may possibly be markers of early imaging-invisible HCC. In addition, active immunotherapy using GPC3 peptides may prevent the development of both non-neoplastic and neoplastic lesions of the liver.

Acknowledgements

We thank Ms. Chinatsu Kojima for her help with the preparation of this manuscript. This study was supported in part by Health and Labor Sciences Research Grants for Research on Hepatitis from the Ministry of Health, Labour and Welfare, Japan, and a Grant-in-Aid for the Third-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare, Japan, the Foundation for Promotion of Cancer Research in Japan, the Japan Research Foundation for Clinical Pharmacology, and a Research Resident Fellowship from the Foundation for Promotion of Cancer Research, Japan (E.H.).

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World J Surg (2009) 33:312–317
DOI 10.1007/s00268-008-9831-2

World Journal of Surgery

Risk Factors of Surgical Site Infection After Hepatectomy for Liver Cancers

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Published online: 21 November 2008
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Abstract
Background Risk factors of surgical site infection (SSI) after hepatectomy under the guideline of Centers for Disease Control and Prevention (CDC) are not well examined.
Methods Hospital records of consecutive patients who underwent hepatectomy without biliary reconstruction for liver cancers were reviewed retrospectively. Prophylactic antibiotics were given to patients just before skin incision and every 3 hours during the operations. Clinicopathological factors were compared between patients who developed SSI and those without it.
Results There were 405 patients identified, and the incidence of SSI was 23 cases (5.8%). In multivariate analysis, intraoperative bowel injury, blood loss >2000 ml, and age older than 65 years were significant risk factors of SSI after hepatectomy.
Conclusions Prophylactic antibiotics were necessary only during the operation for most patients who underwent hepatectomy without biliary reconstruction. However, patients with intraoperative bowel injury, blood loss >2000 ml, and age older than 65 years are at risk to develop SSI and might need additional administration of prophylactic antibiotics after surgery.

Introduction

Use of antibiotics is one of the main techniques to prevent surgical site infection (SSI) after surgery. There has been tremendous accumulation of evidence during the last three decades with regard to the optimal methods of its administration [1]. The Centers for Disease Control and Prevention (CDC) recommended in its 1999 guideline to maintain therapeutic levels of prophylactic antibiotic during the operation and, at most, a few hours after closure of incisions [2]. However, it is well known that incidence of SSI is greatly influenced by patients' underlying general status and perioperative factors [3]. Disease and procedure-specific risks and use of prophylactic antibiotics are not well examined, except for colorectal surgery [4, 5], open heart surgery [6], cholecystectomy [7, 8], etc.

It is suggested that hepatectomy suppresses Kupffer cell and T-cell function significantly, which renders patients immunosuppressive [9]. Postoperative infection, including SSI, deteriorates hepatic failure in cases with limited hepatic functional reserve. There is a wide variety in operation time, blood loss, transfusion requirement, etc., depending on the extent of parenchymal resection. Underlying cirrhosis and hypoalbuminemia inhibits normal wound healing [10]. However, perioperative factors that should be considered a significant risk to develop SSI after hepatectomy have not been clear. The purpose of this study was to analyze the risk factors of SSI after hepatectomy with prophylactic antibiotics under CDC guideline and to clarify who might benefit from additional administration of prophylactic antibiotics after operation.

Materials and methods

Patients who underwent hepatectomy for liver cancers from November 2002 to December 2006 at National Cancer Center East Hospital, Kashiwa, Japan, were identified and reviewed retrospectively. Patients who

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underwent hepatectomy without biliary reconstruction regardless of diagnosis were included in the study. Patients who underwent cholecystectomy along with hepatectomy were included in the study, but those who underwent simultaneous procedures, such as colorectal resection or stoma closure, were excluded from the study.

The extent of hepatectomy was evaluated according to the disease progression, liver function, and general condition of patients [11]. Tumor progression and resectability was assessed by imaging studies, such as contrast enhanced computed tomography (CT) scans, magnetic resonance imaging (MRI), hepatic arterial angiography, ultrasound, and chest x-ray. Liver function was assessed by liver biochemistry test, Child-Pugh grade [12], and the indocyanine green retention rate at 15 minutes [13]. All patients were reviewed before surgery at weekly conferences by hepatic surgeons, medical oncologists, and interventional radiologists to discuss whether the planned procedures were appropriate. Hepatic resection was performed under intraoperative ultrasonographic guidance by the pean fracture method with or without inflow occlusion (Pringle's maneuver). Anatomic hepatectomy was performed whenever possible, whereas partial resection was performed in consideration of limited liver functional reserve or anatomic location of the tumor. During parenchymal resection, all blood vessels and bile ducts were ligated whenever possible with 2-0 or 3-0 braided silk or vessel clip. One or two closed drains were inserted at the end of operation in the right subphrenic space or wherever close to the resected liver parenchyma. Drains were removed when no rebleeding or bile leakage was observed on postoperative day (POD) 3 or 4.

SSI was defined as a condition in which purulent discharge was observed from any incision or space that was manipulated during an operation within 30 days after the operation with or without microbiological evidence as in the guideline issued by CDC [2], and it was identified retrospectively by reviewing clinical records of patients who underwent hepatectomy. Remote site infection was defined as a condition in which fever and leukocytosis were present with bacteria in sputum, urine, catheter-tip, blood, or other body fluid/space, or according to the physician's judgment regardless of microbiological evidence.

Patients were usually given two doses of cefazolin as prophylactic antibiotics. One gram of cefazolin was administered to patients within 30 minutes before skin incision and another dose 3 hours later. When the operation lasted more than 3 hours, additional doses were given every 3 hours thereafter during the operation. No antibiotics were given after incisions were closed if patients had already received two doses of cefazolin.

All data were compiled in a database for analysis (Microsoft Excel and SPSS 11.0 J for Windows). Differences between numerical variables were tested with Mann-Whitney *U* test and those between categorical variables were tested with χ^2 statistics. Multivariate analysis was performed with logistic regression test. $p < 0.05$ was deemed significant.

Results

During the period of study, 405 patients underwent hepatectomy without biliary reconstruction for primary or secondary liver cancers at National Cancer Center East Hospital, Kashiwa, Japan. Of these 405 patients, 23 patients (5.8%) developed SSI (incisional, 20; organ/space, 3). Incisional SSIs were treated by opening incisions and organ/space SSIs were treated by drainage under ultrasound guidance. The patient characteristics and demographic variables are listed in Table 1. No differences in these basic characteristics, except age, were observed between patients with SSI and those without it. Mean age of patients with SSI was 68.2 years and was statistically older than those without SSI. A cutoff value of aged 65 years had the highest statistical power ($p = 0.016$). Patients' ASA score, comorbidities, and underlying liver pathology were statistically similar between the two groups.

Culture results of infecting organisms included *Bacteroides faragilis* (n = 3), *Staphylococcus aureus* (n = 2), *Klebsiera oxytoca* (n = 1), *Serratia marcescens* (n = 1), *Escherichia coli* (n = 1), *Streptocccocus anginosus* (n = 1), *Streptococcus constellatus* (n = 1), *Enterobacter cloacae* (n = 1), *Citrobacter braakii* (n = 1), *Citrobacter freundii* (n = 1), Corynebacterium species (n = 1), and Candida species (n = 1).

The perioperative variables are listed in Table 2. Operation time, red blood cell (RBC) transfusion requirement, RBC transfusion volume, and intraoperative bowel injury were statistically different between the two groups. Blood loss did not reach statistical significance, but cutoff value of 2000 ml had the significant power to predict SSI ($p = 0.003$). Multivariate analysis of those variables found that intraoperative bowel injury, blood loss >2000 ml, and age older than 65 years were the significant risk factors to develop SSI after hepatectomy without biliary reconstruction (Table 3). Rates of SSI increased dramatically with the number of risk factors present (Fig. 1). Patients with two or more risk factors were statistically more likely to develop SSI than those with none or only one risk factor.

During the same period, three patients died within 30 days from the operations. One patient died from pulmonary embolism on POD 3, another died from brain stroke on POD 3, and the other died from esophageal varix rupture on POD 9. Incidence of remote site infection was

Table 1 Patient characteristics and demographic variables for patients with SSI compared with those without it

	SSI (−) (N = 382)	SSI (+) (N = 23)	*P* value
Age (yr)[a]	63.7 ± 0.5	68.2 ± 2	*0.034*
≥65[b]	194 (50.9)	18 (78.3)	*0.016*
<65	188 (49.1)	5 (21.7)	
Gender[b]			0.809
Male	285 (74.6)	18 (78.3)	
Female	97 (25.4)	5 (21.7)	
Body mass index (kg/m^2)[a]	23.8 ± 0.6	23.6 ± 0.7	0.583
Diabetes mellitus[b]	75 (19.6)	1 (4.5)	0.095
ASA score[b]			0.488
1	111 (29.5)	7 (30.4)	
2	243 (64.6)	16 (69.6)	
3	22 (5.9)		
Diagnosis[b]			0.566
HCC	239 (62.6)	13 (56.5)	
Metastases	126 (33)	8 (34.8)	
Others	16 (4.5)	2 (8.7)	
Viral hepatitis serology[b]			0.858
HBV	51 (14)	3 (13)	
HCV	141 (38.7)	8 (34.8)	
HBV and HCV	7 (1.9)		
Liver parenchyma[b]			0.758
Chronic hepatitis	105 (29.6)	9 (39.1)	
Liver cirrhosis	93 (26.2)	5 (21.7)	
Child class[b]			0.634
A	355 (94.4)	21 (91.3)	
B	21 (5.6)	2 (8.7)	
ICG15R[a]	14.6 ± 0.4	15.5 ± 1.6	0.571

[a] Mann-Whitney *U* test

[b] χ^2 test

Data are numbers with percentages in parentheses or means ± standard error of the mean

ASA American society of anesthesiology, *HCC* hepatocellular carcinoma, *HBV* hepatitis B virus, *HCV* hepatitis C virus, *ICG15R* indocyanin green 15 min retention rate

11 (2.5%) (pneumonia (n = 6), urinary tract infection (n = 1), catheter infection (n = 1), epididymitis (n = 1), unknown origin (n = 2)). Other morbidities included bile leak (n = 9), retractable ascites (n = 6), ileus (n = 4), transient renal insufficiency (n = 4), rebleeding (n = 3), pleural effusion (n = 3), skin rash (n = 2), poor oral intake (n = 2), delirium (n = 1), transient heart failure (n = 1), pulmonary embolism (n = 1), upper gastrointestinal bleeding (n = 1), wound dehiscence (n = 1). There were four reoperations for three rebleedings and one wound dehiscence.

Discussion

Our study clearly demonstrated the risk factors of SSI after hepatectomy with prophylactic antibiotics under the CDC guideline. Intraoperative bowel injury, blood loss >2000 ml, and age older than 65 years were the significant risk factors. Although both alimentary tract surgery and hepatobiliary surgery are classified as clean-contaminated [14], biliary tract without calculus is normally sterile contrary to the alimentary tract, which has high bacterial densities [15, 16]. Intraoperative bowel injury is suspected to contaminate surgical field of hepatectomy without biliary reconstruction and to increase the risk of SSI. Blood loss reduces the concentration of antibiotics and is found to be a risk factor of SSI [17, 18]; 1500 ml to 2000 ml of blood loss is the suggested threshold to administer additional doses of cefazolin to maintain a concentration higher than the minimum inhibitory concentration for the common infecting organisms [19, 20]. Our threshold of 2000 ml of blood loss is compatible with previous findings. Elderly patients also are reported to be susceptible to SSI [18, 21]. Because aging involves complex physiologic changes, it is difficult to clarify a definitive mechanism of the vulnerability of elderly patients. Reduction in immune function is one suggested mechanism [10].

Rates of SSI increased dramatically with the number of the three risk factors present (Fig. 1). According to the National Nosocomical Infections Surveillance (NNIS) report, rates of SSI after hepatopancreaticobiliary complex

Table 2 Perioperative variables for patients with SSI compared with those without it

	SSI (−) (N = 382)	SSI (+) (N = 23)	*P* value
Operation time (min)[a]	210 ± 19	269 ± 23	*0.021*
≥300[b]	68 (17.8)	9 (39.1)	*0.017*
<300	313 (82.2)	14 (60.9)	
Pringle time (min)[a]	63.3 ± 2.1	75.9 ± 9.7	0.259
None[b]	26 (7.3)	0 (0)	0.23
>0	331 (92.7)	20 (100)	
Repeat resection[b]	110 (28.8)	4 (17.4)	0.338
Blood loss (ml)[a]	1070 ± 69	1928 ± 470	0.068
≥2000[b]	50 (13.2)	9 (39.1)	*0.003*
<2000	332 (86.8)	14 (60.9)	
RBC transfusion (ml)[a]	177 ± 29	537 ± 192	*0.003*
None[b]	297 (78.2)	12 (52.2)	*0.009*
>0	83 (21.8)	11 (47.8)	
Intraoperative bowel injury[b]	3 (0.8)	4 (17.4)	*<0.001*
Bile leak[b]	7 (1.8)	2 (22.2)	0.087
Resected segments (Couinaud)[b]			0.96
<2	285 (74.8)	16 (69.6)	
2–3	42 (11)	3 (13)	
≥4	54 (14.2)	4 (17.4)	
Resected weight (g)[a]	221 ± 19	269 ± 77	0.281
Largest tumor size (cm)[a]	3.8 ± 0.2	3.7 ± 0.4	0.253
NNIS index[b]			0.184
0	293 (76.9)	14 (60.9)	
1	86 (22.6)	9 (39.1)	
2	2 (0.5)		
Postoperative length of stay[a]	10.2 ± 0.2	23.7 ± 5.7	*<0.001*

[a] Mann-Whitney *U* test

[b] χ^2 test

Data are numbers with percentages in parentheses or means ± standard error of the mean

RBC red blood cell, *NNIS* national nosocomical infection surveillance

Table 3 Multivariate analysis of SSI risk factors

	P value	Odds ratio (95% confidence intervals)
Age ≥65 yr	*0.027*	3.4 (1.15–10.05)
Blood loss ≥2000 ml	*0.004*	4.4 (1.63–11.91)
Intraoperative bowel injury	*<0.001*	20.08 (4–100.8)
RBC transfusion	0.62	1.51 (0.31–7.42)
Operation time >300 min	0.67	1.35 (0.34–5.32)

SSI risk factors identified by univariate analysis were compared by multivariate analysis (logistic regression test)

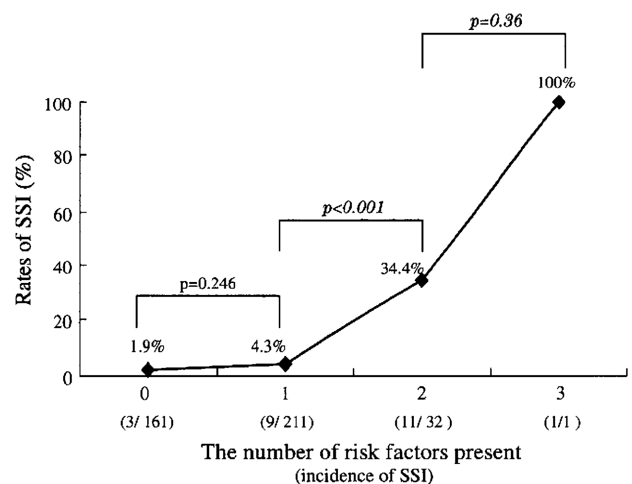

Fig. 1 Rates of SSI increased with the number of risk factors present. Rates of SSI were not statistically different between patients with one risk factor and those without any factors. However, patients with two or more risk factors developed SSI at a significantly higher rate than those with none or only one risk factor

surgery range from 3.24–7.04% [22]. Other reported rates of SSI after hepatectomy range from 4.6–25.2% [23, 24]. Compared with those previously reported rates, the rates of SSI for patients with none or only one risk factor, 1.9% and 4.3% respectively, are considered allowable. Prophylactic antibiotics for hepatectomy without biliary reconstruction are necessary only during operations for patients with none or only one risk factor. However, patients with two or more risk factors developed SSI at statistically higher rates. Fujita et al. [4] reported that two additional doses of postoperative antibiotics reduced the incidence of incisional SSI from 14.2% to 4.3% compared with single-dose preoperative administration in elective colorectal surgery

[4]. Additional administration of postoperative antibiotics maintains therapeutic levels for longer hours and reduces the incidence of SSI more effectively for patients at higher risk. Although there have been no published data concerning the effectiveness of postoperative administration of antibiotics in hepatectomy, Fig. 1 illustrates that patients with two or more risk factors may receive some additional doses of postoperative antibiotics as in colorectal surgery. Appropriate doses of additional antibiotics are matters to be discussed.

There were five infecting organisms that were resistant to cefazolin: *Bacteroides fragilis*, *Enterobacter, cloacae*, *Serratia marcescens*, Corynebacterium species, and Citrobacter species. Because some patients lack microbiologic data, a definitive conclusion about the optimum choice of prophylactic antibiotics was not possible. However, it is evident that cefazolin alone was effective for most patients who underwent hepatectomy without biliary reconstruction. Two of the seven patients with intraoperative bowel injury developed SSI with *Bacteroides fragilis*. Because likely pathogens in alimentary tract surgery are gram-negative bacilli and anaerobes [2], postoperative antibiotics with anaerobic coverage might be more effective for patients with intraoperative bowel injury.

Postoperative infections, especially organ/space SSI, sometimes deteriorate hepatic function and may cause mortalities. We experienced 23 SSIs and 11 remote site infections, but none of the patients died from those infections. We speculate that our strict evaluation of extent of hepatectomy using CT volumetry and liver function test precluded some excessive hepatic resection and saved postoperative hepatic function. Postoperative infection is more likely to occur in patients with hepatic dysfunction [25]. Our relatively low rate of major hepatectomy in consideration of hepatic functional reserve might be related to the fewer incidence of SSI.

RBC transfusion requirement and operation time were significant risk factors of SSI in univariate analysis, but not in multivariate analysis. Transfusion has immunosuppressive effects on postoperative patients via reductions in natural killer cell number and cytotoxic T-cell function [26, 27] and is reported to be a risk factor of SSI in colorectal surgery [28, 29]. However, controversy exists concerning the causal relationship between transfusion and SSI [30], and a recent meta-analysis denies the association between transfusion and postoperative infection [31]. Our result is consistent with the meta-analysis. Operation time is another reported risk factor of SSI [18]. Cefazolin exhibits time-dependent decrease in serum and tissue concentration, and additional administrations are recommended every 3 or 4 hours during operation to maintain therapeutic levels of cefazolin [2]. Because all of our patients received a second dose of cefazolin at 3 hours from incision, serum and tissue concentration of cefazolin was expected to exceed therapeutic levels during the whole time of operations for most patients. Influence of operation time on the incidence of SSI was suspected to be minimized with additional dose of cefazolin at 3 hours from incision.

Abdominal drainage after elective hepatectomy is controversial. Some randomized, controlled trials (RCTs) reported increased incidence of SSI and other morbidities associated with abdominal drainage and denied the routine placement of drainage catheters [32, 33]. However, the routine drainage group in those RCTs had drainage catheters placed for at least 5 to 9 days, which was unnecessarily long. We almost routinely placed drainage catheters but removed them on POD 3/4 or earlier if postoperative bleeding and bile leakage were denied. Early removal of prophylactic drains prevents intra-abdominal infections [34]. We do not consider that abdominal drainage causes more infections if drains are removed on POD 3/4 or earlier.

Our study has several limitations. First, SSI was detected indirectly by retrospectively reviewing patient records and laboratory data. It has been suggested to be a less accurate method than prospective direct observation of surgical sites [2]. Some SSI might be possibly undetected because of inappropriate patient records. However, indirect case-finding by reviewing daily records and laboratory data is the most widespread method of surveillance in the medical literature. Its reported sensitivity is as high as 83.8–92.3% compared with prospective direct finding of SSI [35]. Since then, we do not consider that our surveillance method precludes the importance of our findings. Second, it is a single-center study. Our department is one of the highest volume centers in Japan and performs 250 hepatopancreaticobiliary cancer surgeries in a year. Also, we do not perform operations on patients with end-stage renal disease on dialysis due to inadequacies of dialysis facilities. Our relatively low rate of SSI incidence may be attributable to the high volume of cases and to the patient selection.

Conclusions

Our study demonstrated that prophylactic antibiotics were necessary only during operations and, at most, a few hours after closure of incisions in most of the patients who underwent hepatectomy without biliary reconstruction. However, patients with intraoperative bowel injury, blood loss >2000 ml, and age older than 65 years were at risk for developing SSI. Patients with two or more risk factors may receive additional doses of postoperative antibiotics to prevent SSI more effectively.

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J Gastroenterol (2010) 45:60–67
DOI 10.1007/s00535-009-0109-8

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Transcatheter arterial infusion chemotherapy with cisplatin–lipiodol suspension in patients with hepatocellular carcinoma

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Received: 25 October 2008 / Accepted: 12 July 2009 / Published online: 5 August 2009
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Abstract
Purpose The aim of this study was to investigate the antitumor efficacy of treatment, identify prognostic factors, and construct a prognostic index in patients with hepatocellular carcinoma treated by transcatheter arterial infusion chemotherapy (TAI) using cisplatin suspended in lipiodol.
Methods We analyzed the outcomes in a total of 94 consecutive patients with previously untreated hepatocellular carcinoma who were treated by TAI using cisplatin suspended in lipiodol.
Results Twenty-seven patients (29%) showed complete response and 21 patients (22%) showed partial response, with an overall response rate of 51% (95% confidence interval, 41–61%). The median survival time was 2.5 years and the proportions of survivors at 1, 2, and 5 years were 81.6, 65.2, and 18.3%, respectively. The results of multivariate analysis indicated a significant association of serum albumin $\geq$3.0 g/dL, maximum tumor size $\leq$3.0 cm, absence of ascites, and unilateral distribution of the tumors with a favorable survival. For clinical application, we also propose a prognostic index based on a combination of these prognostic factors. Based on this index, the patients were classified into three groups: those with good, intermediate, and poor prognosis. The median survival times in these three groups were 4.3, 2.7, and 1.1 years, respectively ($p < 0.01$).
Conclusions TAI with cisplatin suspended in lipiodol exhibited favorable tumor efficacy and survival in patients with hepatocellular carcinoma. The prognostic factors identified and the index proposed based on these factors may be useful for predicting life expectancy, determining treatment strategies, and designing future clinical trials.

Keywords Hepatocellular carcinoma · Transcatheter arterial infusion chemotherapy · Cisplatin · Prognosis

Abbreviations

HCC	Hepatocellular carcinoma
TAE	Transcatheter arterial chemoembolization
TAI	Transcatheter arterial infusion chemotherapy
CT	Computed tomography
AFP	Serum alpha-fetoprotein
PIVKA II	Protein induced by vitamin K absence or antagonist-II
CR	Complete response
PR	Partial response

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world, and its incidence is continuing to increase worldwide. However, the prognosis of advanced HCC remains unsatisfactory [1]. Curative therapies such as resection, liver transplantation, and local ablative treatments may offer a chance of improved life

expectancy, but these treatment modalities are applicable to only a small proportion of all HCC patients. Transcatheter arterial chemoembolization (TAE) has been recognized as an effective palliative treatment option for patients with advanced HCC, because two meta-analyses [2, 3] of seven randomized controlled trials [4–10] showed that TAE significantly improves the survival of unresectable HCC patients with preserved hepatic function [1]. Transcatheter arterial infusion chemotherapy (TAI) is also often used for the treatment of advanced HCC, but a consensus regarding the most effective chemotherapeutic regimen has not yet been reached [11, 12]. Lipiodol, a lipid lymphographic agent, is selectively retained by HCC tissues for prolonged periods in comparison with non-cancerous tissues, and is therefore commonly mixed with anticancer agents to allow these agents to be retained for prolonged periods of time in the target tumor [13–15]. In a randomized controlled trial of TAE and TAI with zinostatin stimalamer and lipiodol, TAE did not yield superior survival as compared to TAI in patients with advanced unresectable HCC [16]. Our previous analysis also revealed that TAE did not significantly improve the survival of patients with HCC in comparison with TAI using cisplatin suspended in lipiodol, even though TAE is known to have higher antitumor efficacy than TAI [17]. Thus, TAI may have a higher efficacy on survival compared to TAE. If the appropriate indications for TAI can be expanded, additional embolization may not be necessary in some patients, considering that TAE has more deleterious effects on the liver functions than TAI [17, 18]. However, proper patient selection for TAI with lipiodol has not yet been fully investigated, although those for TAI without lipiodol [19–21] and for TAE [22–24] have been frequently analyzed. Analysis of prognostic factors would suggest appropriate patient selection for TAI. The present study was conducted to investigate the antitumor efficacy of the treatment, and to evaluate a number of variables that may affect survival in patients with HCC treated by TAI using cisplatin suspended in lipiodol; we have proposed a prognostic index in patients treated with TAI based on the results of our analyses.

Materials and methods

Patients

Between October 1987 and May 1996, 94 consecutive patients with previously untreated HCC were treated by transcatheter arterial infusion chemotherapy using cisplatin suspended in lipiodol at Kumamoto University Hospital, Japan. The study subjects were patients who were judged to be suitable candidates for TAI (Table 1). HCC was diagnosed on the basis of histological examination or distinctive findings on computed tomography (CT) and/or angiography, associated with elevated serum levels of serum alpha-fetoprotein (AFP) or protein induced by vitamin K absence or antagonist-II (PIVKA II). Pretreatment evaluation included a complete medical history and careful physical examination. The laboratory procedures included complete

Table 1 Patient characteristics

	No of patients (%)
Host-related variables	
Age (years)	
Median [range]	64 [41–81]
Gender	
Male	62 (66%)
Blood transfusion	
Present	28 (30%)
Alcohol abuse[a]	
Present	11 (12%)
Smoking habit[b]	
Present	31 (33%)
Hepatitis B surface antigen	
Positive	14 (15%)
Hepatitis C antibody	
Positive	76 (81%)
Ascites	
Present	14 (15%)
Child-Pugh class	
A	45 (48%)
B	48 (51%)
C	1 (1%)
Tumor-related variables	
Number of tumors	
Multiple	53 (56%)
Tumor distribution	
Unilateral	70 (74%)
Maximum tumor size (cm)	
Median [range]	2.9 [1.5–12.0]
Portal vein invasion	
Present	7 (7%)
Alpha-fetoprotein (ng/mL)	
Median [range]	36.9 [1.9–17,100]
PIVKA II (mAU/mL)	
Median [range]	30 [0–6,000]
Other variables	
Modified Japan Integrated Stage	
Median [range]	2 [0–5]

PIVKA II protein induced by vitamin K absence or antagonist-II

[a] Ethanol intake $\geq$80 g/day for $\geq$5 years

[b] $>$20 cigarettes/day for $>$10 years

differential blood count, biochemistry tests, viral markers, including serum hepatitis B surface antigen and serum hepatitis C antibody, and tumor markers, including the serum levels of AFP and PIVKA II. Before treatment, a chest X-ray and ultrasonography and CT of the abdomen were obtained to evaluate the extent and size of the tumors and to exclude the presence of extrahepatic metastasis. The number, size, and distribution of the tumors were examined by CT and/or angiography. Written informed consent was obtained from all the patients prior to the start of the treatment.

Treatment procedure

Following conventional visceral angiography, TAI was performed by selectively introducing a catheter into the proper, right or left hepatic artery, or a branch of the artery feeding the tumor and injecting cisplatin suspended in lipiodol (iodized oil; Guerbet, Paris, France). The dose of the drug was determined based on the tumor size and liver function. The cisplatin suspension in lipiodol was prepared by the following procedure [25]: cisplatin powder, produced by evaporating water and sodium chloride from cisplatin solution, was sterilized by heating and subsequently suspended in lipiodol with a mortar and pestle under sterile conditions. The content of cisplatin in the lipiodol was adjusted to 20 mg/mL.

After the treatment, follow-up examinations, including CT, tumor marker measurement, and serum biochemistry, were performed, first at one month after the treatment completion and subsequently every 3–4 months. The transcatheter arterial treatments were repeated when relapse of the treated lesions and/or new hepatic lesions were seen.

Evaluation of the antitumor efficacy

The antitumor effect was assessed by contrast-enhanced CT or magnetic resonance imaging at one month after the treatment. Lipiodol accumulation in the tumor was regarded as representing necrotic tissue, because earlier studies have shown that areas on the CT showing lipiodol retention correspond to necrotic areas in the tumors [13–15]. We defined complete response (CR) as disappearance or 100% necrosis of all tumors, and partial response (PR) as >50% reduction and/or necrosis in the sum of all measurable tumors. Progressive disease was defined as more than 25% enlargement in the sum of all lesions and/or the appearance of any new lesions. Stable disease was considered as any disease that did not qualify for classification as CR, PR or progressive disease.

Factors analyzed

The relationships of pretreatment clinical variables to survival were investigated by univariate and multivariate analyses. The pretreatment variables were chosen based on their possible effects on the prognosis and tumor response indicated by previous investigations [1–12, 16–30] or suggested by our own clinical experience. Each of the variables, which were classified as host-related or tumor-related, was divided into two subgroups in accordance with clinically meaningful values for easy application in clinical practice, as shown in Table 2.

Overall survival was measured from the date of initial treatment to the date of death or last follow-up. Survival curves were calculated by the Kaplan–Meier method, and differences in survival were evaluated by the log rank test. The Cox proportional hazard model was used to determine the most significant variables related to survival. Forward and backward stepwise regression procedures based on the partial likelihood ratio were used to determine the major independent predictors of survival. A prognostic index based on the regression coefficients derived from all variables identified by the multivariate analysis was constructed. Stratification of the patients was conducted on the basis of this prognostic index. All p values presented in this report are of the two-tailed type. Differences at $p < 0.05$ were considered to be significant.

Results

Patient characteristics

The characteristics of all the 94 patients are shown in Table 1. There were 62 males (66%) and 32 females (34%), with a median age of 64 (range 41–81) years. There were 45 patients (48%), 48 patients (51%) and 1 patient (1%) with Child-Pugh stage A, B, and C [29], respectively. Fifty-three patients (56%) had multiple tumors, and the median maximum tumor size was 2.9 (range 1.5–12.0) cm. The median modified Japan Integrated Stage [30] was 2 (range 0–5). The median number of courses of TAI was two (range 1–9) during the follow-up period, and the median follow-up duration was 2.5 years (range 0.2–8.4 years). The median dose of cisplatin at first TAI was 50 (range 20–150) mg per treatment.

Treatment efficacy and survival

Twenty-seven patients (29%) showed CR and 21 patients (22%) showed PR, with an overall response rate of 51% (95% confidence interval, 41–61%). The median survival time was 2.5 years, and the proportions of survivors at 1, 2, 3, and 5 years were 81.6, 65.2, 39.8, and 18.3%, respectively (Fig. 1). The cause of death was tumor progression in 47 patients, hepatic failure in 25 patients, rupture of esophageal varices in 4 patients, and other causes in 6

Table 2 Univariate analysis of prognostic factors in patients with hepatocellular carcinoma treated by transcatheter arterial infusion chemotherapy using cisplatin suspended in lipiodol

	n	Median survival (years)	2-year survival (%)	Hazard ratio	*p* value
Host-related variables					
Age (years)					
≥60	67	2.5	65		
<60	27	2.6	54	0.98 (0.60–1.59)	0.93
Gender					
Female	32	2.7	66		
Male	62	2.4	60	0.99 (0.61–1.56)	0.97
Blood transfusion					
Present	28	2.5	60		
Absent	66	2.7	63	0.77 (0.48–1.24)	0.28
Alcohol abuse[a]					
Present	11	2.0	55		
Absent	83	2.6	63	0.63 (0.33–1.20)	0.16
Smoking habit[b]					
Absent	63	2.5	59		
Present	31	3.4	69	0.79 (0.50–1.27)	0.33
HBs Ag					
Negative	80	2.5	64		
Positive	14	1.8	46	0.77 (0.40–1.49)	0.45
HCV Ab					
Negative	18	1.9	47		
Positive	76	2.5	65	0.93 (0.53–1.64)	0.81
Ascites					
Present	14	1.4	21		
Absent	80	2.8	69	0.29 (0.16–0.53)	**<0.01**
WBC ($\times 10^4$/mm^3)					
≤4.0	51	2.5	61		
>4.0	43	2.5	64	0.76 (0.49–1.19)	0.23
Hemoglobin (g/dL)					
<10	17	2.4	59		
≥10	77	2.6	63	0.69 (0.40–1.19)	0.18
Platelet ($\times 10^4$/mm^3)					
<7.5	36	2.5	67		
≥7.5	58	2.5	59	0.89 (0.57–1.37)	0.59
Total bilirubin (mg/dL)					
≥2.0	13	1.8	46		
<2.0	81	2.7	65	0.59 (0.32–1.09)	0.09
Albumin (g/dL)					
<3.0	33	1.6	35		
≥3.0	61	4.0	76	0.29 (0.18–0.47)	**<0.01**
AST (U/L)					
≥85	24	2.4	58		
<85	70	2.8	63	0.63 (0.38–1.04)	0.07
ALT (U/L)					
≥92	21	2.4	57		
<92	73	2.7	63	0.74 (0.44–1.24)	0.25
LDH (U/L)					
≥500	9	1.8	44		
<500	85	2.5	64	0.76 (0.36–1.58)	0.46
Prothrombin time (%)					
<70	41	2.4	58		
≥70	53	2.7	65	0.93 (0.60–1.45)	0.76
ICG R15 (%)					
≥30	46	2.2	52		
<30	43	3.4	71	0.68 (0.43–1.07)	0.09
Tumor-related variables					
Number of tumors					
Multiple	53	2.0	51		
Single	41	2.8	76	0.63 (0.41–0.98)	**<0.05**
Tumor distribution					
Bilateral	24	1.1	27		
Unilateral	70	2.8	73	0.39 (0.24–0.65)	**<0.01**
Maximum tumor size (cm)					
>3.0	40	1.6	42		
≤3.0	54	3.2	76	0.41 (0.26–0.66)	**<0.01**
Portal vein invasion					
Present	7	1.0	17		
Absent	87	2.6	65	0.36 (0.15–0.84)	**<0.05**
Alpha-fetoprotein (ng/mL)					
≥100	46	2.4	57		
<100	48	2.6	67	0.66 (0.42–1.02)	0.06
PIVKA II (mAU/mL)					
≥100	14	1.1	34		
<100	80	2.7	67	0.53 (0.29–0.97)	**<0.05**

p values lesser than 0.05 are given in bold

HBs Ag hepatitis B surface antigen, *HCV Ab* hepatitis C antibody, *WBC* white blood cell count, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *LDH* lactic dehydrogenase, *ICG* indocyanine green test, *PIVKA II* protein induced by vitamin K absence or antagonist-II

[a] Ethanol intake ≥80 g/day for ≥5 years

[b] >20 cigarettes/day for >10 years

patients. Neither severe toxicity including renal dysfunction or thrombocytopenia, nor complication or treatment related death were seen in the present study.

Univariate and multivariate analysis

The median survival times, two-year survival, hazard ratios and *p* values of the survival time for univariate analysis are shown in Table 2. Among the host-related factors, absence of ascites and a serum albumin level of >3.0 g/dL were

significantly associated with a longer survival time. Among the tumor-related factors, single nodule, unilateral distribution of tumors, maximum tumor size <3.0 cm, absence of portal vein invasion, and PIVKA II level <100 mAU/mL were significantly associated with a longer survival time. The results of multivariate analysis using the Cox proportional hazard model are shown in Table 3. In the multivariate analyses, only those variables identified as significant by the univariate analysis were entered. Serum albumin ≥3.0 g/dL, maximum tumor size <3.0 cm, absence of ascites, and unilateral distribution of the tumors were significantly associated with favorable survival.

Risk groups based on the regression model

For the clinical application of these findings, a prognostic index was calculated based on the regression coefficients derived from the four variables identified by multivariate analysis (Table 3), as follows: prognostic index = score for albumin (0 for ≥3.0, 1 for <3.0 g/dL) + score for ascites (0 for absence, 1 for presence) + score for maximum tumor size (0 for ≤3.0, 1 for >3.0 cm) + score for tumor distribution (0 for unilateral, 1 for bilateral). The index values ranged from 0 to 4. The patients were then classified into three groups according to the prognostic index, as follows: good prognosis group (Group A: prognostic index = 0, $n = 31$ patients) (equivalent to patients with none of the four prognostic factors); intermediate

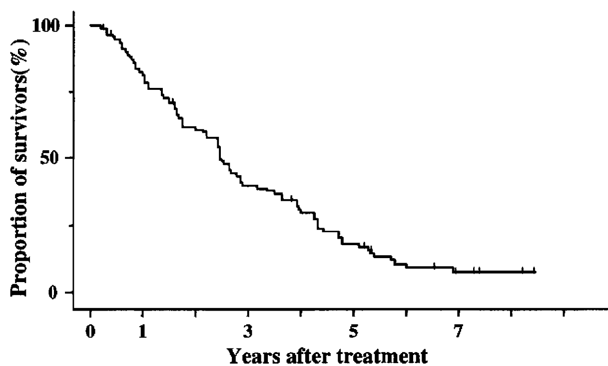

Fig. 1 Overall survival curve for all patients with hepatocellular carcinoma treated by transcatheter arterial infusion chemotherapy using cisplatin suspended in lipiodol. *Tick marks* indicate censored cases

prognosis group (Group B: prognostic index = 1, $n = 28$ patients) (equivalent to patients with one of the four prognostic factors); poor prognosis group (Group C: prognostic index ≥ 2, $n = 35$ patients) (equivalent to patients with two or more of the four prognostic factors). The survival curves for the three groups are shown in Fig. 2. The median survival times in the good, intermediate, and poor prognosis groups were 4.3, 2.7, and 1.1 years, respectively. There were significant differences in the survival time among the three groups ($p < 0.01$).

Discussion

TAE has been widely used for cases with unresectable HCC and is currently the mainstay of non-surgical treatment for HCC, because it has been shown to exert a marked antitumor effect against HCC and can be administered for any type of HCC, regardless of the size, location or number of tumors [1]. In addition, the survival benefit of this treatment modality has been verified by two meta-analyses [2, 3] of seven randomized controlled trials [4–10]. However, TAE has deleterious effects on liver functions, thereby impairing the baseline prognosis. On the other hand, TAI has milder hepatotoxicity, but also shows a lower antitumor efficacy against advanced HCC than TAE. However, in a randomized controlled trial of TAE versus TAI with zinostatin-stimalamer and lipiodol, TAI and TAE were reported to yield comparable survival [16]. Moreover, the result of our retrospective analysis of TAE versus TAI using cisplatin–lipiodol suspension indicated similar outcomes for the two modalities [17]. From the results of these two studies, we could not conclude that additional embolization is not necessary for the treatment of advanced HCC, but there may be a subset of patients of advanced HCC in which TAI alone may yield sufficient treatment efficacy and survival. Therefore, this analysis of prognostic factors was carried out to enable identification of appropriate candidates for TAI using cisplatin–lipiodol suspension among HCC patients with no prior treatment. This single-institution study was undertaken using a unified method for tumor staging and identical procedures for treatment, follow-up, and supportive care throughout the duration of the study, to enable us to obtain reliable results for confirming important

Table 3 Significant prognostic factors determined by multivariate analysis with the Cox proportional hazard model

Variable	Coefficient	Hazard ratio (95% confidence intervals)	p value
Albumin ≥3.0 g/dL	0.94	0.39 (0.23–0.66)	<0.001
Maximum tumor size ≤3.0 cm	1.01	0.37 (0.19–0.69)	0.001
Absence of ascites	0.81	0.45 (0.11–0.40)	0.002
Unilateral tumor distribution	0.77	0.46 (0.27–0.79)	0.004

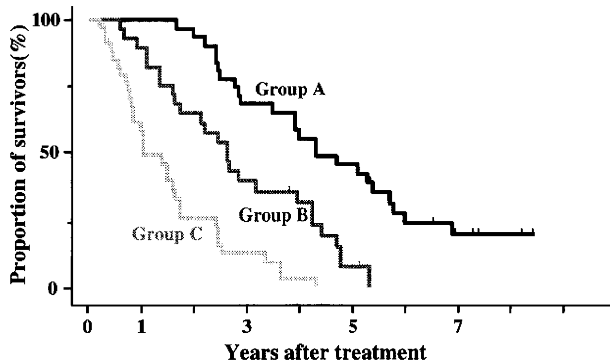

Fig. 2 Survival curves for the three groups determined by a prognostic index. *Group A* good prognosis (31 patients), *Group B* intermediate prognosis (28 patients), *Group C* poor prognosis (35 patients). *Tick marks* indicate censored cases

prognostic factors, predicting life expectancy and designing future clinical trials of TAI for HCC.

In this study, cisplatin was administered as the anti-cancer agent for TAI. Cisplatin has been reported to exert its actions by binding to the DNA in cancer cells, inhibiting DNA synthesis and subsequent cellular division. It is one of the key drugs for advanced HCC, that constituted a component of the combined chemotherapeutic regimen used in three of the seven randomized controlled trials of TAE reported until date [6, 7, 9]. In Japan, a favorable tumor response (33.8%) was reported in a clinical study of intra-arterial administration of cisplatin for advanced HCC [21], and the treatment has been approved for the treatment of HCC by the Ministry of Health, Labour and Welfare of Japan. Lipiodol has been used as a carrier for anticancer agents in targeting chemotherapy [13–15], and a suspension of cisplatin powder in lipiodol was used in this study. It has been reported that stronger antitumor effect is obtained by hepatic arterial administration of a combination of lipiodol and an anticancer agent than by that of an anticancer agent alone [26]. Recently, a lipophilic cisplatin derivative that can be suspended in lipiodol, SM-11355, was reported to show promising tumor efficacy (CR rate: 56%) in a phase II trial, and further trial is ongoing [27]. Therefore, combined therapy with cisplatin and lipiodol has been expected to become established as a valid option for the treatment of HCC. The response rate (51%: 95% confidence interval, 41–61%) at one month obtained in this study was more favorable than that in a clinical study of cisplatin alone, because TAI with an emulsion of an anti-cancer agent and lipiodol could be expected to exert more potent effects than an anticancer agent alone. However, follow-up at one month might be insufficient for evaluation of the rate/pattern of recurrence of HCC.

The median survival time and survival rates at two years in the current study were 2.5 years and 65.2%, respectively. These results were comparable or superior to those of TAE reported from the aforementioned seven randomized controlled trials [4–10]. Although the study was based on a retrospective cohort design, the treatment efficacy of TAI with cisplatin–lipiodol suspension was promising and comparable to that of TAE for HCC.

In regard to the host-related factors, absence of ascites and a serum albumin level >3.0 g/dL were found to be favorable prognostic factors by multivariate analysis. Ascites and albumin are the most important factors to consider when evaluating the hepatic reserve, being included in both the Okuda staging system [28] and Child-Pugh classification [29], and have been shown to be prognostic factor in previous studies of patients with advanced HCC [19, 20, 22–24]. In regard to the tumor-related factors, a maximum tumor size ≤3.0 cm and unilateral distribution of the tumors were identified as being significantly associated with a longer survival time by multivariate analysis. Increased tumor size and bilateral distribution of tumors are the well-known unfavorable prognostic factors in HCC patients, and have been shown to be correlated with increased tumor volume and poorer differentiation of HCC, which reflect a more advanced stage and higher malignant potential of the tumors [22]. However, these prognostic factors for TAI with lipiodol in this study were similar to those identified for TAI without lipiodol [19–21] or TAE in previous reports [22–24], and no specific prognostic factors for TAI could be identified in this study.

For clinical application of these findings, we propose a prognostic index based on the independent prognostic factors identified in this study. Patients could be classified into three groups: those with good, intermediate, and poor prognosis ($p < 0.0001$) (Fig. 2). This index consists of both hepatic reserve and tumor stage, like the modified JIS score [30], and it differs from the Child-Pugh stage or TNM stage which are, respectively, based on either only the hepatic reserve or tumor stage. An index based on both the hepatic reserve and tumor stage might enable a more accurate prediction of life expectancy and stratification of the group into more distinct prognoses. This index can be easily calculated, because it is based on variables obtained during routine examinations before TAI. It can, therefore, be used to stratify patients with HCC before TAI according to the predicted survival. Accordingly, patients with good prognosis may obtain sufficient treatment efficacy and survival with TAI alone. In contrast, patients with a poor prognosis may be treated with supportive care only because of the extremely short median survival (1.1 years) expected, or may be treated other more aggressive treatments, such as more intensive chemotherapy. Recently, systemic chemotherapy for advanced HCC has become an important treatment modality, because sorafenib has been proven to confer a survival benefit and to show promise as a standard

treatment for patients with advanced HCC [31]. To improve the treatment efficacy, further chemotherapy regimens, such as the combination therapy comprising TAI with cisplatin suspended in lipiodol and sorafenib or other molecularly targeted agents, remain as challenges to be met following further detailed investigations. These findings may be helpful in predicting the life expectancy in HCC patients treated with TAI and provide more information to stratify patients in future TAI trials. It is also important to validate this prognostic index by applying it to other populations of HCC patients.

In conclusion, TAI with cisplatin suspended in lipiodol exhibited favorable tumor efficacy and survival in patients with HCC. Although no specific prognostic factors for TAI could be identified in this study, the results of the prognostic factors and the prognostic index may be helpful for predicting life expectancy, determining the most appropriate treatment strategies, and designing future clinical trials.

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Characterization of Dendritic Cells and Macrophages Generated by Directed Differentiation from Mouse Induced Pluripotent Stem Cells

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Key Words. Induced pluripotent stem cells • Dendritic cells • Macrophages • MHC • Embryonic stem cells • Cell therapy

ABSTRACT

Methods have been established to generate dendritic cells (DCs) from mouse and human embryonic stem (ES) cells. We designated them as ES-DCs and mouse models have demonstrated the induction of anti-cancer immunity and prevention of autoimmune disease by in vivo administration of genetically engineered ES-DCs. For the future clinical application of ES-DCs, the histoincompatibility between patients to be treated and available human ES cells and the ethical concerns associated with human ES cells may be serious obstacles. However, recently developed induced pluripotent stem (iPS) cell technology is expected to resolve these issues. This report describes the generation and characterization of DCs derived from mouse iPS cells. The iPS cell-derived DCs (iPS-DCs) possessed the characteristics of DCs including the capacity of T-cell-stimulation, antigen-processing and presentation and cytokine production. DNA microarray analyses revealed the upregulation of genes related to antigen-presenting functions during differentiation into iPS-DCs and similarity in gene expression profile in iPS-DCs and bone marrow cell-derived DCs. Genetically modified iPS-DCs expressing antigenic protein primed T-cells specific to the antigen in vivo and elicited efficient antigen-specific antitumor immunity. In addition, macrophages were generated from iPS cells (iPS-MP). iPS-MP were comparable with bone marrow cell-derived macrophages in the cell surface phenotype, functions, and gene expression profiles. STEM CELLS *2009;27:1021–1031*

Disclosure of potential conflicts of interest is found at the end of this article.

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INTRODUCTION

Dendritic cells (DCs) are the most potent antigen-presenting cells (APC) which are known to play major roles in the priming of naive T-cells and also in the maintenance of immunological self-tolerance, by promoting T-cells with regulatory functions or by inducing anergy of T-cells. Several groups have previously established methods to generate APC or DCs from mouse [1, 2] and human [3–5] embryonic stem (ES) cells (ES-DCs). Genetic engineering of ES-DCs can readily be done by the introduction of transgenes into undifferentiated ES cells and subsequent differentiation of the ES cell clones into ES-DCs. By genetic engineering, we can generate ES-DCs capable of modulating immune response in an antigen-specific manner. Mouse systems have demonstrated the induction of anti-cancer immunity [6–10] and the prevention of autoimmune disease [11, 12] by in vivo administration of genetically engineered ES-DCs.

Considering the future clinical application of ES-DCs technology, however, the unavailability of human ES cells genetically identical to the patients to be treated is a problem. In addition, ethical concerns related to the use of human ES cells are anticipated to be serious obstacles which will hinder the realization of the medical use of human ES-DCs.

It was recently revealed that ES cell-like pluripotent stem cells, designated as induced pluripotent stem (iPS) cells, can be generated by the simultaneous introduction of several genes for reprogramming factors, such as Oct3/4, Sox2, Klf4, and c-Myc, into somatic cells [13–20]. The issue of histoincompatibility between patients to be treated and ES cells may be overcome by the generation of iPS cells from somatic cells of the patients such as fibroblasts. The major ethical issues related to human ES cells would be avoided by aid of iPS cell technology, because the use of human embryos is not necessary for the generation of iPS cells.

Author contributions: S.S.: conception and design, collection of data, manuscript writing, final approval of manuscript; M.H., Y.M., S.F., and T.I.: collection of data, final approval of manuscript; K.T., K.O., and S.Y.: provision of study material, final approval of manuscript; Y.N.: manuscript writing, final approval of manuscript.

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Differentiation of iPS cells into various cells belonging to the three germ layers has been demonstrated by the analysis of teratomas generated from mouse and human iPS cells. In addition, the pluripotency of iPS cells is obvious by the contribution of iPS cell-derived cells to various organs of the chimeric mice developed from iPS cell-introduced blastocysts [14]. As for the in vitro generation of cells of mesodermal lineage from iPS cells, differentiation into cardiac myocytes and endothelial cells from mouse iPS cells has been recently reported [21–23]. However, it remains to be elucidated whether fully differentiated and functional hematopoietic cells can be generated from iPS cells by directed differentiation in vitro. In the present study, we generated DCs and macrophages from mouse iPS cells (iPS-DCs and iPS-MP), and characterized them by morphological, functional, and gene-expression analyses.

Materials and Methods

Cell Lines, Cytokines, Chemicals, and Peptides

The mouse embryonic fibroblast-derived iPS cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 15% ES cell-qualified fetal calf serum (FCS; Gibco-Invitrogen, Carlsbad, CA; http://www.invitrogen.com), 1,000 U/ml leukemia inhibitory factor, 50 U/ml penicillin, 50 mg/ml streptomycin, nonessential amino acids, and 50 μM 2-mercaptoethanol (2-ME) on feeder cell layers of mitomycin C-treated mouse primary embryonic fibroblasts (PEF). Mouse bone marrow stromal cells, OP9 [24], were maintained in DMEM supplemented with 20% FCS and seeded onto gelatin-coated dishes before used as feeder cells. The T-cell hybridomas, RF33.70 [25], recognizing ovalbumin $(OVA)_{257-264}$ in the context of K^b and DO11.10[26], recognizing $OVA_{323-339}$ in the context of $I\text{-}A^d$, were maintained in RPMI-1640 medium supplemented with 10% FCS. MO4 [27], a C57BL/6-derived B16 melanoma cell line expressing OVA, was maintained in RPMI-1640 medium supplemented with 10% horse serum. Recombinant mouse interleukin (IL)-4, tumor necrosis factor (TNF)-α, granulocyte macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF) were purchased from Peprotec (London, U.K.; http://www.peprotechec.com). Lipopolysaccharide (LPS) from *Escherichia coli* and OVA protein was purchased from Sigma Chemical (St. Louis, MO; http://www.sigma-chem.com.au), agonistic anti-CD40 monoclonal antibody (mAb, clone HM-40-3) was from PharMingen (San Jose, CA; http://www.bdbiosciences.com) and OK432 was purchased from Chugai Pharmaceutical (Tokyo, Japan; http://www.chugai-pharm.co.jp/hc/chugai_top_en.jsp). $OVA_{257-264}$ peptide, SIINFEKL, was synthesized on an automatic peptide synthesizer (PSSM8; Shimadzu, Kyoto, Japan; http://www.shimadzu.com) and purified by HPLC.

Differentiation Culture

The procedure for induction of differentiation of iPS cells into DCs is composed of three steps (supporting information Fig. 1). Step 1: iPS cells were suspended in α-MEM supplemented with 20% FCS and seeded (1×10^5 cells per dish) onto OP9 cell layers in 100-mm dishes. On day 6 or 7, the cells were treated with PBS/0.25% trypsin/1 mM ethylenediaminetetraacetic acid (EDTA; trypsin/EDTA) for 10 minutes, recovered with medium containing FCS, and subjected to step 2 culture or stocked frozen for future use. Step 2: Cells harvested from step 1 culture were suspended in α-MEM supplemented with 20% FCS, GM-CSF (1,000 U/ml), and 2-ME (50 μM) and plated onto freshly prepared OP9 cell layers. Cells recovered from one dish of step 1 culture were seeded onto 8–10 dishes. Thereafter, at 6 days after the passage, floating cells were recovered by pipetting and then were subjected to step 3 culture or stocked frozen. Step 3: The cells were transferred to bacteriological Petri dishes (5×10^5 cells/100-mm dish) without feeder cells and cultured in RPMI-1640 medium supplemented with 10% FCS, GM-CSF (1,000 U/ml) and 2-ME. To induce complete maturation of iPS-DCs, cells cultured for 10–14 days in Petri dishes were transferred to new dishes and cultured in RPMI-1640/10% FCS supplemented with GM-CSF (1,000 U/ml), IL-4 (10 ng/ml), TNF-α (5 ng/ml), and anti-CD40 mAb (10 μg/ml). For the generation of macrophages, the cells recovered from step 2 were cultured in bacteriological Petri dishes or tissue culture-coated dishes ($1–2 \times 10^6$ cells/100-mm dish) in RPMI-1640 medium supplemented with 10% FCS, 5% horse serum, M-CSF (100 ng/ml), and 2-ME.

Generation of DCs and Macrophages From Bone Marrow Cells

Femoral and tibial bone marrow cells were obtained from DBA/2 mice. To generate DCs (BM-DCs), cells were cultured in RPMI-1640 medium supplemented with 10% FCS, 1,000 U/ml GM-CSF, and 50 μM 2-ME for 7 days in Petri dishes. To generate macrophages (BM-MP), cells were cultured in RPMI-1640 medium supplemented with 10% FCS, 5% horse serum, 100 ng/ml M-CSF, and 50 μM 2-ME for 7–10 days in Petri dishes. For the analysis, macrophages were harvested using trypsin/EDTA.

Microscopic Analysis

Unfixed cells in the culture plates were stained with phycoerythrin (PE)-conjugated anti-Flk1 monoclonal antibody (1.25 μg/ml) in DMEM supplemented with 10% FCS for 1 hour and washed three times. Cytospin specimens were stained with May-Grünwald-Giemsa and mounted in Entellan *neu* (Merck, Darmstadt, Germany; http://www.merck.com). Bright-field, phase-contrast, and fluorescence microscopic analysis were done on an inverted microscope (IX70, Olympus, Tokyo, Japan, http://www.olympus-global.com/en/global) and microscopic images were captured by using a digital camera unit DP70 (Olympus).

Flow Cytometric Analysis

The staining of cells and analysis on a flow cytometer (FACScan, Becton Dickinson, San Jose, CA; http://www.bd.com) was done as described previously [2]. The following monoclonal antibodies (mAb) conjugated with fluorescence isothiocyanate (FITC) or PE were used for staining: anti-mouse Flk-1 (clone Avas12a1, rat IgG2a, eBioscience, San Diego, CA; http://www.ebioscience.com), anti-mouse CD45 (clone 30-F11, rat IgG2b, eBioscience), anti-mouse CD11b (clone M1/70, IgG2b, Pharmingen), anti-mouse CD11c (clone N148, hamster IgG, Chemicon, Temecula, CA, http://www.chemicon.com), anti-mouse CD80 (clone RMMP-1, rat IgG2a, Caltag), anti-mouse CD86 (clone RMMP-2, rat IgG2a, Caltag), anti-F4/80 (A3-1, rat IgG2b, Serotec Ltd., Oxford, U.K., http://www.serotec.com), mouse IgG2a control (clone G155-178, Pharmingen), mouse IgG2a control (clone G155-178, Pharmingen), rat IgG2a control (clone LO-DNP-16, Caltag), rat IgG2a control (clone LODNP-57, Beckman-Coulter, Fullerton, CA, http://www.beckmancoulter.com), and hamster IgG control (clone 530-6, Caltag). Intracellular staining with FITC-conjugated Fab fragment of anti-influenza virus hemagglutinin (HA) antibody (clone 3F10, rat IgG_1, Roche Diagnostics, Basel, Switzerland, http://www.roche-applied-science.com) was done using IntraPrep (Immunotech, Marseillu, France, http://www.beckmancoulter.com/products/pr_immunology.asp). Two-color staining with PE-conjugated tetramer of $H\text{-}2K^b\text{-}OVA_{257-264}$-complex (MBL, Nagoya, Japan; http://www.mbl.co.jp/e/index.html) in combination with FITC-conjugated anti-CD8 (clone KT15, Beckman-Coulter) was done according to the manufacturer's instructions.

Mixed Lymphocyte Reaction

Splenic T-cells were isolated from female C57BL/6 mice by using a pan-T-cell isolation kit (Miltenyi Biotec, Belgish-Bladbach, Germany, http://www.miltenyibiotec.com) and then they were used as responders. Graded numbers of stimulator cells were X-ray irradiated (35 Gy) and cocultured with responders (1.5×10^5) in wells of 96-well round-bottomed culture plates for

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4 days. [^{3}H]-methyl-thymidine (247.9 Gbq/mmol) was added (0.037 Mbq per well) during the last 16 hours of the culture. At the end of the culture, the cells were harvested onto glass fiber filters (Wallac, Turku, Finland; http://www.perkinelmer.com) and the incorporation of [^{3}H]-thymidine was measured by scintillation counting.

Antigen Presentation Assay

iPS-DCs were seeded into 96-well flat-bottomed culture plates (1×10^4 cells per well) with indicated concentrations of OVA protein, IL-4 (10 ng/ml), and anti-CD40 mAb (10 μg/ml) and cultured overnight. Subsequently, DO11.10 hybridoma cells were added to the wells (5×10^4 cells per well) and the culture was continued for further 24 hours. At the end of the culture, the concentration of IL-2 in the culture supernatant was measured by ELISA (eBioscience). For the assay with OVA-transfectant iPS-DCs, the indicated numbers of iPS-DCs were cocultured with hybridoma cells, DO11.10 or RF33.70, in the absence of exogenously added antigen for 24 hours and production of IL-2 was also measured by ELISA.

Analysis of the Activation of NKT Cells by iPS-DCs Loaded with α-Galactosylceramide

Splenic T-cells of (C57BL/6 × BALB/c) F1 (CBF1) mice were isolated using nylon-wool columns. Mature iPS-DCs were cultured in the presence of α-galactosylceramide (α-GalCer; 100 ng/ml) or vehicle (0.00025% Polysorbate-20) alone for 18 hours, washed, and cocultured with splenic T-cells (1.6×10^5 DCs + 4×10^6 T-cells per well in 24-well culture plates). After 24 hours, the cells were recovered and analyzed on their cytotoxic activity by a 4-hour ^{51}Cr-release assay using YAC-1 cells (5×10^3 cells per well) as targets in 96-well round-bottomed culture plates. In the analysis of the stimulation of NKT cells in vivo, iPS-DCs loaded with either α-GalCer or vehicle alone were intraperitoneally injected into H-2-matched CBF1 mice (1.2×10^6 cells per mouse). After 24 hours, the mice were sacrificed and the cytotoxic activity of whole spleen cells pooled from three mice for each group were analyzed using YAC-1 cells as targets, as described earlier.

Quantitation of Cytokine Production by iPS-DCs

iPS-DCs were cultured in 48-well culture plates (1.5×10^5 cells/200 μl per well) in the presence or absence of IL-4 (10 ng/ml), anti-CD40 mAb (10 μg/ml), TNF-α (10 ng/ml), LPS (1 μg/ml), and OPK432 (5 or 25 μg/ml). After 3 days of culture, culture supernatant was collected and concentration of IL-12p70 and TNF-α was determined by using ELISA kits (eBioscience).

cDNA Microarray Analysis

iPS cell-derived cells in culture step 2, iPS-DCs, iPS-MP, BM-DCs, and BM-MP were recovered from the culture and subjected to cDNA microarray analysis, without purification of specific cell fractions. Total cellular RNA was extracted by using an RNeasy kit (Qiagen, Hilden, Germany, http://www1.qiagen.com). Integrity of RNA samples was verified by using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, http://www.agilent.com) and the purity and concentration was checked based on A260/280 (A260/280: 1.98∼2.04). For each cell sample, cDNA was synthesized by using MMLV-RT using 300 ng of RNA as template and the cDNA was used as template for synthesis of cyanine-3 (Cy-3)-labeled cRNA by using T7 RNA polymerase. A total of 1.65 μg each of Cy-3-labeled cRNA was hybridized to a microarray slide (Whole Mouse Genome Oligo Micoarray 4 × 44K; Agilent Technologies; http://www.home.agilent.com) for 17 hours and washed, according to the manufacturer's instructions (Protocol: One-Color Microarray-based Gene Expression Analysis, version 5.7). The slides were scanned on Agilent DNA Microarray Scanner and data were processed and normalized using the GeneSpring GX9.0 software program.

Plasmid Construction

A cDNA fragment coding for a truncated form of ovalbumin, $OVA_{241-340}$, was prepared by PCR amplification using full length cDNA for OVA as a template with PCR primers 5′-*cctcgag*cccgccgccaccatgg*ctagc*atgttggtgctgttgcctgat-3′ (Xho I and Nhe I sites are indicated by underline) and 5′-cttaagcgtagtctgggacgtcgtatgggtactctctgcctgcttcattgatttc-3′. The design of these primers results in the cloning of OVA downstream of the Kozak sequence and the addition of the HA epitope (MYPYDVPDYA) to the carboxyl terminus of OVA fragment. The cDNA fragment for $OVA_{241-340}$-HA was cloned into pCAG-INeo, a mammalian expression vector driven by a CAG promoter and containing the internal ribosomal entry site (IRES)-neomycin resistance gene cassette to generate pCAG-OVA-INeo. A cDNA fragment for amino terminal portion of invariant chain (Ii_{1-80}) was prepared by PCR amplification using full-length cDNA for human Ii as a template with PCR primers, 5′-ac*ctcgag*cccgccgccaccatggatgaccagcgcgaccttatctc-3′ and 5′-aa*gctagc*aagcttcatgcgcaggttctccag-3′, and inserted into the Xho I-Nhe I site of pCAG-OVA-INeo to generate the Ii-$OVA_{241-340}$ expression vector, pCAG-Ii-OVA-INeo.

Transfection of iPS Cells by Electroporation

iPS cells maintained on layers of PEF were harvested by using trypsin/EDTA and suspended in DMEM (3×10^7 cells per milliliter) and 1.2×10^7 cells were electroporated in a 4-mm-gap cuvette under 225 V and 600 μF with 30 μg of plasmid DNA. After electroporation, cells were cultured on neomycin-resistant PEF feeder layers in 100-mm culture dishes in the presence of G418 (500 μg/ml) for 9–10 days. Subsequently, drug-resistant colonies were picked up and transferred into 24-well culture plates. iPS cell transfectant clones with high levels of expression of the transgene were selected based on the resistance to high-dose (3 mg/ml) of G418. Thereafter, the expression of the transgene after differentiation of the transfectants was examined by intracellular staining with anti-HA mAb and flow cytometric analysis.

Analysis of In Vivo Priming of Antigen-Specific T-Cells by iPS-DCs

Transfectant or nontransfectant iPS-DCs were stimulated with IL-4, TNF-α, and anti-CD40 mAb and injected intraperitoneally into C57BL/6 mice (1.5×10^5 cells per mouse). Eight days after the injection, spleen cells were isolated from the injected mice and pooled for each group of three mice. After hemolysis, spleen cells were cultured in 24-well culture plates (3×10^6 cells/2 ml per well) in RPMI-1640 supplemented with horse serum (10%), recombinant human IL-2 (100 U/ml), and $OVA_{257-264}$ peptide (0.001 μM). After 5 days, the cells were harvested and OVA-specific cytotoxic T lymphocyte (CTL) activity was analyzed by 5 hour-^{51}Cr-releasae assay using OVA-peptide-pulsed or unpulsed EL-4 cells as targets. The percentage of specific lysis was calculated as: 100 × (experimental release − spontaneous release)/(maximal release − spontaneous release). Spontaneous release and maximal release were determined in the presence of medium or 1% Triton X-100, respectively. The experiments using mice were done according to the plan approved by animal research committee of Kumamoto University. The frequency $CD8^+$ T-cells specific to $OVA_{257-264}$ was analyzed using the PE-labeled tetramer of K^b-$OVA_{257-264}$-complex as described earlier.

Tumor Challenge Experiments

OVA-transfectant or nontransfectant iPS-DCs were injected intraperitoneally into C57BL/6 mice (1.0×10^5 cells per mouse) on day-10, and MO4 cells (2 × or 3×10^5 cells per mouse) were inoculated subcutaneously into the shaved right flank region on day 0. The tumor size was measured on days 11, 15, and 18 and the tumor volume was calculated as follows: tumor volume (mm^3) = (length × width × height).

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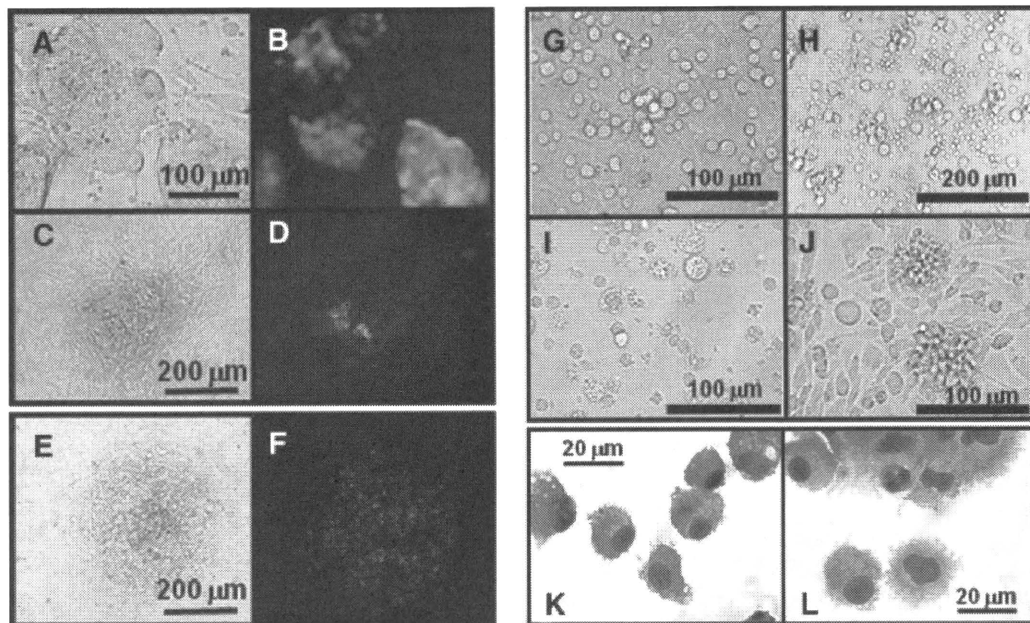

Figure 1. Morphological changes from iPS cells to iPS-DC. Phase-contrast images (**A, C**) and fluorescence images showing expression of green fluorescence protein (**B, D**) of undifferentiated Nanog-iPS cell colonies on primary embryonic fibroblasts feeder layer (**A, B**) and a differentiating colony on OP9 feeder layer at day 3 of the step 1 culture (**C, D**). A phase-contrast image (**E**) and a fluorescence image showing expression of Flk-1 (**F**) of a colony at day 6 of the step 1 culture. Phase-contrast images of iPS cell-derived hematopoietic cells at day 3 (**G**) and day 6 (**H**) in step 2 and day 15 (**I, J**) in step 3 are shown. (**K, L**): Cytospin specimens of iPS-DC recovered from step 3 culture were stained with May-Grünwald-Giemsa. Cells in (**I–L**) had been stimulated for 2–4 days with IL-4, TNF-α, and anti-CD40 mAb.

Chemotaxis Assay

A multiwell chemotaxis assay was done by using 24-well Transwell permeable support chamber (pore size 5 μm; Corning, NY; http://www.corning.com/index.aspx). Cell suspensions (1×10^6 cells per ml) in serum-free culture medium (AIMV, Gibco-Invitrogen) were added to the upper compartments (0.1 ml per well) and the indicated concentration of C5a in AIMV medium was added to the lower compartments (0.6 ml per well). Assay plates were incubated at 37°C for 90 minutes and then the cells on the upper surface of the microporous membranes were removed by using swabs. Subsequently, the membranes were fixed with methanol for 5 minutes and stained with May-Giemsa solution (Muto Chemicals, Tokyo, Japan). The stained cells on the lower surface of the membranes were counted and the data were indicated as the number of cells per 1 mm^2.

Phagocytosis Assay

The cell suspensions in RPMI-1640/10% FCS/2-ME were added to 48-well culture plates (2×10^5 cells/200 μL per well) and incubated at 37°C for 2 hours to allow the cells to adhere to the plates. FITC-labeled Zymosan A particles (Molecular Probes Inc., Eugene, OR, http://probes.invitrogen.com) were added to the wells (4.8×10^6 particles/200 μL per well) and, after incubation for the indicated period, cells were rinsed with PBS and harvested by using trypsin/EDTA. The cells were treated with trypan blue to quench FITC of the cell-surface attached particles, washed and then analyzed on FACScan flow cytometer.

Measurement of Nitric Oxide Production

The cells suspended in phenol-red-free DMEM/5% FCS were seeded into 96-well plates (1×10^5 cells/0.2 ml per well) in the presence or absence of IFN-γ (200 U/ml) and LPS (100 ng/ml). After 24 hours of incubation, concentration of $NO_2 + NO_3$ in the culture supernatant was determined based on Griess method by using a nitric oxide assay kit (Dojindo, Kumamoto, Japan; http://www.dojindo.com).

Results

Generation of DCs From iPS Cells

The present study mainly examined iPS-MEF-Ng-38C-2 (38C-2), one of the previously established mouse iPS cell clones [14], for the capacity to differentiate into functional DCs. 38C-2 was developed by introduction of the four genes (Oct3/4, Sox2, Klf4, and c-Myc) for reprogramming factors into embryonic fibroblasts and subsequent selection based on the expression of the Nanog gene. The procedure to induce the differentiation of iPS cells into DCs, composed of three steps as shown in supporting information Figure 1, was basically the same as that developed previously using mouse ES cells [2].

Undifferentiated iPS cells were maintained on the feeder layers of PEF. They were similar to ES cells in morphology (Fig. 1A) and growth properties. Nanog-selected iPS cells carry a transgene of the Nanog genome inserted with a cDNA coding for green fluorescence protein (GFP) in the 5′-untranslated region. The expression of GFP was observed in approximately half of the undifferentiated Nanog-iPS cells (Fig. 1B), as reported previously [14]. To initiate the differentiation, iPS cells were transferred onto OP9 feeder layers (step 1). After 3 days, mesodermally differentiated flat colonies appeared. The expression of GFP was scarcely observed in the differentiated colonies (Fig. 1C, 1D), thus indicating that the promoter of Nanog gene was turned off along with the mesodermal differentiation of iPS cells. On day 6, most of the colonies exhibited a differentiated morphology. They were completely negative for the expression of GFP and positive for cell surface expression of Flk-1/VEGFR2 (Fig. 1E, 1F).

On day 6 or 7 of step 1, cells were harvested by using trypsin/EDTA and dissociated into single cells. Subsequently,

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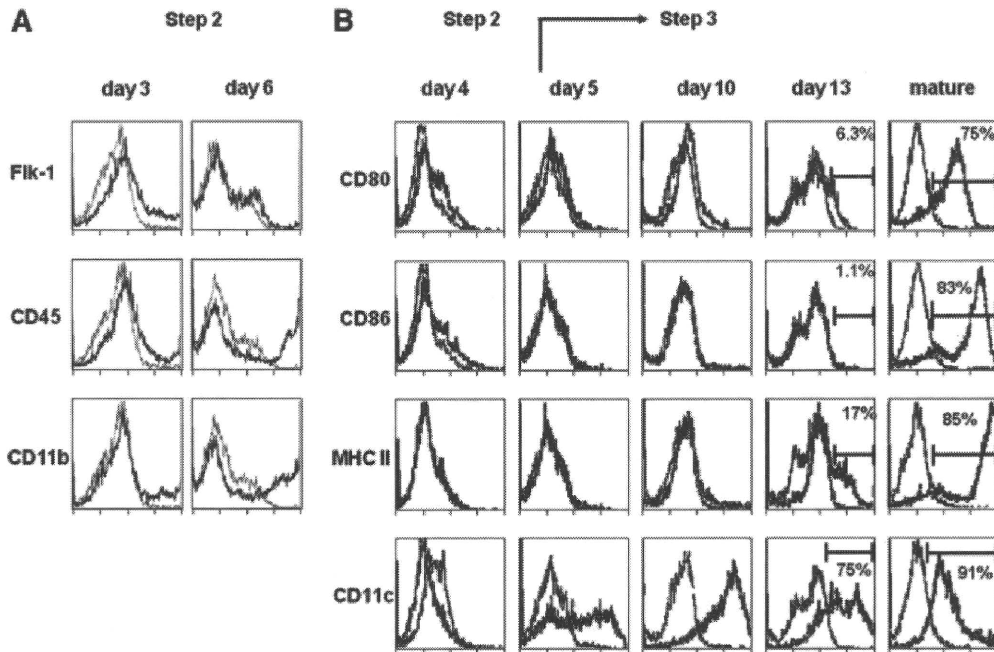

Figure 2. Surface phenotypes of iPS-derived cells in differentiation culture. (A): The cells at days 3 and 6 in step 2 culture were examined for the expression of Flk-1, CD45, and CD11b. (B): The cells in step 2 and step 3 culture were examined for the expression of CD80, CD86, MHC class II, and CD11c. The staining patterns of specific antibodies (thick lines) and isotype-matched controls (thin lines) are shown. The numbers in the panels of day13 and mature DC indicate percentages of cells positive for CD80, CD86, or MHC class II.

the cells were transferred onto freshly prepared OP9 feeder layers and cultured in the presence of GM-CSF, to start step 2. On the next day, homogenous small cells, resembling primitive hematopoietic progenitor cells, appeared (Fig. 1G). The iPS cell-derived round cells, expressing Flk-1 and CD45 (Fig. 2A), gradually increased and became morphologically heterologous (Fig. 1H). The addition of exogenous GM-CSF was essential for the propagation of the cells, thus indicating that the cells proliferated in response to GM-CSF. At day 6 in culture step 2, more than half of the floating cells highly expressed CD11b (Fig. 2A), thus suggesting their commitment to myeloid cell lineage. Step 2 culture was continued for 6–7 days.

At the end of step 2, floating or loosely adherent cells were recovered by pipetting and transferred them into bacteriological Petri dishes without feeder cells (step 3). After 5–7 days, most of floating cells showed irregular shape with some protrusions (supporting information Fig. 2). In addition, some of the transferred cells adhered to the dish surfaces like macrophages. Based on the morphology, the floating cells with protrusions were named iPS-DCs (iPS cell-derived dendritic cells). iPS-DCs expressed CD11c, but did not express CD80, CD86, and MHC class II until day 10 of the step 3 culture (Fig. 2B). At day 13, some of them expressed CD80 and MHC class II, thus suggesting spontaneous partial maturation. After day 10 of the step 3 culture, 1–2 × 10^6 iPS-DCs were recovered from one Petri dish. The number of cells increased about 400–600 times from the initiation of the differentiation until differentiation into iPS-DCs.

To induce full maturation of iPS-DCs, we transferred the floating cells into new Petri dishes, and added IL-4, TNF-α, and anti-CD40 mAb to the cells simultaneously. In 2 or 3 days, most of the cells exhibited morphology of typical mature DCs, with many long protrusions or veil-like protrusions and some of the cells formed clusters (Fig. 1I–1L). Flow cytometric analysis demonstrated that high levels of cell-surface expression of CD80, CD86, and MHC class II in the mature iPS-DCs (Fig. 2B).

One of the Fbx15-selected iPS cell clones, iPS-MEF-FB-20A-10[13] (20A-10), was also subjected to the differentiation culture. The 20A-10 iPS cells differentiated and proliferated well in the culture steps 1 and step 2, resulting in appearance of a large number of myeloid lineage cells at the end of step 2 (supporting information Fig. 3A). In the step 3 culture with GM-CSF intended for generation of DCs, 20A-10-derived myeloid cells further grew, exhibited some protrusions, and expressed CD11c. However, they were refractory to maturation. Even when stimulated with the simultaneous addition of IL-4, TNF-α and anti-CD40 mAb, they expressed very low level of cell-surface MHC class II and did not express CD80 and CD86 (supporting information Fig. 3B).

Functions and Gene-Expression of iPS-DCs

Nanog-selected iPS cells including the clone 38C-2 were derived from PEF with a mixed genetic background composed of 75% DBA (H-2^d), 12.5% C57BL/6 (H-2^b), and 12.5% 129S4 (H-2^b) [14], and thus their H-2 haplotype could be b/b, b/d, or d/d. Before the analyses of the immunological functions of iPS-DCs, we determined the H-2 haplotype of 38C-2 iPS cells by flow cytometric and PCR-based analyses and that was found to be d/b (supporting information Fig. 4).

To examine the capacity of iPS-DCs to stimulate T-cells, allogeneic mixed lymphocyte reaction (MLR) assay was conducted using iPS cell-derived cells of several different differentiation stages as stimulators. We used splenic T-cells isolated from naive C57BL/6 mice (H-2^b) as allogeneic responder T-cells. As shown in Figure 3A, the floating cells harvested from step 2 culture (pre-iPS-DCs) or at day 8 of step 3 (immature iPS-DCs) exhibited either no or a very low level of activity to stimulate naive T-cells. Partially maturated iPS-DCs harvested on day 15 of the step 3 culture induced a small but definite proliferative response of T-cells. In contrast,

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