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ORIGINAL ARTICLE

Branched-chain amino acid-enriched nutrients improve nutritional and metabolic abnormalities in the early post-transplant period after living donor liver transplantation

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Abstract
Background/purpose Malnutrition and metabolic disorder of patients undergoing living donor liver transplantation (LDLT) can affect post-transplant prognosis. The aim of this study was to establish whether perioperative usage of branched-chain amino-acid (BCAA)-enriched nutrients improve metabolic abnormalities of patients undergoing LDLT.
Methods We designed a randomized pilot study (UMIN registration number; 000004323). Twenty-five consecutive adult elective LDLT recipients were enroled and divided into two groups: the BCAA group (BCAA-enriched nutrients, $n = 12$) and the control group (standard diet, $n = 13$). Metabolic and nutritional parameters, including BCAA-to-tyrosine ratio (BTR), retinol binding protein (RBP), and prealbumin were regularly measured from 1 week before to 4 weeks after LDLT. Non-protein respiratory quotient (npRQ) was measured before and 4 weeks after LDLT.
Results BTR and RBP improved considerably in the BCAA group compared with the controls. npRQ significantly increased from 1 week before LDLT to 4 weeks after LDLT in the BCAA group (0.77 ± 0.05 to 0.84 ± 0.06, $P = 0.002$), but not in the control group (0.78 ± 0.04 to 0.81 ± 0.05).
Conclusions Supplementation with BCAA-enriched nutrients might improve persistent nutritional and metabolic disorders associated with end-stage liver disease in the early post-transplant period, and consequently shorten the post-transplant catabolic phase after LDLT. A larger multicenter trial is needed to confirm these findings.

Keywords Branched-chain amino-acid-enriched nutrients · Living donor liver transplantation · Non-protein respiratory quotient · Energy metabolism · Perioperative nutrition

Introduction

It is well known that the liver plays a central role in nutrient metabolism. End-stage liver diseases display a wide range of nutritional and metabolic abnormalities, such as protein-energy malnutrition and amino acid imbalance due to progressive liver metabolic disorders [1–6]. A severe cirrhotic liver often lacks adequate glycogen stores and progresses into a severe catabolic state with such patients using free fatty acids from adipose tissue as their energy source. Thus, most liver transplantation candidates show some kind of nutritional depletion and metabolic disorders at their initial evaluation [7]. To improve these abnormalities in patients with liver cirrhosis, many studies have been reported and both branched-chain amino-acid (BCAA)-enriched nutrients and BCAA granules have recently been used [8–16]. Therefore, we hypothesize that perioperative administration of BCAA-enriched nutrients may improve the metabolic abnormalities of patients undergoing living donor liver transplantation (LDLT).

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In Japan, approximately 98% of liver transplantations are of the LDLT type because of the chronic shortage of deceased donors, and more than 5000 LDLTs were performed from 1998 to 2008 [17]. Despite improvements in surgical techniques and the innovation of perioperative management, LDLT still has relatively high post-operative mortality and morbidity rates compared with other general surgeries [18]. Although many investigators have reported a close relationship between nutritional state and post-operative morbidity and mortality after liver transplantation, there have been few studies concerning the perioperative changes in metabolic/nutritional parameters including rapid turnover of proteins and non-protein respiratory quotient (npRQ) during the early post-operative period after liver transplantation [19–22].

In this pilot study, we prospectively analyzed the effect of enteral administration of BCAA-enriched nutrients in LDLT patients on post-operative metabolic abnormalities.

Materials and methods

Patients

Because there had been no study concerning the perioperative changes in metabolic/nutritional parameters including npRQ during the early post-operative period after LDLT, we designed a pilot randomized control trial (RCT) over a limited period of time. Of 25 consecutive patients from March 2009 to October 2010 at Okayama University Hospital who were scheduled for elective LDLT and who were invited to take part in this study, all of the patients agreed to participate (Fig. 1).They were randomly divided into two groups: a 'control' group ($n = 13$) and a 'BCAA' group ($n = 12$). Randomization was performed using a computer-generated random permuted block method. One patient in the control group developed severe liver graft failure on post-operative day (POD)7 from refractory steroid-resistant rejection and died on POD23, so we could not measure npRQ and liver regeneration ratio after LDLT. Thus, we excluded this patient from the analysis and a total of 24 patients were analyzed in the per-protocol analysis. Clinical features of the subjects in both groups are shown in Table 1. All subjects provided written consent to participate in the study, and the Ethics Committee at Okayama University Hospital approved the protocol. This trial was registered with UMIN Clinical Trials Registry (http://www.umin.ac.jp/ctr, UMIN000004323).

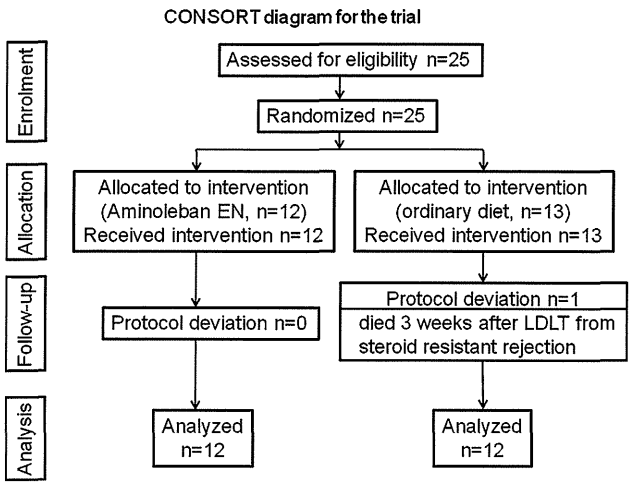

Fig. 1 CONSORT diagram for the trial. Flow diagram of subject progress through the phase of this pilot randomized trial. *CONSORT* consolidated standards of reporting trials

Protocol

Figure 2 shows the nutritional intervention schedule in this prospective study. After hospitalization, well-educated dietitians summarized the descriptions of the nutritional guidance for daily intake of energy and excess or deficient protein intake in each patient, and this information was provided to the patient via the physician in charge of the trial at Okayama University Hospital. In the control group, ordinary diet was adjusted to 30–35 kcal and 1.2–1.3 g of protein per kilogram of ideal body weight per day according to the European Society for Parenteral and Enteral Nutrition (ESPEN) guidelines for patients who could eat sufficient amounts of food before and 1 week after LDLT [23]. In the BCAA group, Aminoleban EN (Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan) was administered as a BCAA-enriched nutrient twice a day from 7 days until 1 day before LDLT and was continued from 3 days until 4 weeks after LDLT. One package of Aminoleban EN contains 210 kcal of energy (13.5 g of protein, 3.5 g of fat, and trace minerals and vitamins) and 6.075 g of BCAAs (isoleucine 2.04 g, leucine 2.25 g, and valine 1.785 g) [24]. Thus, in the BCAA group, total energy intake was adjusted by subtracting 420 kcal for energy and 27 g for protein from the total calorie and protein allowances per day. If any of the patients had difficulties with oral administration of Aminoleban EN after LDLT, Aminoleban EN was administered via nasogastric tube. Throughout the study, the control group did not receive a placebo because of the difficulty of finding a suitable placebo with a taste similar to Aminoleban EN [12]. The primary endpoint was improvement in the npRQ and the secondary end point was improvement in biochemical measurements and the liver regenerative ratio.

Table 1 Preoperative recipient status, graft variables, operative data, and total insulin usage

Variable	Control group ($n = 12$)	BCAA group ($n = 12$)	P
Age (years)	48.5 ± 4.4	52.6 ± 10.2	0.71*
Sex (male/female)	4/8	7/5	0.41$
Diagnosis			
Hepatitis (B/C/alcoholic)	8 (1/6/1)	9 (2/5/2)	0.57$
Autoimmune hepatitis	1	0	
Cholestatic disease	3	2	
Other	0	1[a]	
Preoperative status			
Child–Pugh score	10.0 ± 2.4	10.8 ± 2.1	0.54*
MELD score	15.8 ± 4.5	15.8 ± 5.2	0.75*
Donor and graft characteristics			
Donor age (years)	43.4 ± 14.2	42.5 ± 10.9	0.84*
Graft: right lobe/left lobe/posterior segment	4/6/2	8/4/0	0.15$
GRBW ratio (%)	0.85 ± 0.19	0.95 ± 0.24	0.3*
GW/SLV ratio (%)	44.5 ± 9.3	49.4 ± 11.2	0.18*
Surgical data			
Blood loss/body weight (mL/kg)	67 ± 49	132 ± 96	0.07*
Operative time (min)	525 ± 71	576 ± 146	0.4*
CIT (min)	57.7 ± 37.2	81.3 ± 63.8	0.54*
WIT (min)	51.9 ± 21.9	43.6 ± 15.6	0.31*
Total insulin usage (IU)	350 ± 185	483 ± 258	0.46*

MELD model end-stage liver disease, *GRBW* graft-to-recipient body weight ratio, *GW/SLV* graft weight to recipient standard liver volume ratio, *CIT* cold ischemic time, *WIT* warm ischemic time

* P values were evaluated by the Mann–Whitney U test

$ P values were evaluated by the chi-square test

[a] Budd–Chiari syndrome

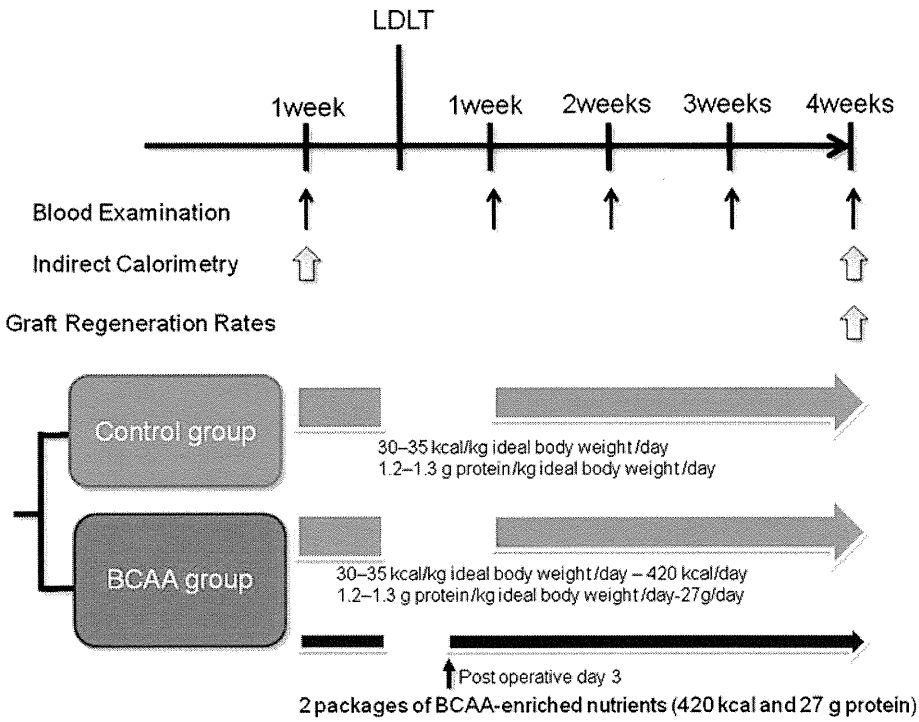

Fig. 2 Schematic presentation of the intervention schedule used in this prospective study. *Gray-filled lines* represent the intake duration of an ordinary diet adjusted to 30–35 kcal and 1.2–1.3 g of protein per kilogram of ideal body weight (BW) per day according to the ESPEN guideline before and 1 week after LDLT. The *black-filled line* represents the intake duration of Aminoleban EN, administered twice a day from 7 days to 1 day before LDLT and then restarted from 3 days to 4 weeks after LDLT. In the BCAA group, total energy intake was adjusted with a concomitant reduction of 420 kcal for energy and 27 g for protein from the total calorie and protein allowances per day

Measurements

Biochemical parameters

Serum total bilirubin (T.Bil), prothrombin time-international normalized ratio (PT-INR), BCAA-to-tyrosine ratio (BTR), and the rate of turnover of proteins, prealbumin, and retinol binding protein (RBP), were measured weekly from 1 week before up to 4 weeks after LDLT. BTR is a parameter that reflects the intake of BCAAs and has been reported to be a good indicator of the severity of hepatic parenchymal injury in patients with chronic liver disease [25]. Assessment of prealbumin and RBP are used to determine visceral protein mass associated with the current nutritional condition [26]. T.Bil and PT-INR were measured to evaluate the liver graft function.

Liver regeneration rates

Liver regeneration rates (LRR) were calculated using the following formula:

LRR = volume of the liver graft (g) calculated by a computed tomography obtained at 4 weeks after LDLT/the actual liver graft weight (g) measured at LDLT.

NpRQ and oxidative rates of carbohydrates, protein and fat

Non-protein respiratory quotient by an indirect calorimeter (Aeromonitor AE-300S, Minato Medical Science, Osaka, Japan) was measured twice per patient throughout the hospitalization period: 1 week before and 4 weeks after LDLT. Dietitians who were well acquainted with the Aeromonitor AE-300S used throughout this prospective study measured npRQ and the oxidative rates of carbohydrates, proteins and fat after overnight fasting of the patients. To examine the effect of BCAA-enriched supplements on each patient's metabolic status, we calculated ΔnpRQ (npRQ increase in value) using the following formula:

ΔnpRQ = the value of each patient's npRQ measured at 4 weeks after LDLT (POW4) − the value of each patient's npRQ measured at 1 week before LDLT.

Calculation of energy intake

Total energy intake (oral and parenteral intake), oral energy intake, oral protein intake and parental energy intake on post-operative weeks (POW) 1, 2, 3, and 4 was calculated in both groups.

Infectious complications

Infectious complications, bacterial, fungal and cytomegalovirus (CMV) infections, were investigated after LDLT. Infections were diagnosed using the criteria proposed by the Centers for Disease Control and based on previous reports regarding liver transplant recipients. Bacterial and fungal infections were diagnosed based on their clinical manifestations and the isolation of organisms. CMV infection was diagnosed as the presence of CMV antigenemia in patients in whom CMV antigens were not detectable previously.

Clinical outcomes

Clinical outcomes, such as the amount of fresh frozen plasma used during the post-transplant period, the length of intensive care unit and hospital stays after LDLT, and in-hospital mortality, were investigated in both groups.

Statistical analysis

Continuous values are presented as the mean ± standard deviation (SD). Differences in continual values between the control and BCAA groups were evaluated by the Mann–Whitney U test. Differences in frequency were analyzed by the chi-square test. All statistical analyses were performed with JMP software (Release 8.0.1, SAS Institute Japan). All reported P values were two-sided, and a P value <0.05 was considered statistically significant.

Results

Clinical background

There were no significant differences in age, sex, or underlying diseases between the two groups (Table 1). Preoperative examination of the hepatic reserve demonstrated similar Child–Pugh scores (control group 10.0 ± 2.4, BCAA group 10.8 ± 2.1). In addition, there was no difference in the model for end-stage liver disease (MELD) score, which represents the general preoperative status of the recipient, between the control group (15.8 ± 4.5) and the BCAA group (15.8 ± 5.2). With regard to the status of the liver grafts, there were no differences in donor age, graft weight/recipient body weight ratio and graft weight/recipient standard liver volume (GW/SLV) ratio. Furthermore, there were no significant differences in surgical invasiveness between the two groups, such as blood loss, operation time, or the duration of graft ischemia. In addition, there was no significant difference in the total insulin requirement from 7 days

before LDLT to 4 weeks after LDLT between the control group (350 ± 185 IU) and the BCAA group (483 ± 258 IU).

Changes in biochemical parameters

Serum levels of biochemical parameters such as BTR, BCAA, tyrosine, prealbumin and RBP were considerably improved 1 week after LDLT in both groups (Table 2). BTR was significantly improved throughout POW1–4 and reached the normal range at POW1 in the BCAA group (Table 2; Fig. 3a). BCAA, which is the numerator of BTR, was significantly increased at POW2 in the BCAA group (Table 2). Tyrosine, which is the denominator of BTR, was significantly decreased at POW1 and POW3 in the BCAA group (Table 2). Concerning rapid turnover proteins, prealbumin was significantly elevated at POW2 (Table 2), RBP was also improved significantly at POW2 and POW3 and reached the normal range at POW2 in the BCAA group (Table 2; Fig. 3b). The PT-INR improved significantly at POW3 and POW4 in the BCAA group (Table 2).

Liver regeneration after LDLT

Next, we calculated LRR in each patient of both the control and BCAA groups. The mean values of LRR in the control and BCAA groups were 1.81 ± 0.48 SD and 1.75 ± 0.37 SD, respectively. The mean volumes of liver grafts measured at 4 weeks after LDLT in the control and BCAA groups were 882 ± 148 SD (g) and 998 ± 204 SD (g), respectively. There were no differences between the two groups in both LRR and volume of the transplanted liver grafts (Table 3).

Changes in npRQ

In this study, well-trained dietitians successfully measured oxidative rates of carbohydrates, protein and fat in all patients at 1 week before and 4 weeks after LDLT. In the control group, the mean values of npRQ 1 week before and 4 weeks after LDLT were 0.789 ± 0.04 SD and 0.804 ± 0.06 SD, respectively, showing no significant increase in the mean value of npRQ. In contrast, a significant elevation of the mean value of npRQ was observed in the BCAA group from 1 week before to 4 weeks after LDLT (0.777 ± 0.05 SD to 0.835 ± 0.06 SD, $P = 0.022$, Table 4). Also, as shown in Fig. 4, ΔnpRQ clearly increased in the BCAA group. In addition, the mean oxidative rate of carbohydrates at 4 weeks after LDLT compared with that at 1 week before LDLT was significantly increased in the BCAA group (20.5–39.1%, $P = 0.016$) but not in the control group (24.7–30.1%, $P = 0.223$, Table 4; Fig. 5).

Table 2 Metabolic, nutritional and liver functional parameters measured in perioperative periods

	Pre	POW1	POW2	POW3	POW4
BTR					
Control group	1.68 ± 0.57	3.90 ± 1.34	3.72 ± 0.97	4.04 ± 1.63	4.17 ± 1.22
BCAA group	2.18 ± 0.77	5.24 ± 1.05*	5.49 ± 1.53*	5.70 ± 1.26*	6.04 ± 2.29*
BCAA (μmol/L)					
Control group	351 ± 64	473 ± 78	334 ± 98	362 ± 59	372 ± 69
BCAA group	369 ± 82	471 ± 87	418 ± 57*	385 ± 76	377 ± 96
Tyrosine (μmol/L)					
Control group	211 ± 76	124 ± 39	99 ± 67	97 ± 35	84 ± 25
BCAA group	183 ± 56	93 ± 23*	82 ± 26	70 ± 17*	67 ± 18
Prealbumin (mg/dL)					
Control group	5.25 ± 2.22	11.50 ± 3.23	11.83 ± 4.34	12.33 ± 5.74	14.33 ± 6.47
BCAA group	5.25 ± 2.34	12.75 ± 1.71	15.08 ± 4.56*	15.91 ± 5.55	16.50 ± 7.15
RBP (mg/dL)					
Control group	0.61 ± 0.20	1.93 ± 0.87	1.85 ± 0.93	1.89 ± 1.06	2.33 ± 1.39
BCAA group	0.75 ± 0.25	2.46 ± 0.85	3.03 ± 1.06*	3.05 ± 1.19*	3.16 ± 1.37
Total bilirubin (mg/dL)					
Control group	5.58 ± 3.57	3.82 ± 3.02	7.07 ± 9.43	7.47 ± 11.21	6.37 ± 10.05
BCAA group	5.19 ± 4.51	5.84 ± 3.89	6.19 ± 4.93	5.91 ± 5.99	4.88 ± 6.55
PTINR					
Control group	1.40 ± 0.39	1.23 ± 0.18	1.18 ± 0.20	1.32 ± 0.52	1.24 ± 0.32
BCAA group	1.33 ± 0.32	1.18 ± 0.14	1.03 ± 0.13	1.00 ± 0.09*	0.99 ± 0.07*

BTR BCAA-to-tyrosine ratio, *BCAA* branched-chain amino acid, *RBP* retinol binding protein, *PT-INR* prothrombin time-international normalized ratio, *Pre* 1 week before LDLT, *POW* post-operation weeks

* Statistically significant differences were observed in the mean values between the control and BCAA groups using the Mann–Whitney *U* test

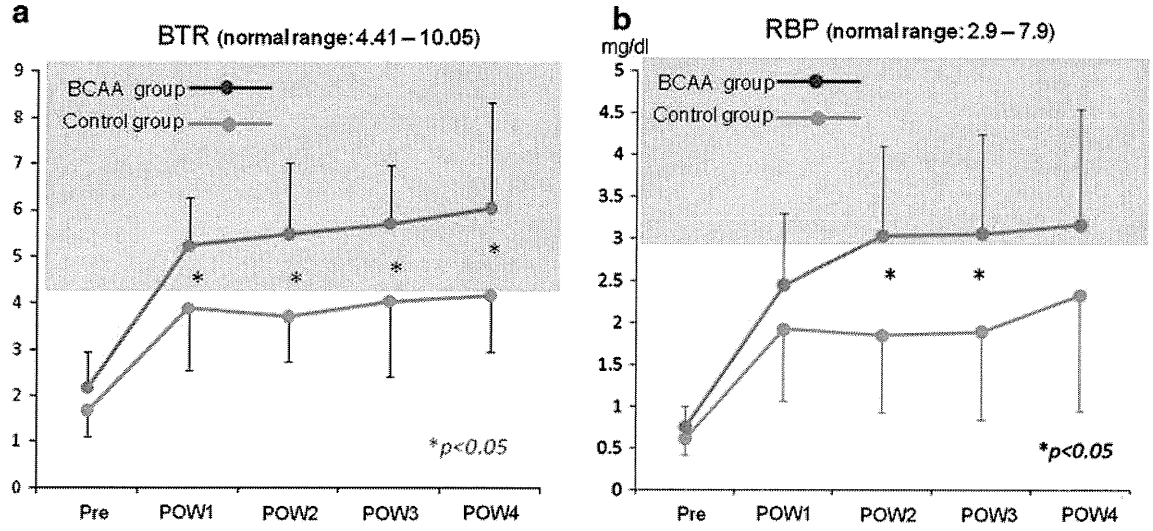

Fig. 3 BTR and RBP measured in the perioperative periods of LDLT. BTR and RBP were measured weekly from 1 week before and up to 4 weeks after LDLT. *Pre* 1 week before LDLT, *POW* post-operation weeks

Table 3 LRRs and volume of liver grafts at 4 weeks after LDLT

Variable	Control group ($n = 12$)	BCAA group ($n = 12$)	P
LRR	1.81 ± 0.48	1.75 ± 0.37	0.729
Graft volume (g)	882 ± 148	998 ± 204	0.119

LRR liver regeneration rates

P values were evaluated by the Mann–Whitney *U* test

Energy intake

Table 5 shows the average values of energy intake in both groups. In the BCAA group, the values of oral energy and protein intake include 420 kcal and 27 g protein per day provided by Aminoleban EN. There were no significant differences between the groups in total energy intake and parenteral energy intake throughout POW1–4. Oral energy and protein intake were significantly greater in the BCAA group only at POW1.

Infectious complications and clinical outcomes

Table 6 shows the infectious complications and clinical outcomes in both groups. Eight episodes of bacterial infections occurred in 4 recipients (33.3%) in the control group and five episodes of bacterial infections occurred in three recipients (25%) in the BCAA group. There were no significant differences in the occurrence of bacterial, CMV, and fungal infections between the two groups. The post-operative use of fresh frozen plasma did not differ significantly between groups. Hepatic artery thrombosis occurred in one patient 2 weeks after LDLT in the control group. This patient had complicated severe liver failure with cholangitis and died 5 months after LDLT. Although there were no significant differences, the length of intensive care unit and hospital stays after LDLT tended to be shorter in the BCAA group.

Discussion

This study was performed to test the feasibility of short-term administration of BCAA-enriched enteral nutrients to patients who had undergone LDLT. To the best of our knowledge, this is the first prospective pilot study demonstrating the efficacy of BCAA-enriched nutrients in patients with LDLT. Our results indicate that the administration of BCAA-enriched nutrients throughout the perioperative period significantly improved the post-operative metabolic abnormalities in patients with LDLT, and that there were no significant differences in LRR of grafts between the two groups.

Generally, the liver plays an important role in energy metabolism. End-stage liver diseases often result in liver cirrhosis lacking in adequate glycogen stores that develops into a severe catabolic state. Most of the patients needing LDLT have end-stage liver disease and, therefore, require appropriate nutritional management throughout the perioperative period for early recovery from the post-transplant catabolic phase. Kaido et al. [27] retrospectively examined 576 cases of adult LDLT and reported that enteral nutrition could be a promising strategy to improve post-operative mortality and morbidity rates. However, there have been no studies investigating the types of enteral nutrition that provide better outcomes for patients after liver

Table 4 NpRQ, oxidation rates of carbohydrate, fat and protein in the control and BCAA groups

	Control group		*P*	BCAA group		*P*
	Pre	POW4		Pre	POW4	
npRQ	0.789 ± 0.04	0.804 ± 0.06	0.318	**0.777 ± 0.05**	**0.835 ± 0.06**	**0.022***
%C	24.7 ± 12.1	30.1 ± 18.6	0.223	**20.5 ± 15.6**	**39.1 ± 16.8**	**0.016***
%F	58.8 ± 12.7	56.5 ± 19.2	0.704	57.3 ± 15.3	46.8 ± 16.9	0.178
%P	16.5 ± 9.8	13.4 ± 4.7	0.339	22.3 ± 12.9	14.1 ± 4.1	0.076

The %C, %F and %P denote the oxidation rates of carbohydrate, fat and protein, respectively

npRQ non-protein respiratory quotient, *Pre* 1 week before LDLT, *POW* post-operation weeks

P values were evaluated by the Mann–Whitney *U* test

* Statistically significant differences (*bold*) in the mean values at 4 weeks after LDLT compared with those at 1 week before LDLT

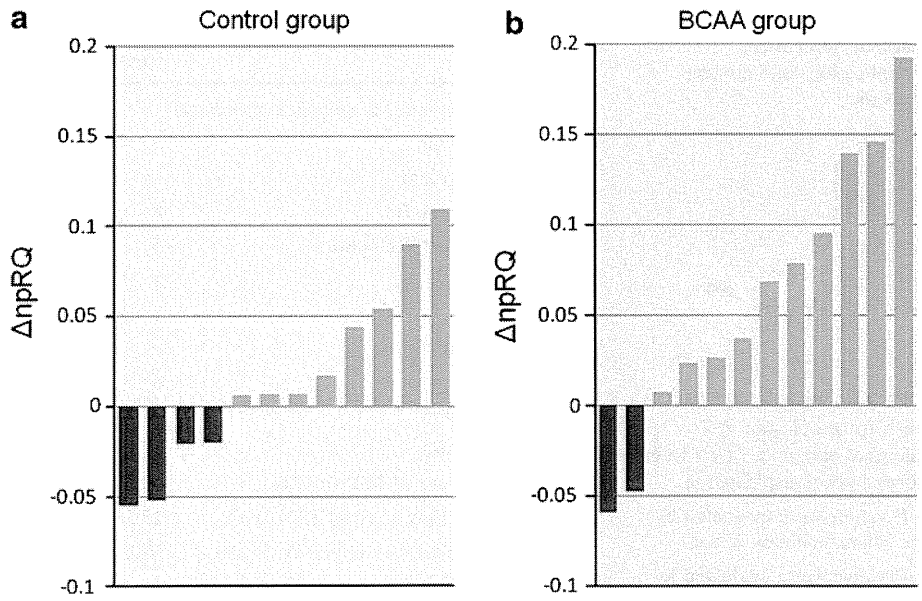

Fig. 4 Waterfall graph showing changes in non-protein respiratory quotient (npRQ) for each patient in both groups. Changes in npRQ of each patient before and after LDLT are represented by ΔnpRQ, which was obtained by subtracting the value of npRQ measured at 1 week before LDLT from that measured at 4 weeks after LDLT

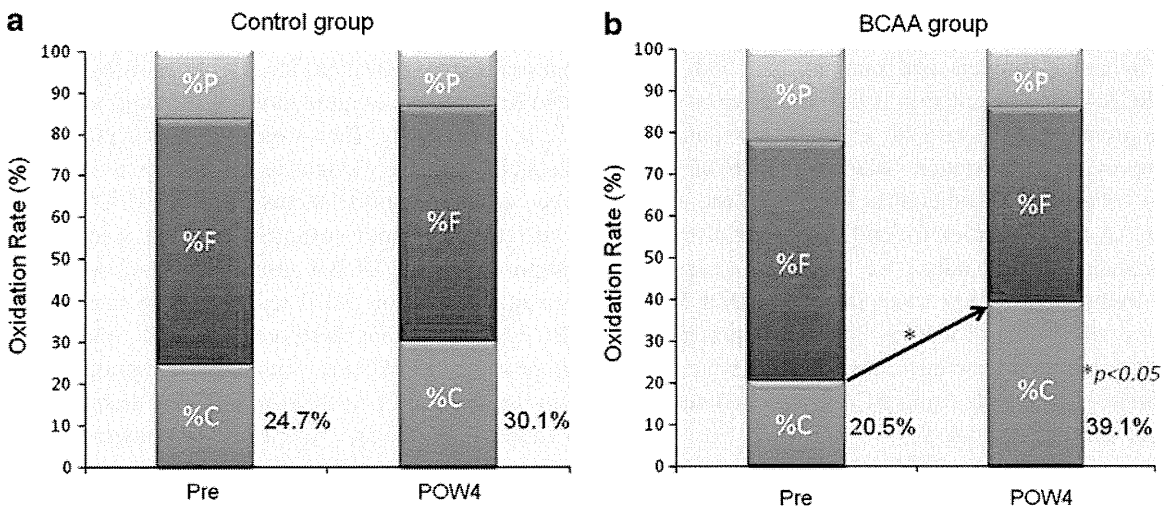

Fig. 5 Analysis using indirect calorimeter for oxidation rates of carbohydrate, fat, and protein. *%C*, *%F* and *%P* denote the oxidation rates of carbohydrate, fat and protein, respectively. Values are expressed as the % means. *Pre* 1 week before LDLT, *POW* post-operation weeks

Table 5 Energy intakes in the control and BCAA groups

	POW1	POW2	POW3	POW4
Total energy intake (kcal/day)				
Control group	1123 ± 221	1289 ± 550	1320 ± 320	1474 ± 613
BCAA group	1295 ± 406	1280 ± 433	1376 ± 404	1516 ± 390
Oral energy intake (kcal/day)				
Control group	810 ± 340	1014 ± 511	1139 ± 454	1262 ± 652
BCAA group	1092 ± 342*	1099 ± 427	1144 ± 464	1302 ± 469
Oral protein intake (g/day)				
Control group	32.8 ± 17.6	45.0 ± 21.2	49.0 ± 20.3	55.2 ± 28.1
BCAA group	49.4 ± 14.7*	50.2 ± 18.3	53.2 ± 21.2	59.5 ± 18.0
Parental energy intake (kcal/day)				
Control group	313 ± 223	275 ± 250	181 ± 238	212 ± 364
BCAA group	203 ± 188	181 ± 141	232 ± 348	214 ± 229

POW post-operation weeks

* Statistically significant differences in the mean values between the control and BCAA groups using the Mann–Whitney *U* test

Table 6 Infectious complications and clinical outcomes

Variable	Control group ($n = 12$)	BCAA group ($n = 12$)	P
Infectious complications (number of patient)			
Bacterial infection			
Number of patient	4	3	0.653$^{\$}$
Number of episodes	8	5	
Intra-abdominal infection	5	3	
Sepsis	1	1	
Pneumonia	1		
Catheter infection	1	1	
CMV infection	5	5	1.000$^{\$}$
Fungal infection	1	0	0.307$^{\$}$
Clinical outcomes			
Post-operative use of FFP (mL/kg)	19.3 ± 14.0	19.5 ± 13.8	0.922*
Length of ICU stay after LDLT (days)	13.3 ± 7.9	11.8 ± 3.1	0.594*
Length of hospital stay after LDLT (days)	65.3 ± 42.9	56.4 ± 37.1	0.527*
In hospital mortality	1	0	0.307$^{\$}$

CMV cytomegalovirus, *FFP* fresh frozen plasma, *ICU* intensive care unit, *LDLT* living donor liver transplantation

* *P* values were evaluated by the Mann–Whitney *U* test

$^{\$}$ *P* values were evaluated by the chi-square test

transplantation. It has been reported that the administration of BCAA could improve malnutrition associated with liver cirrhosis in animal models and humans [28, 29]. Additionally, BCAA supplementation is also effective in down-regulating protein catabolism with a reduction in ammonia, and in improving the nitrogen balance in liver cirrhosis, leading to better clinical outcomes. Therefore, the usefulness of BCAA administration for end stage liver diseases is widely known. Additionally, for patients undergoing deceased living donor liver transplantation (DDLT), the usefulness of early enteral feeding has been reported [30]. Taking these points of view into consideration, we planned this prospective pilot study using BCAA-enriched nutrients for patients undergoing LDLT.

To determine the benefit of BCAA-enriched nutrients to patients with LDLT, we measured serum biochemical nutritional parameters, such as BTR, prealbumin, and RBP (Table 2; Fig. 3). In the BCAA group, BTR was significantly improved throughout POW1–POW4 and reached the normal range. Thus, appropriate administration of BCAA-enriched nutrients was confirmed. The plasma half-life of prealbumin and RBP, known as rapid turnover proteins, are 2.5 days and 12 h, respectively. Their concentrations in plasma reflect the capacity for protein synthesis in hepatocytes which represent the current nutritional status [26, 31–33]. Our results demonstrate that rapid turnover proteins were significantly improved in the BCAA group compared with the control group. In addition, RBP reached the normal range in the BCAA group. Furthermore, PT-INR was significantly improved at POW3 and 4 in the BCAA group in spite of the similar amount of fresh frozen plasma used in both groups (Tables 2, 6). Even though the administration of BCAA reportedly promotes liver regeneration in the rat model [34], there is no evidence from clinical studies on a correlation between the regeneration of liver grafts and the management of perioperative nutrition. In the present study, we found no

differences in the LRRs or in the volume of the transplanted grafts at 4 weeks after LDLT between the two groups. Based on our results, it is suggested that the improvement in nutritional abnormalities due to the administration of BCAA-enriched nutrients throughout the perioperative period may be due to the functional development of hepatocytes rather than the regeneration of liver grafts. Thus, our results may indicate that the BCAA-enriched nutrients may act not only as an energy source or a stabilizer of amino acid imbalance but as some kind of signal transmitter in hepatocytes. Indeed, Matsumura et al. reported that oral administration of BCAA augments increased albumin synthesis in rat hepatocytes by the induction of an mTOR-signaling pathway [35–37].

In this study, we observed a clear improvement in npRQ status in patients receiving BCAA-enriched nutrients (Fig. 4). Commonly, npRQ reflects the status of energy metabolism in the entire body, which is at a low level in patients with end-stage liver disease. Such patients display carbohydrate metabolic disorders and increased usage of free fatty acids from adipose tissues as their energy source [38, 39]. Reduced glucose oxidation and enhanced lipid oxidation are responsible for the decrease in npRQ. Actually, in this study npRQs measured 1 week before LDLT were at low levels in both groups. Our prospective pilot study demonstrated that the administration of BCAA-enriched nutrients significantly improved, almost to a normal range, the npRQ and the rates of carbohydrate oxidation measured 4 weeks after LDLT (Table 4; Fig. 5).

The metabolic and nutritional state of recipients should gradually improve after LDLT over a long period of time due to the newly transplanted liver grafts. However, the LDLT recipients tend to fall into a severe and prolonged post-transplant catabolic state because of the invasiveness of the operative procedure and the necessity for regeneration of the partial liver graft. Plevak et al. [40] reported that a negative nitrogen balance persisted for 28 days after DDLT. Indeed, in this study, the mean value of npRQ and the average rate of carbohydrate oxidation had not improved at 4 weeks after LDLT in the control group. A successful nutritional goal after liver transplantation is to change from the post-transplant catabolic state to reach an anabolic state [19]. As our results demonstrate, the npRQ values and the rates of carbohydrate oxidation had recovered to near the normal range at 4 weeks after LDLT in the BCAA group. Therefore, the administration of BCAA-enriched nutrients may contribute to the change from a severe and prolonged post-transplant catabolic state to an anabolic state at an earlier stage.

The ESPEN guideline (published in 2006) recommended the use of immunonutrition 5–7 days before LDLT. In this study, BCAA-enriched nutrients were administered for 7 preoperative days to patients undergoing LDLT, improving nutritional and metabolic disorders during the early post-transplant period after LDLT. Cell culture and animal feeding studies indicated that an adequate supply of BCAA is necessary to support the efficient immune function of cytotoxic T lymphocytes and natural killer cells [41]. These results indicate that the requirement for BCAAs of immune cells is related to the synthesis of proteins such as cytokines, cytokine receptors, acute phase proteins, and immune-globulins [42, 43]. Therefore, we think that perioperative administration of BCAA might contribute to the encouragement of both innate and acquired immunity to pathogens after liver transplantation.

There are several limitations of the present study. Firstly, although there was no statistical significance, the proportion of the right lobe graft to the non-right lobe graft and the GRBW ratio were higher in the BCAA group than in the control group. Therefore, improvements in the nutritional and metabolic parameters in this study might be derived from these factors. Secondly, the insulin requirement was higher in the BCAA group than in the control group even though there was no statistical significance. Thus, the improvement in npRQ and the rates of carbohydrate oxidation might be influenced by the total dose of insulin during this trial. Thirdly, it is unclear whether improvements in nutritional and metabolic parameters were due to the BCAA-rich environment during the early perioperative period or early forced enteral feeding because enteral energy and protein intake on POW1 were greater in the BCAA group. A large-scale and well-designed RCT is anticipated to minimize the effects of such confounding factors.

In conclusion, BCAA-enriched enteral nutrients could improve the nutritional and metabolic disorders associated with end-stage liver disease in the early post-transplant period, and consequently shorten the post-transplant catabolic phase after LDLT. This study involved only a small number of patients, and a larger multicenter trial is needed to confirm these findings.

Appendix: Discussion at the plenary session of the 23rd Congress of the Japanese Society of Hepato-Biliary-Pancreatic Surgery, 9 June 2011

Comments by T. Beppu

In the performance of a randomized controlled trial (RCT), it is important to clearly state the study protocol, including the calculation of sample size. How did you calculate the number of patients needed in this trial?

Answer by R. Yoshida

According to previous data of the non-protein respiratory quotient (npRQ) measured in our hospital before this trial, the calculated sample size was 146 patients for each group (the alpha error was set at 5% with a power of 80%).

Our hypothesis should be proven by a quantitative RCT with adequate case numbers, but it is almost impossible to perform an RCT dealing with such a large number of patients undergoing living donor liver transplantation (LDLT) in a single center, unless it is a specialized institute. In addition, there has been no report concerning the perioperative serial changes in metabolic and nutritional parameters, including npRQ, during the early post-operative period after LDLT. Therefore, we designed a pilot RCT study during the limited period of time.

Comments by T. Beppu

Medication compliance often becomes the problem in taking nutrients during the early post-operative period. How was the compliance of taking the Aminoleban EN oral formula during this trial?

Answer by R. Yoshida

Because well-educated dietitians advised each patient before the trial on the importance of taking Aminoleban EN, almost all of the patients took the Aminoleban EN correctly, which was better than we expected. However, if patients had difficulties with the oral administration of Aminoleban EN just after LDLT, it was administered through a nasogastric tube.

Comments by T. Beppu

Did you estimate the difference in the liver regeneration rates in both groups after this trial, for example 3 months after LDLT or later?

Answer by R. Yoshida

We did not calculate the long term liver regeneration rates after this trial. However, we would expect to find no differences in the long-term liver regeneration rates or volume of the transplanted grafts between the two groups, because we believe that the improvement of nutritional abnormalities by the administration of branched chain amino acid-enriched (BCAA-enriched) nutrients was due to the functional development of the hepatocytes rather than the regeneration of liver grafts.

Comments by F. Kimura

Did you analyze for what kind of patient Aminoleban EN is effective?

Answer by R. Yoshida

Because the number of patients enrolled in this trial was small, we did not perform subgroup analysis in this study. However, we would expect that perioperative nutritional management is more useful for recipients with more severe conditions. With that in mind, we will perform subgroup analysis when the number of cases increases.

Comments by M. Kayahara

In a clinical study dealing with glucose metabolism, it is important to consider the dosage of insulin used in both groups. Did you investigate these factors in this trial?

Answer by R. Yoshida

I agree with your opinion, so we would like to verify whether our results are associated with those factors (the result of this analysis is shown in Table 1).

Comments by K. Kubota

It is difficult to understand the detailed mechanism by which the BCAA-rich environment leads to the improvement of metabolic abnormalities such as the low oxidative rate of carbohydrates. What do you consider to be the reason for these improvements?

Answer by R. Yoshida

It is unclear why a BCAA-rich environment results in an improvement in the rate of carbohydrate oxidation. However, we considered that the BCAA-enriched nutrients may act not only as an energy source or a stabilizer of an amino acid imbalance but also as some kind of signal transmitter in hepatocytes. As a result, BCAA-enriched nutrients improved the rate of carbohydrate oxidation. It has been reported that the oral administration of branched chain amino acids augments increased albumin synthesis in rat hepatocytes by the induction of the mTOR-signaling pathway.

Comments by J. Fujimoto

In daily clinical practice, general recovery of the graft function is also important for deviation from the catabolic state. Did you survey the changes in the parameters of the liver function such as serum bilirubin level and coagulation function?

Answer by R. Yoshida

We investigated the serial changes in total bilirubin level and prothrombin time–international normalized ratio (PT-INR). The total bilirubin level did not show a significant difference between the groups, but PT-INR improved significantly at POW3 and POW4 in the BCAA group. This result might indicate that graft function recovered earlier in the BCAA group.

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ORIGINAL ARTICLE

Enhanced antitumor efficacy of telomerase-specific oncolytic adenovirus with valproic acid against human cancer cells

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Replication-selective oncolytic viruses are being developed for human cancer therapy. We previously developed an attenuated adenovirus (OBP-301, Telomelysin), in which the human telomerase reverse transcriptase promoter element drives expression of *E1A* and *E1B* genes linked with an internal ribosome entry site. OBP-301 can replicate in, and causes selective lysis of, human cancer cells. Valproic acid (VPA), which is an effective antiepileptic drug, is known to inhibit the histone deacetylase activities. We determined whether the antitumor effect of OBP-301 could be enhanced by VPA in human lung cancer cells. In an *in vitro* cell viability assay, OBP-301 infection killed four human lung cancer cell lines, H1299, H1299-R5 (a subline of H1299 with a low level of the coxsackievirus and adenovirus receptor (CAR) expression), H460, and A549, more efficiently in the presence of VPA than in its absence. VPA treatment increased CAR expression in all the four lung cancer cells. Consistent with their CAR upregulation, the infection efficiency of adenoviruses in the presence of VPA was significantly higher than that in its absence. The molecular mechanism of this combined effect could be explained by an increase in adenovirus infectivity via VPA-mediated upregulation of CAR. These results suggest that treatment with OBP-301 in combination with VPA is a promising strategy for human lung cancer.

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Keywords: oncolytic adenovirus; telomerase; valproic acid

INTRODUCTION

Exploitation of new platforms for treatment of a variety of human malignant cancers is an urgent issue worldwide. Replication-selective oncolytic viruses are being aggressively developed and studied *in vitro* and *in vivo* as a promising new therapeutic approach for cancer treatment.[1–3] A variety of mutant or genetically modified adenoviruses has been engineered and these viruses have shown antitumor potency and safety in clinical trials.[4–7] We previously constructed an adenovirus vector (OBP-301, Telomelysin), in which the human telomerase reverse transcriptase promoter element drives expression of *E1A* and *E1B* genes linked with an internal ribosome entry site. We showed that OBP-301 causes efficient selective killing of human cancer cells, but not of normal cells.[8] Although OBP-301 demonstrated a broad-spectrum antitumor efficacy, infectivity of the currently available adenoviral agent, which is derived from human adenovirus serotype 5, varies widely depending on the expression level of coxsackievirus and adenovirus receptor (CAR) on the surface of target cells.[9,10] Therefore, the improvement and augmentation of adenoviral infectivity is an important challenge to ensure maximal antitumor activity.

Histone deacetylase (HDAC) inhibitors have been proposed as a new type of antitumor agent that have a potential role in the epigenetic modulation of gene expression, induction of cell death, apoptosis and cell cycle arrest in tumor cells. Several HDAC inhibitors are currently being investigated in clinical trials in the cancer therapy as single agents or in combination with different agents.[11–13] We previously showed that FR901228 (depsipeptide, FK228), a novel type of HDAC inhibitor isolated from the fermentation broth of *Chromobacterium violaceum*, preferentially increased adenovirus infectivity via upregulation of CAR expression on target tumor cells, leading to a profound oncolytic effect of OBP-301;[14] however, clinical use of FR901228 is limited to the treatment of cutaneous T-cell lymphoma.

Valproic acid (VPA) is a synthetic derivative of propylpentanoic acid that is clinically and widely used as an anticonvulsant and mood-stabilizing drug, mainly in the treatment of epilepsy and bipolar disorder. VPA is also known to inhibit HDAC activities and is undergoing several clinical trials as an antitumor drug in various cancers.[15,16] Recent studies have demonstrated that VPA improves the antitumor efficacy of oncolytic herpes simplex virus in human glioma cells[17] and of adenovirus-mediated gene therapy in human head and neck squamous cell carcinoma.[18] Although OBP-301 is currently being evaluated as a monotherapy in clinical trials,[19] multimodal therapeutics aimed at enhancing antitumor efficacy might be essential for a successful clinical outcome.

In this study, we examined whether pretreatment with VPA can upregulate CAR levels on the target tumor cells, thereby enhancing the antitumor effect of OBP-301 against human lung cancer cells.

MATERIALS AND METHODS

Cell culture and reagents

The human lung cancer cell lines H1299 (a human non-small-cell lung cancer cell line), H1299-R5 and H460 (a human large-cell lung cancer cell line) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units ml^{-1} penicillin, and 100 mg ml^{-1} streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. H1299-R5 is a subline of H1299 that is refractory to adenovirus infection due to a low level of CAR expression.[20] A549 (a human lung adenocarcinoma cell line) was cultured

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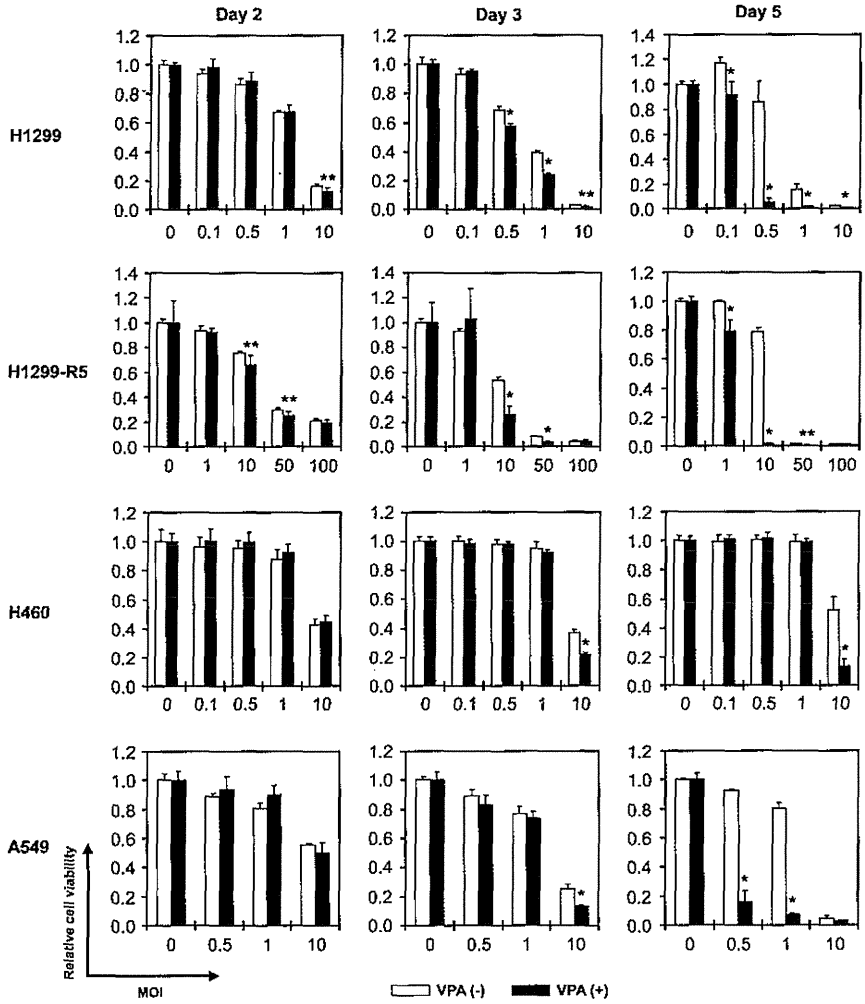

Figure 1. Antitumor efficacy of OBP-301 plus valproic acid (VPA) against human lung cancer cells. To evaluate the antitumor efficacy of the combination of OBP-301 and VPA *in vitro*, H1299, H1299-R5 (a subline of H1299 with a low level of coxsackievirus and adenovirus receptor (CAR) expression), H460 and A549 cells were treated with or without 1 mM of VPA, and then infected with the indicated multiplicity of infection (MOI) of OBP-301 at 24 h after VPA treatment. Cell viability was assessed using the XTT assay at 2, 3 and 5 days after OBP-301 infection. Data are means ± s.d. (*) and (**) indicate statistical significance of $P<0.01$ and $P<0.05$, respectively vs the viability of OBP-301-infected cells without VPA treatment.

in DMEM-F12 medium supplemented with 10% fetal bovine serum, 100 units ml^{-1} penicillin, and 100 mg ml^{-1} streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. H1299 and A549 cell lines were obtained from the American Type Culture Collection (Manassas, VA). The sodium salt of VPA was purchased from Sigma-Aldrich (St Louis, MO) and was prepared as a 1 M stock solution.

Recombinant adenoviruses

OBP-301 (Telomelysin) is a telomerase-specific replication-component adenovirus variant, in which the human telomerase reverse transcriptase promoter element drives the expression of *E1A* and *E1B* genes linked with an internal ribosome entry site.[8] A replication-deficient adenoviral vector containing the green fluorescent protein (*GFP*) gene inserted under the cytomegalovirus promoter (Ad-GFP) was also used.

Cell viability assay

The XTT (sodium 3′-[1-(phenylaminocarbonyl)-3, 4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate) assay was performed to measure the cell viability. Briefly, the cells were seeded at a density of 1×10^3 cells per well in 96-well plates 16–20 h before viral infection and were infected with OBP-301 at a multiplicity of infection (MOI) of 0, 1, 10 or 50 plaque-forming units per cell. Cell viability was determined at the indicated times by using a Cell Proliferation Kit II (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol.

Flow cytometric analysis

The cells (2×10^5 cells) were labeled with a mouse monoclonal anti-CAR antibody (RmcB; Upstate Biotechnology, Lake Placid, NY) for 30 min on ice. An isotype-matched normal mouse IgG1 conjugated to fluorescein isothiocyanate (Serotec, Oxford, UK) was used as a control. The cells were then incubated with a fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG second antibody (Zymed Laboratories, South San Francisco, CA) and were analyzed using flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA). Relative mean fluorescence intensity was determined by calculating the quotient of the mean fluorescence intensity in antibody-treated cells and nontreated cells.

Quantitative real-time PCR analysis

The DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA), and quantitative real-time PCR analysis of *E1A* gene expression was performed using a LightCycler instrument (Roche Molecular Diagnostics, Indianapolis, IN). The sequences of the specific primers used for E1A amplification were: sense: 5′-CCT GTG TCT AGA GAA TGC AA-3′ and antisense: 5′-ACA GCT CAA GTC CAA AGG TT-3′. PCR amplification began with a 600 s denaturation step at 95 °C, which was followed by 40 cycles of

denaturation at 95 °C for 10 s, annealing at 58 °C for 15 s, and extension at 72 °C for 8 s. Data analysis was performed using LightCycler Software. Expression levels of the treated cells were normalized to those of untreated cells for each sample.

Fluorescent microscopy and fluorescent microplate reader

Human lung cancer cells were infected with Ad-GFP at an MOI of 1 for 24 h. Cells were photographed using an IX71 fluorescent microscopy (Olympus, Tokyo, Japan). The level of expression of GFP fluorescence was measured using fluorescent microplate reader (DS Pharma Biomedical, Osaka, Japan) with excitation/emission at 485 nm/528 nm.

Statistical analysis

Significant differences among the groups were assessed by Student's *t*-test using values from the original data analysis. *P*-values <0.05 were considered statistically significant.

RESULTS

Antitumor efficacy of OBP-301 plus VPA against human lung cancer cells

We first evaluated the antitumor efficacy of a combination of OBP-301 and VPA *in vitro*. H1299, H1299-R5 (a subline of H1299 with a low level of CAR expression), H460 and A549 cells were treated with or without 1 mM of VPA, and then infected with the indicated MOI of OBP-301 at 24 h after VPA treatment. Cell viability was assessed using the XTT assay at 2, 3 and 5 days after infection (Figure 1). VPA treatment increased the antitumor efficacy of OBP-301 in all of the cell lines that we tested. In particular, VPA treatment dramatically increased the antitumor efficacy of OBP-301 at 5 days after infection when OBP-301 was used at 0.5 MOI in H1299 and A549 cells, at 1 MOI in A549 cells and at 10 MOI in H460 cells. These results suggested that VPA enhanced the antitumor efficacy of OBP-301 against human lung cancer cells.

Expression of CAR on human lung cancer cells after VPA treatment

To explore the mechanism by which VPA enhanced the antitumor efficacy of OBP-301, we next examined whether VPA upregulates CAR expression on the cell surface. H1299, H1299-R5, H460 and A549 cells were treated with or without 1 mM of VPA for 24 h and 72 h. Cancer cells were then subjected to flow cytometric analysis. VPA treatment induced CAR expression in a time-dependent manner in all the four lung cancer cell lines (Figure 2). The CAR expression was increased in the VPA-treated cells by 1.3 to 3.1-fold compared with the VPA-nontreated cells (Figure 2b).

Infectivity of the oncolytic adenovirus in human lung cancer cells after VPA treatment

We further examined the effect of VPA on OBP-301 infectivity by measuring E1A DNA copy number. H1299, H1299-R5, H460 and A549 cells were treated with or without 1 mM VPA, and infected with 1 MOI of OBP-301 24 h later. The cells were harvested at 2 h after infection and E1A copy number was analyzed using quantitative real-time PCR analysis. As shown in Figure 3a, the E1A copy number in the four lung cancer cell lines was increased by VPA treatment: the VPA-treated to VPA-nontreated ratio was 1.8, 2.1, 1.2 and 1.2 for H1299, H1299-R5, H460 and A549 cells, respectively. Moreover, to confirm the effect of VPA on OBP-301 infectivity, we investigated the expression of adenovirus-derived protein in cancer cells. Using fluorescent microscopy and fluorescent microplate reader, the expression of GFP was evaluated in the cells treated with or without 1 mM VPA, followed by the replication-defective GFP-expressing adenovirus, Ad-GFP, infection 24 h later (Figure 3b). The fluorescence intensity of GFP in VPA-treated H1299, H1299-R5, H460 and A549 cells was significantly higher than that in control cells (Figure 3c). These findings were consistent with the results of E1A copy number as analyzed using quantitative real-time PCR. These results suggest that the infectivity of OBP-301 for H1299, H1299-R5, H460 and A549 cells was increased by VPA treatment.

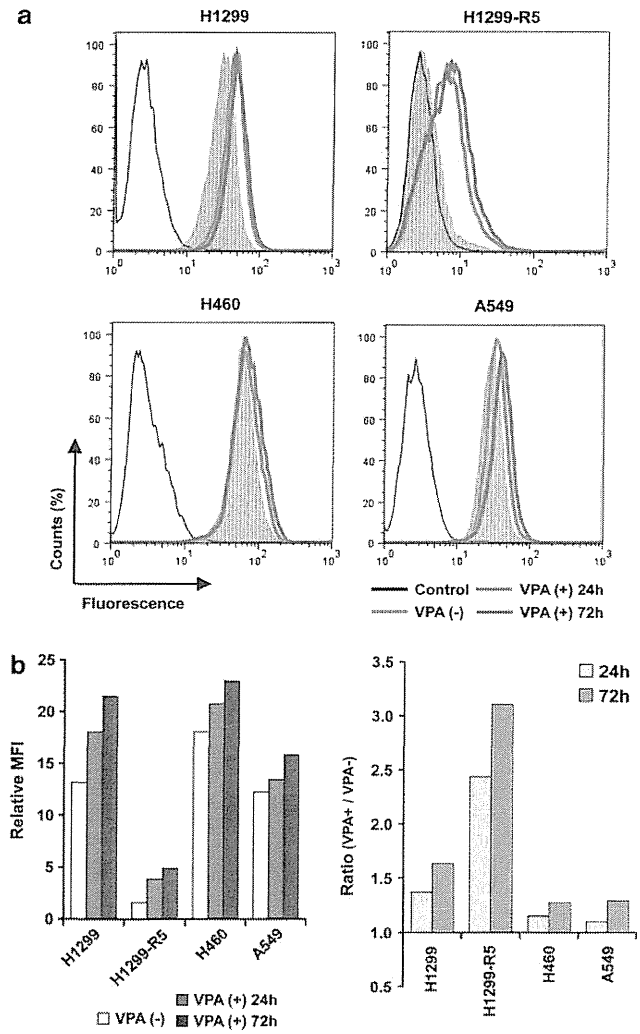

Figure 2. Expression of coxsackievirus and adenovirus receptor (CAR) on human lung cancer cells after valproic acid (VPA) treatment. The indicated cells were treated with or without 1 mM of VPA for 24 h or 72 h and were then subjected to flow cytometric analysis. Both treated and untreated cells were incubated with mouse monoclonal anti-CAR antibody (RmcB), whose cell binding was detected with a fluorescein isothiocyanate-labeled secondary antibody. An isotype-matched normal mouse IgG1 conjugated to fluorescein isothiocyanate was used as a control in all experiments. (**a**) The rightward shift of the histogram after exposure to VPA indicates increased CAR expression. (**b**) Relative mean fluorescence intensity (MFI) was calculated using the following formula; relative $MFI = MFI_{VPA(-) \text{ or } VPA(+)}/MFI_{control}$. The CAR expression ratio of VPA(+) to VPA(−) was also shown.

Replication of OBP-301 in human lung cancer cells after VPA treatment

To determine the effect of VPA on OBP-301 replication, we measured the E1A copy number in the presence and absence of VPA treatment. H1299, H1299-R5, H460 and A549 cells were infected with OBP-301 24 h after VPA treatment, were harvested at various time points over 72 h after infection of OBP-301 and E1A copy number was then analyzed using quantitative real-time PCR analysis. The ratios were normalized by dividing the value of

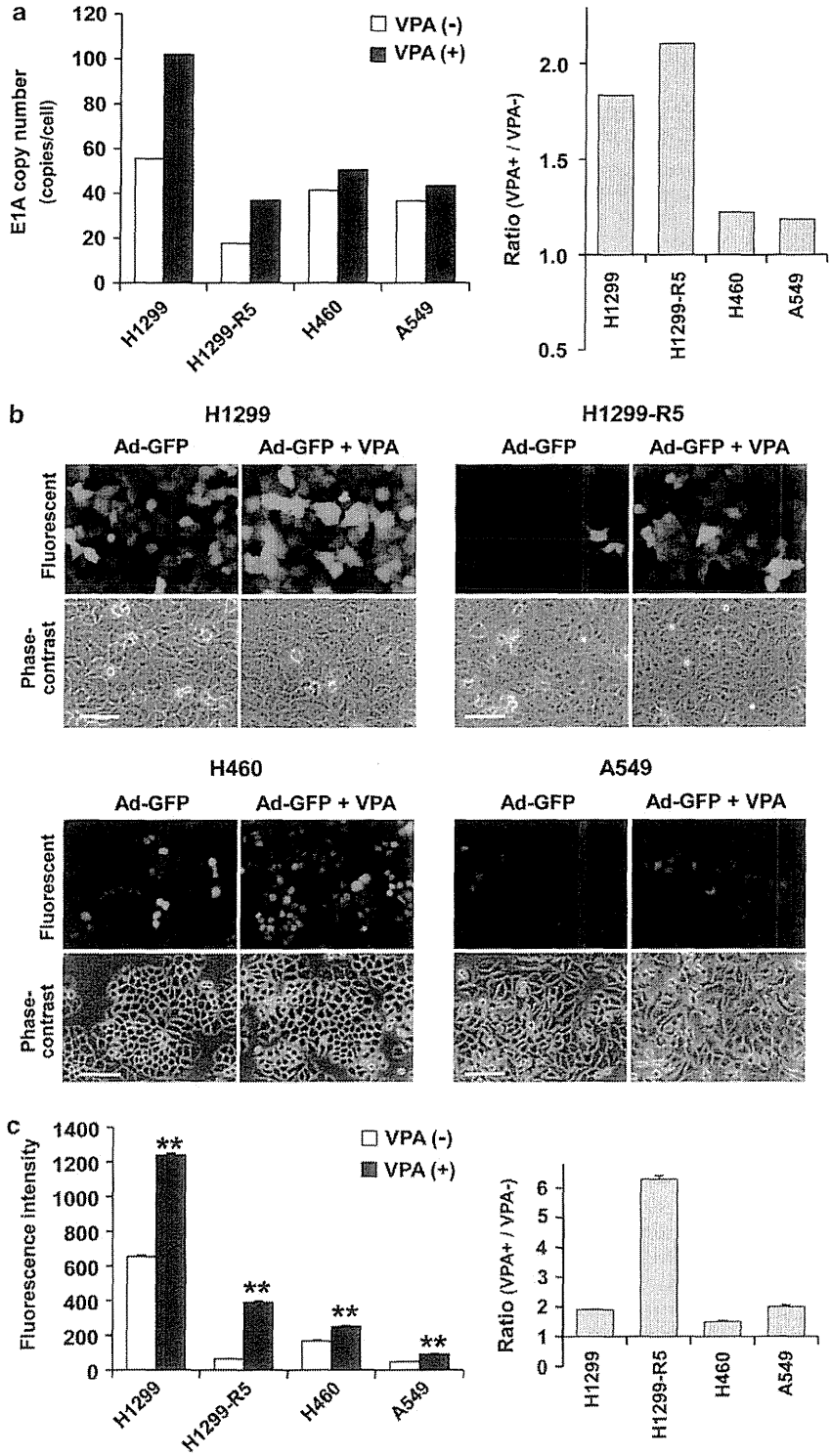

Figure 3. Infectivity of OBP-301 in human lung cancer cells after valproic acid (VPA) treatment. The indicated cells were treated with or without 1 mM of VPA, and infected with 1 multiplicity of infection (MOI) of OBP-301 (**a**) or 1 MOI of Ad-green fluorescent protein (GFP) (**b**, **c**) 24 h later. Viral infectivity was assessed by measuring the E1A copy number using real-time quantitative PCR (**a**), observation of cellular GFP fluorescence using fluorescent microscopy (**b**), and by measurement of GFP fluorescence intensity using fluorescent microplate reader (**c**). Scale bars = 100 μm. (**) indicates statistical significance of $P<0.01$.

cells 2 h after viral infection. As shown in Figure 4, OBP-301 had replicated 3–4 logs by 72 h after infection in all OBP-301-infected cells with or without VPA treatment, suggesting that VPA had no apparent effect on OBP-301 replication in all of the tested lung cancer cells.

DISCUSSION

Virotherapy by oncolytic viruses including adenoviral agent OBP-301 is quite a promising therapeutic approach for cancer and OBP-301 has been shown to efficiently kill a variety of human cancer cells.[8] Virus infectivity is an essential aspect in achieving the

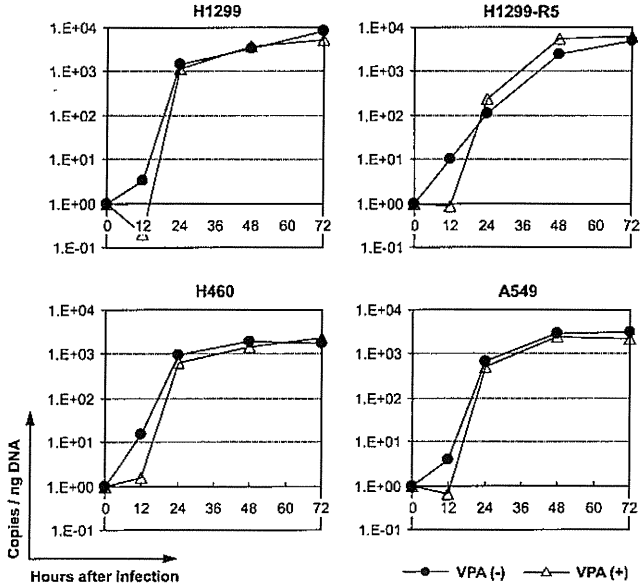

Figure 4. Replication of OBP-301 in human lung cancer cells after valproic acid (VPA) treatment. The indicated cells were treated with or without 1 mM of VPA, and infected with one multiplicity of infection (MOI) of OBP-301 24 h later. Viral E1A copy number in the cells was then assayed using real-time quantitative PCR. Viral E1A copy number of each sample is defined as the fold increase relative to that at 2 h (the 2 h value is designated as 1).

maximal effect of oncolytic viruses. In terms of viral infectivity, increasing the expression of the adenovirus receptor CAR on the surface of target cancer cells is important and increases the efficacy of cancer therapy by oncolytic adenoviral agents. Here, we showed that VPA, an HDAC inhibitor that is well established as a treatment for epilepsy and bipolar disorder, enhanced the expression level of CAR on the surface of human lung cancer cells. Our findings are consistent with the results of Kothari *et al.*,[18] who reported that VPA increases the mRNA level of CAR in NT8e head and neck squamous cell carcinoma. We previously showed that FR901228, a novel type of HDAC inhibitor, increases the CAR expression level on the surface of H460 and A549 cells, but not of H1299 cells.[14] In the present study, VPA increased the CAR expression level on the surface of H1299, H460 and A549 cells, and H1299 cells was more sensitive to VPA than H460 and A549 cells (Figure 2). The reason why the CAR expression in these lung cancer cell lines treated with VPA is different from the result treated with FR901228 is unclear. However, it is possible that the difference in sensitivity to FR901228 and VPA between these cell lines is due to differences in specificity for HDACs; for example, FR901228 specifically inhibits HDAC1 and 2, and VPA inhibits class I and IIα HDACs.[12]

One of the advantages of replication-competent oncolytic adenoviruses is that less virus particles are required for treatment, because viruses can be produced in tumor tissues by replication. We used the replication-defective Ad-GFP at a MOI of 1 to directly evaluate the effect of VPA on increased virus infectivity of the oncolytic virus, and we confirmed increased infection efficiency of the adenovirus following VPA treatment (Figures 3b and c). These results are also consistent with the results reported by Kothari *et al.*[18] Regarding the effect of VPA on viral replication, Höti *et al.*[21] reported that VPA inhibited adenoviral replication by induction of p21, which is cell-cycle-regulating protein. However, in our study, VPA treatment did not affect the replication of OBP-301 (Figure 4) though VPA upregulated p21 expression in H1299 and H1299-R5 cells (Supplementary Figure S1a). Additionally, the VPA-mediated p21 expression was markedly reduced in OBP-301-infected cells (Supplementary Figure S1b). These data are consistent with our recent report that adenoviral E1A suppresses the expression of p21,[22] suggesting that E1A would inhibit antagonistic effect of VPA on antitumor effect of oncolytic virus through reduction of p21 expression.

It has been reported that VPA itself has several antitumor effects including alteration of transcriptional activity,[15,16] stimulation of antitumor immune systems[23] and inhibition of tumor angiogenesis.[24] We previously showed that OBP-301 replication produced the endogenous danger signaling molecule, uric acid, in infected human cancer cells. This uric acid stimulated dendritic cells to produce interferon-γ (IFN-γ) and interleukin 12. Subsequently, IFN-γ release upregulated expression of the endogenous proteasome activator PA28 in cancer cells and resulted in the induction of cytotoxic T-lymphocytes.[25] Moreover, OBP-301 induces an antiangiogenic effect by stimulating host immune cells to produce antiangiogenic mediators such as IFN-γ.[26] These findings suggest that combining OBP-301 with VPA should exert a stronger antitumor effect than either agent alone. Furthermore, VPA has been shown to enhance radiosensitization in human cancer cells.[27] We previously reported that OBP-301 enhances radiosensitization in human cancer cells by inhibition of the DNA repair machinery.[28] Based on these findings, combination therapy with OBP-301, VPA and radiation is an exceedingly promising antitumor therapy.

In summary, we showed that VPA increases cellular CAR expression, which, in turn, facilitates virus infection, thereby enhancing antitumor effects in cancer cells. Our data support the possibility that combination of the telomerase-specific adenovirus agent OBP-301 with VPA is a promising strategy for cancer treatment.

CONFLICT OF INTEREST

Hitoshi Kawamura, Katsuyuki Nagai, and Yasuo Urata are the employees of Oncolys BioPharma, Inc., the manufacturer of OBP-301 (Telomelysin).

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Supplementary Information accompanies the paper on Cancer Gene Therapy website (http://www.nature.com/cgt)