT, since case 5: −29 days (case 5), −36 days (case 6), and −22 days (case 7) before LDLT, respectively. IVIG (0.8 g/kg, Sanglopor, CSL Behring, Tokyo, Japan) was administered scheduled after LDLT on days 1, 3, and 5 (case 5), and on days 1 and 4 (cases 6 and 7). The current protocol for scheduled ABOi LDLT at Kyushu University Hospital is shown in Figure 1. For ABOi-LDLT for FHF, our protocol needs to be modified in minor. Rituximab (375 mg/m^2) is given as soon as a suitable donor is selected, followed by starting mycophenolate mofetil and PEs every 12 hr to decrease isoagglutinin titers less than or equal to 64 at the time of LDLT, and IVIG is given scheduled after LDLT on days 1 and 4. If LDLT needs to be performed with

high isoagglutinin titer more than or equal to 128 because of the acute deterioration of the recipient's condition, post-LDLT PEs need to be added (*8*).

RESULTS

Donor and Recipient Data

The recipients who received ABOi LDLT included 5 men and 2 women, and all of them were adult patients (43±16 years, Table 1). The primary disease included FHF (n=3), liver cirrhosis due to hepatitis B with hepatocellular carcinoma (HCC, n=2), and liver cirrhosis due to hepatitis C with HCC (n=2). The mean model for end-stage liver disease score was 16±4. The first three patients received ABOi LDLT in association with Protocol I (the old protocol using GLI before 2007), and the last four patients received Protocol II (the new protocol without using GLI, but with rituximab and IVIG, since 2007). The donors included wives or husbands (n=3), parents (n=2), and children (n=2), with a mean age of 41±10 years. The procured grafts included five right lobe grafts and two left lobe grafts. The mean graft volume was 557±95 g, and the mean graft volume/standard liver volume was 48.6±10.5%.

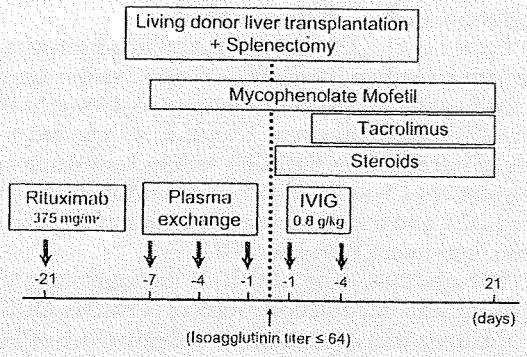

FIGURE 1. New protocol (Kyushu University Protocol) with rituximab, plasma exchanges, splenectomy, intravenous immune globulin, and immunosuppressive medications. No local infusion treatment is used.

Isoagglutinin Titers and CD20 Positive Cells

PE effectively lowered the isoagglutinin titers before LDLT in all the cases (Table 3). No rebound elevation of the isoagglutinin titers was seen in any of the patients except the two FHF cases, regardless of the use of portal infusion, IVIG, and rituximab. However, the two FHF cases showed prominent rebound elevation of the isoagglutinin titers after LDLT with complete suppression of the peripheral CD20 positive cells. They required 5-day course of post-LDLT PE (Table 2). The rebound was uncontrollable by serial PE in case 4, and it was controlled by high-dose IVIG (*7*). Therefore, the isoagglutinin titers were stable in all the patients, 2 weeks after from the LDLT. CD20 positive lymphocytes were suppressed at the time of LDLT after administration of rituximab in the four patients treated with the new protocol, even it was given just 3 days before LDLT. A small population of CD20 positive cells was positive in half of the patients (0.3% in case 4 and 1.4% in case 5) at 3 months after LDLT.

Recipient Outcomes

During the use of the Protocol I, one patient (case 1) encountered catheter-related portal vein thrombosis. Direct hepatic arterial cannulation was attempted several times during LDLT in case 3, resulting in an uncannulated hepatic artery and portal venous cannulation instead. The patient had hepatic artery dissection 16 days after LDLT and underwent hepatic artery revision.

Cases 3 and 4 had clinical AMR, treated by PE in case 3 and PE and IVIG in case 4. Cases 3, 4, and 5 had acute cellular rejection (ACR) at 3 years, 1 year, and 3 months after LDLT, and were treated by regular steroid pulse therapy. Case 3 had ACR during her pregnancy.

In case 1, the patient died because of portal vein thrombosis followed by systemic Aspergillosis. The other six patients are alive with normal liver function tests. Cases 2 and 5 were on entecavir and hepatitis B immune globulin and free from recurrent hepatitis B. Cases 5 and 6 have been treated with pegylated-interferon to treat recurrent hepatitis C, since 3 months after LDLT. Case 5 has been negative for hepatitis C virus RNA, since 8 weeks after the induction of pegylated-interferon. No patients have so far experienced recurrent HCC.

TABLE 3. Serial changes in isoagglutinin titers and CD20 positive lymphocytes

Protocol	ABOi No.	Isoagglutinin titers: Initial (day)	At LDLT	Rebound titer (day)	1 mo	6 mo	CD 20 positive lymphocytes (% all lymphocytes): Initial	At LDLT	3 mo	6 mo	Rejection episodes
I	1	128 (day −3)	32	—	8	N/A	N/A	N/A	N/A	N/A	—
	2	1024 (day −19)	128	—	8	8	N/A	5.3	2.5	N/A	—
	3	64 (day −3)	2	1024 (day 7)	64	64	N/A	0	0	4.1	AMR (day 7, PE) ACR (3 yr, steroid)
II	4	2048 (day −3)	128	2048 (day 7)	512	32	N/A	0	0.3	3.3	AMR (day 4, IVIG+PE) ACR (1 yr, steroid)
	5	512 (day −29)	32	—	16	32	4.0	0	1.4	0.9	ACR (3 mo, steroid)
	6	256 (day −36)	32	—	16	N/A	13.4	0	0	N/A	—
	7	512 (day −22)	8	—	32	N/A	1.9	0	0	N/A	—

ABOi, ABO blood type incompatible; ACR, acute cellular rejection; AMR, antibody mediated rejection; IVIG, intravenous immune globulin; LDLT, living donor liver transplantation; MMF, mycophenolate mofetil; PE, plasma exchange; Tac, tacrolimus.

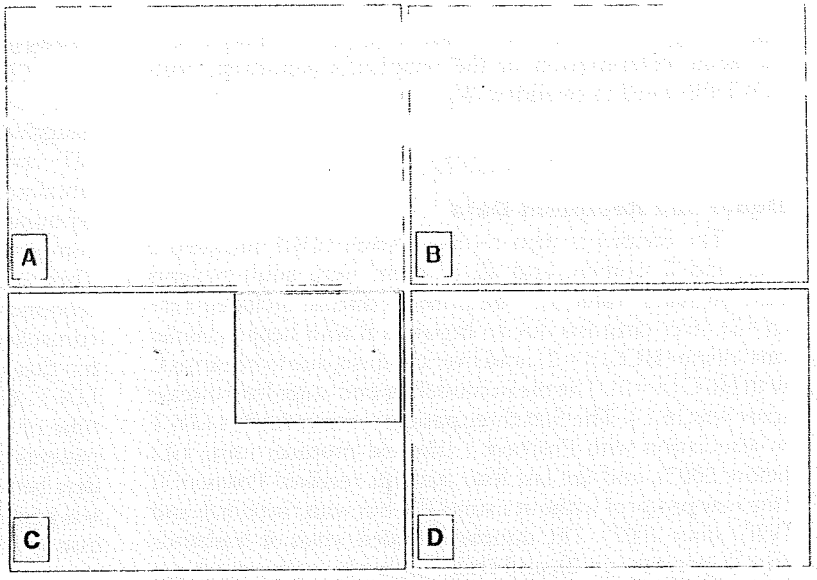

FIGURE 2. Immunohistochemical staining of the explanted spleen (A, B) and an sampled hilar lymph node (C, D) of the case 5, using anti-CD20 antibody (A ×100; C ×100; and ×400) and anti-CD 138 antibody (B, D ×100). CD20 positive B-lymphocytes are present in the lymph node. Rituximab was administered 29 days before transplantation.

During the Era-I with GLI, survival rate was 2 of 3 (67%), and the post-LDLT complications included catheter-associated portal vein thrombosis (n=1), catheter-associated hepatic artery dissection (n=1), ACR (n=1), AMR (n=1), cytomegalovirus infection (n=2), and sepsis (n=2). During the Era-II without GLI but with IVIG, survival rate was 4 of 4 (100%) and the post-LDLT complications included ACR (n=2), AMR (n=1) and cytomegalovirus infection (n=3). Readmission to the intensive care unit after LDLT occurred in 67% (n=2) in the Era-I and 0% in the Era-II. Mean post-LDLT hospital stay was 45 days (23, 31, 80 days) in the Era-I and 31 days (23, 35, 33, 34 days) in the Era-II.

DISCUSSION

Because there has been a little opportunity to perform liver transplantation from deceased donors in Japan, thus the use of ABOi donors are often required in LDLT. Various methods have been implemented to allow these procedures, GLI (*2–7*). The theoretical basis of local administration of prostaglandin, protease inhibitors, and steroids is that the pathologic findings of the failed ABOi liver graft show the features of hepatic disseminated intravascular coagulation (*12*). However, there are problems associated with the application of the GLI through the portal vein or hepatic artery: (i) there is high incidence of catheter-related complications; (ii) relaparotomy may be necessary for the removal of the catheters; (iii) and GLI is not the prime intervention to treat a humoral immune response (*3, 7*). The Japan Study Group for ABO Blood Type Incompatible Transplantation reported that the incidence of catheter-related complications, including vascular thrombosis, bleeding, sepsis, or catheter dislocation, is 37% in portal venous infusion, 22% in portal venous and hepatic arterial dual infusion, and 16% in hepatic arterial infusion (*7*). Moreover, although GLI may slow the secondary cascades in the local disseminated coagulation process, the fundamental antibody-mediated immunologic reaction is not interposed. Even after the application of GLI, a considerable number of ABOi LDLT grafts have been lost due to total graft necrosis associated with the rebound elevation of the isoagglutinin titers, for which PE seemed to be ineffective (*3*).

Rituximab, an anti-CD20 antibody, is a monoclonal antibody that specifically targets the CD20 surface antigen expressed on-B lymphocytes, thus resulting in cell lyses. In the current series, two FHF cases (3 and 4) were given rituximab just 3 days before LDLT and had rebound elevation of isoagglutinin titers with clinical AMR.

Egawa et al. (*13*) has reported that administration of rituximab earlier than 7 days before transplantation significantly depleted B- and memory B-lymphocytes and lowered the peak post-LDLT isoagglutinin titers. Usui et al. (*4*) reported on its use as long as 3 weeks before the LDLT with successful outcomes. In the current cases 5 to 7, we had given rituximab over 3 weeks before LDLT and had satisfactory suppression of serum isoagglutinin titers under IVIG after LDLT.

The administration of IVIG is the significant factor in the new immunomodulation protocol in ABOi LDLT. In the field of kidney transplantation, the effective use of IVIG for the control of acute humoral rejections in sensitized candidates was used as early as 1994 (*14–17*). The proposed mechanisms of action of IVIG on the humoral reaction include B-cell apoptosis through the Fc-receptor dependent pathway, and the inhibition of alloreactive T-cell mediated or complement-mediated allograft injury, although these possibilities have not been confirmed (*16, 17*). The use of IVIG for induction therapy in ABOi liver transplantation has never been reported before, although a limited number of case reports have suggested the use of IVIG for severe AMR after ABOi LDLT (*5, 8*). In emergency transplant settings, such as LDLT for acute liver failure, the impact of IVIG is significant because the early administration of rituximab is not possible. Therefore, what is the role of IVIG in scheduled LDLT in which early administration of rituximab could deplete B-lymphocytes? Among the current series, a hilar lymph node, sampled during the LDLT in the case 5 showed numerous B-lymphocytes in it, although no plasma cells were observed (Fig. 2). The explanted spleen had neither B-lymphocytes nor plasma cells (Fig. 2). Such B-lymphocytes may have survived in the lymph node for 29 days under rituximab or may have been newly differentiated. IVIG might have worked on for these B-lymphocytes, preventing AMR. In scheduled ABOi LDLT, the use of IVIG with

rituximab should also result in better outcomes, because sufficient suppression of humoral reactions can be achieved by earlier administration of rituximab (*4, 13*). Moreover, early administration of rituximab before ABOi-LDLT might replace splenectomy as suggested by Egawa et al. (*13*). Because the major morbidity and mortality after ABOi-LDLT is infectious problems, preservation of a spleen might be beneficial. In the current series, we performed splenectomy for preventing small-for-size graft syndrome in case 5 and for treating pancytopenia for performing interferon treatment for hepatitis C in cases 6 and 7 (*18, 19*).

In a deceased donor liver transplantation setting using ABOi donors, Urbani et al. (*20*) recently reported favorable outcomes. They performed PE and gave IVIG at 1 g/kg after ABOi liver transplantation for initial 14 days when isoagglutinin titer is more than or equal to 8, resulting survival rate of 87.5% (n=8). Although our protocol includes rituximab to deplete B-lymphocytes, the isoagglutinin titer at the time of transplantation depends on a patient's original titer and the possible courses of the PEs before transplantation. Therefore, posttransplant PEs may need to be added, depends on the isoagglutinin titers.

Finally, the major practical problem in the application of IVIG is its high cost. In our protocol, two doses of IVIG for a 60 kg person at 0.8 g/kg would cost 760,000 yen. However, our protocol uses much smaller amount of IVIG than other protocols. Urbani et al. (*20*) used mean 6.6 doses of IVIG at 1g/kg with PEs when isoagglutinin titer is more than or equal to 8 after ABOi liver transplantation from deceased donors (n=8). In a desensitized protocol in kidney transplantation, major centers give IVIG 3 to 5 doses at 2 g/kg with PEs (*16*). Moreover, once AMR occurs after ABOi-LDLT, its treatment would cost at least 1,130,000 yen by five courses of PE, or 610,000 yen by a single PE with a single shot of IVIG (0.8 g/kg). Therefore, we believe that our prophylactic protocol seems to have acceptable cost-outcome balance. Nevertheless, we are still trying to reduce the IVIG dosage to achieve cost-effectiveness.

In conclusion, a new protocol using rituximab, IVIG, and PE seems to protect the graft form AMR in ABOi LDLT. Therefore, GLI via the portal vein or the hepatic artery could be omitted in ABOi LDLT. Continued patient enrollment will allow further validation of these observations.

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Synthetic Alginate is a Carrier of OP-1 for Bone Induction

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Abstract Bone morphogenetic proteins (BMPs) can induce bone formation in vivo when combined with appropriate carriers. Several materials, including animal collagens and synthetic polymers, have been evaluated as carriers for BMPs. We examined alginate, an approved biomaterial for human use, as a carrier for BMP-7. In a mouse model of ectopic bone formation, the following four carriers for recombinant human OP-1 (BMP-7) were tested: alginate crosslinked by divalent cations (DC alginate), alginate crosslinked by covalent bonds (CB alginate), Type I atelocollagen, and poly-D,L-lactic acid-polyethyleneglycol block copolymer (PLA-PEG). Discs of carrier materials (5-mm diameter) containing OP-1 (3–30 μg) were implanted beneath the fascia of the back muscles in six mice per group. These discs were recovered 3 weeks after implantation and subjected to radiographic and histologic studies. Ectopic bone formation occurred in a dose-dependent manner after the implantation of DC alginate, atelocollagen, and PLA-PEG, but occurred only at the highest dose implanted with CB alginate. Bone formation with DC alginate/OP-1 composites was equivalent to that with atelocollagen/OP-1 composites. Our data suggest DC alginate, a material free of animal products that is already approved by the FDA and other authorities, is a safe and potent carrier for OP-1. This carrier may also be applicable to various other situations in the orthopaedic field.

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Introduction

The repair capacity of human bone appears to depend on different very complex processes, such as vascularization, biomechanics, and topography. When damage is severe, as occurs with comminuted fractures or large bone defects after tumor resection, it is difficult for bone union to be achieved [6]. In such cases, autologous or allogenic bone grafting has been used. Autologous bone grafting is common and is still the gold standard, but has several disadvantages, including a limited supply of suitable bone and the risk of chronic pain, nerve damage, fracture, and cosmetic problems at the donor site. Allografts have no donor site problems, but there is the potential risk of disease or an immunologic reaction [10, 21]. For these reasons, the use of bone substitutes such as calcium phosphate-based porous ceramics has been increasing [18, 33]. These bioceramics are highly biocompatible and demonstrate osteoconduction, which is the ability to bind to bone matrix directly. However, they have no osteoinduction, which is the ability to induce new bone formation at ectopic sites.

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor superfamily, are known to elicit new bone formation in vivo, and may play a leading role in

bone tissue engineering [36, 38]. To date, three types of BMP-based bone tissue engineering have been tried, which are cell therapy, gene therapy, and cytokine therapy [27]. Cell therapy involves the transplantation of autologous bone marrow mesenchymal cells after differentiation has been induced by BMP, but considerable resources and time are needed to culture the necessary cells [22, 34]. Gene therapy involves the transduction of genes encoding BMPs into cells at the site of damage [2, 7]. BMP-transduced cells may work more efficiently, compared with a single dose of recombinant cytokine therapy. However, gene therapy still has unsolved problems such as tumorigenesis and immunogenicity. Cytokine therapy involves the implantation of BMPs together with a carrier material that acts as a drug delivery system. We believe cytokine therapy is the most promising of these three approaches in terms of practical application. Cytokine therapy seems most convenient and safe, but the cost is very high because a large amount of BMP is required to achieve bone growth in humans. To increase the cost effectiveness of BMP, an appropriate carrier material is necessary.

Previous studies have indicated adequate in vivo new bone formation cannot be obtained by simply injecting a solution of BMP into the area where bone is needed [32]. For cytokine therapy, an appropriate carrier material is needed that retains BMP and releases it slowly, while serving as a scaffold for new bone formation [28, 29]. Several materials have already been evaluated as BMP carriers, including collagen obtained from animal sources [3, 11, 13, 31], synthetic polymers [14, 15, 19], tricalcium phosphate [17], and other inorganic materials [16]. Atelocollagen is a well-established BMP carrier, and has already been used clinically. PLA-PEG [20], one of the synthetic polymers, has been reported as a potent carrier for BMPs [23, 25, 26]. Although all of these materials can induce bone formation at ectopic and orthotopic sites, none of them has achieved widespread use because of disadvantages, such as the potential risk of disease transmission, fragility, stickiness, and difficulty in obtaining approval for clinical use [1, 4, 5, 14]. We therefore focused on alginate, which is already approved by the FDA for human use as a wound dressing and food additive [8, 37].

Alginate is a water-soluble linear polysaccharide extracted from brown seaweed that is composed of one to four linked α-L-gluronic and β-D-mannuronic acid monomers [9]. Gelation of alginate occurs as a result of crosslinking by divalent cations or covalent bonds [30]. Therefore, two types of alginate wound dressing products are available on the market and both effectively promote wound healing by maintaining a moist environment. One is an alginate crosslinked by divalent cations (DC alginate) and the other is an alginate crosslinked by covalent bonds (CB alginate).

To determine whether alginate can be a carrier for BMP, we compared four materials as carriers for OP-1(BMP-7) using the bone mineral content (BMC) measurement and alkaline phosphatase (ALP) activity measurement of the bone nodules ectopically induced by carrier materials/OP-1 composites. The four materials were DC alginate, CB alginate, atelocollagen, and PLA-PEG. We hypothesized: (1) BMC of bone nodules ectopically induced by DC alginate/OP-1 composite and/or CB alginate/OP-1 composite are equivalent or superior to those by atelocollagen and PLA-PEG; (2) ALP activity of bone nodules ectopically induced by DC alginate/OP-1 composite and/or CB alginate/OP-1 composite are equivalent or superior to those by atelocollagen and PLA-PEG by radiographic appearance and histology of the ectopic bone nodules; and (3) DC alginate and/or CB alginate have appropriate in vitro release kinetics of OP-1 equivalent to atelocollagen and PLA-PEG.

Materials and Methods

To verify our first hypothesis, we designed the following experiment (Experiment 1; Table 1). For each dose of OP-1 (3, 10, and 30 μg), 24 4-week-old male ICR mice were assigned to four equally sized independent groups after they were housed and acclimatized in cages with free access to food and water for 1 week. The four independent groups were DC alginate group, CB alginate group, atelocollagen group, and PLA-PEG group. The mice were anesthetized by intraperitoneal injection of pentobarbital. As reported previously [14, 15], carrier material/OP-1 composites were implanted beneath the fascia of the back muscles on the left side (one composite per animal). The experiment was designed under the assumption that the justifiable difference (effect size) between the atelocollagen group as a control and the other groups was 6 mg in BMC and the standard deviation within each group was 3 from the result of the previous study [24]. For the experiment to detect the difference at the 5% significance level with 90% power in the one-way analysis of variance, the necessary number of mice per group was six. Three weeks after implantation, these mice were killed and ectopic bone induced at the implantation site was harvested for further evaluation, including BMC measurement, radiography, and histological examination. The experimental protocol was approved by the Animal Experiment Committee of Osaka University, and the experiments were carried out in accordance with the Osaka University guidelines for care and use of laboratory animals.

To verify the second hypothesis, we repeated the Experiment 1 and obtained radiographs and measured ALP activity (Experiment 2; Table 1).

Table 1. Study groups and experimental design

Experiments	Carrier materials	Dose of OP-1	n	Examination
Experiment 1	DC alginate	3, 10, and 30 μg	18*	BMC, radiography, histology
	CB alginate	3, 10, and 30 μg	18*	BMC, radiography, histology
	Atelocollagen	3, 10, and 30 μg	18*	BMC, radiography, histology
	PLA-PEG	3, 10, and 30 μg	18*	BMC, radiography, histology
Experiment 2	DC alginate	3, 10, and 30 μg	18*	Radiography, ALP activity
	CB alginate	3, 10, and 30 μg	18*	Radiography, ALP activity
	Atelocollagen	3, 10, and 30 μg	18*	Radiography, ALP activity
	PLA-PEG	3, 10, and 30 μg	18*	Radiography, ALP activity

*: n = 6, each dose.

The BMC of the harvested discs was determined by dual-energy xray absorptiometry (DXA) using an animal bone densitometer (PIXImus; Lunar Corp, Madison, WI) and was expressed as milligrams per ossicle. Radiographs were obtained with a soft xray apparatus (MX-20 Faxitron®; Torrex and Micro Focus Systems, Wheeling, IL).

To measure ALP activity, the harvested discs were crushed, homogenized in 0.2% Nonidet® P-40 containing 1 mmol/L $MgCl_2$, and centrifuged at 10,000 rpm for 1 minute at 4°C. The supernatants thus obtained were assayed for ALP activity with an Alkaline Phospha B-Test Wako kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan) using p-nitrophenyl phosphate (p-NP) as a substrate. The protein content was measured with a Pierce® BCA protein assay kit (Thermo Fisher Scientific Inc, Rockford, IL), and ALP activity was standardized by the protein content and expressed as nmol p-NP/minute/mg protein.

After radiography and BMC measurement, the samples were fixed in 10% neutral formalin, decalcified with ethylenediaminetetraacetic acid (pH 7.4), dehydrated in a graded ethanol series, and embedded in paraffin. One section per group with the largest tissue area (5-μm thick) were cut and stained with hematoxylin and eosin for observation under a light microscope. The formation of new bone, new bone marrow, degradation of the materials, and inflammatory change were evaluated by a pathologist (AM) and an orthopaedic surgeon (KN).

To verify the third hypothesis that DC alginate and/or CB alginate have appropriate in vitro release kinetics of OP-1, we incubated carrier materials/OP-1 composites in centrifuge tubes containing 1000 μL phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA) and kept for 21 days at 37°C. For each composite group, three samples were examined. The PBS in the tubes was replaced every 2 days, and then 100 μL was collected for assay after 24 hours. The amount of OP-1 was determined by measurement with a commercial BMP-7 ELISA kit (R&D Systems Inc, Minneapolis, MN) on days 1, 3, 7, 13, and 21 according to the manufacturer's instruction.

OP-1 (BMP-7 in a lyophilized 5% lactose formulation) was provided by Stryker Biotech (Hopkinton, MA). OP-1 was dissolved in distilled water at a concentration of 2 μg/μL. DC alginate (ARGODERM®; crosslinked by Ca^{2+}), CB alginate (KURABIO®), and atelocollagen (INSTAT®) were purchased from Smith & Nephew (London, UK), Koyo Sangyo Co, Ltd (Tokyo, Japan), and Johnson & Johnson (New Brunswick, NJ), respectively. PLA-PEG with a total molecular weight of 11,400 Da and a PLA:PEG molar ratio of 51:49 was synthesized and provided by Taki Chemicals Co, Ltd (Hyogo, Japan).

To prepare carrier material/OP-1 composites, sheets of DC alginate, CB alginate, and atelocollagen were cut into discs (5-mm diameter). Then 25 μL of a solution containing 3, 10, or 30 μg OP-1 was added dropwise to each disc, after which the discs were freeze-dried and stored at −20°C until implantation into mice. All procedures were carried out under sterile conditions.

PLA-PEG/OP-1 composites were prepared as described previously [25]. Briefly, 10 mg of the polymer was liquefied in 50 μL acetone and mixed with 3, 10, or 30 μg OP-1. Each mixture was evaporated to dryness to remove acetone in a safety cabinet, fabricated into a disc-shaped implant, and stored at −20°C until implantation into mice.

To verify the first and second hypotheses, we used one-way analysis of variance (ANOVA), followed by a post hoc Scheffe's test. For each of these statistical analyses, the data sets met the assumptions of normality ($p > 0.15$ by the Jarque-Bera test [12]) justifying the use of parametric models. All analyses were performed using the R software program (Version 2.8.1;R Foundation for Statistical Computing).

Results

With 3 μg OP-1, BMC of the new bone in the DC alginate group was greater than that in atelocollagen group ($p = 0.0234$) and PLA-PEG group ($p = 0.0009$). With

30 μg OP-1, however, we observed no differences among the DC alginate, atelocollagen, and PLA-PEG. On the other hand, BMC of CB alginate group was very low compared with the other groups (Fig. 1). The results suggest that the BMC of DC alginate group was superior to those of atelocollagen and PLA-PEG groups, especially with a low dose of OP-1.

In the DC alginate group, ALP activity was high independent of the OP-1 dose. With 3 μg OP-1, DC alginate/OP-1 composites exhibited higher ALP activity than atelocollagen group ($p = 0.0071$) and PLA-PEG group ($p = 0.0001$) (by Scheffe's test). ALP activity of the CB alginate group was very low compared with the other groups (Fig. 2). The results suggest that ALP activity of the DC alginate group was superior to those of atelocollagen and PLA-PEG groups, especially with a low dose of OP-1.

In the release study of OP-1, the maximum concentration of OP-1 in the supernatant was detected on Day 1, followed by a steady decline. The decline of OP-1 levels in the atelocollagen group was faster than that in the other groups. In the DC alginate group, the decrease of OP-1 levels was the slowest and the concentration of OP-1 was still higher than 200 ng/mL on Day 21 (Fig. 3). These data suggested that DC alginate retains OP-1 and releases it most slowly compared to atelocollagen and PLA-PEG.

In the additionally performed radiographic examination of the bone nodules, obvious bone formation was only detected in the DC alginate and atelocollagen groups with 3 μg OP-1 (Fig. 4A–D). In the CB alginate group, new bone formation was observed only with 30 μg OP-1. The results of the additionally performed histological examination were consistent with the radiographic findings. In the DC alginate and atelocollagen groups, abundant new bone formation that contained normal hematopoietic bone marrow was observed even at low dose of OP-1. In the CB alginate group, however, new bone formation was very poor at low dose of OP-1. With 30 μg OP-1, irrespective of the carrier materials, newly formed bone had a thin cortex

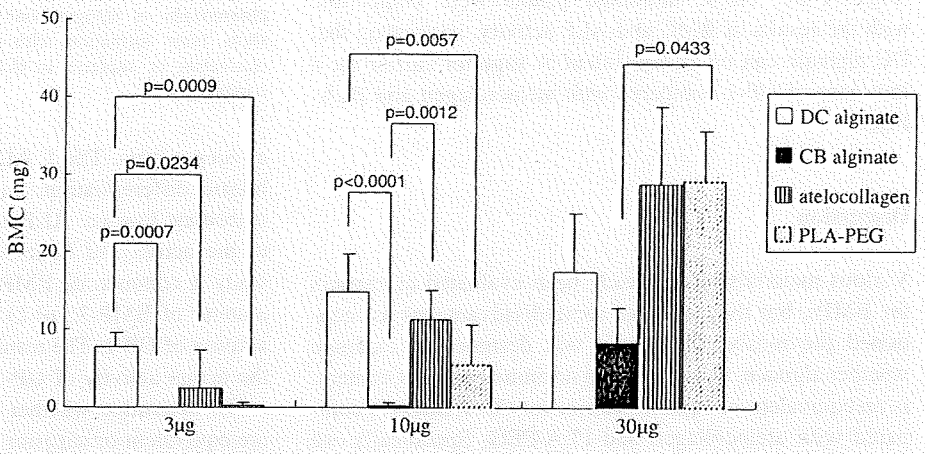

Fig. 1 In each carrier material group, BMC was measured by DXA using a PIXImus animal densitometer. BMC increased in an OP-1-dose dependent manner with every carrier material. With 3 μg and 10 μg OP-1, the BMC of the new bone in the DC alginate group was greater than that in the other groups.

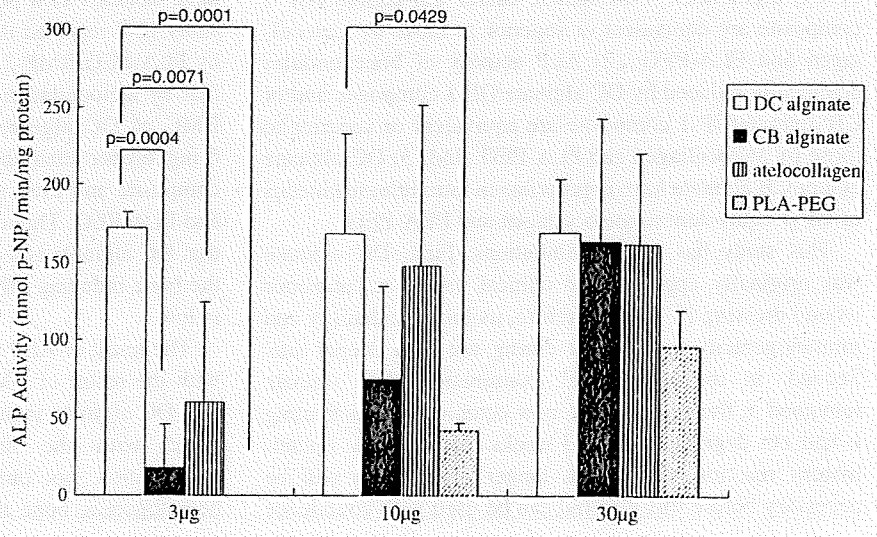

Fig. 2 ALP activity of ectopic bone was measured by using p-NP as a substrate. In the CB alginate, atelocollagen, and PLA-PEG groups, ALP activity increased in a dose-dependent manner. In the DC alginate group, ALP activity was relatively high independent of the dose of OP-1.