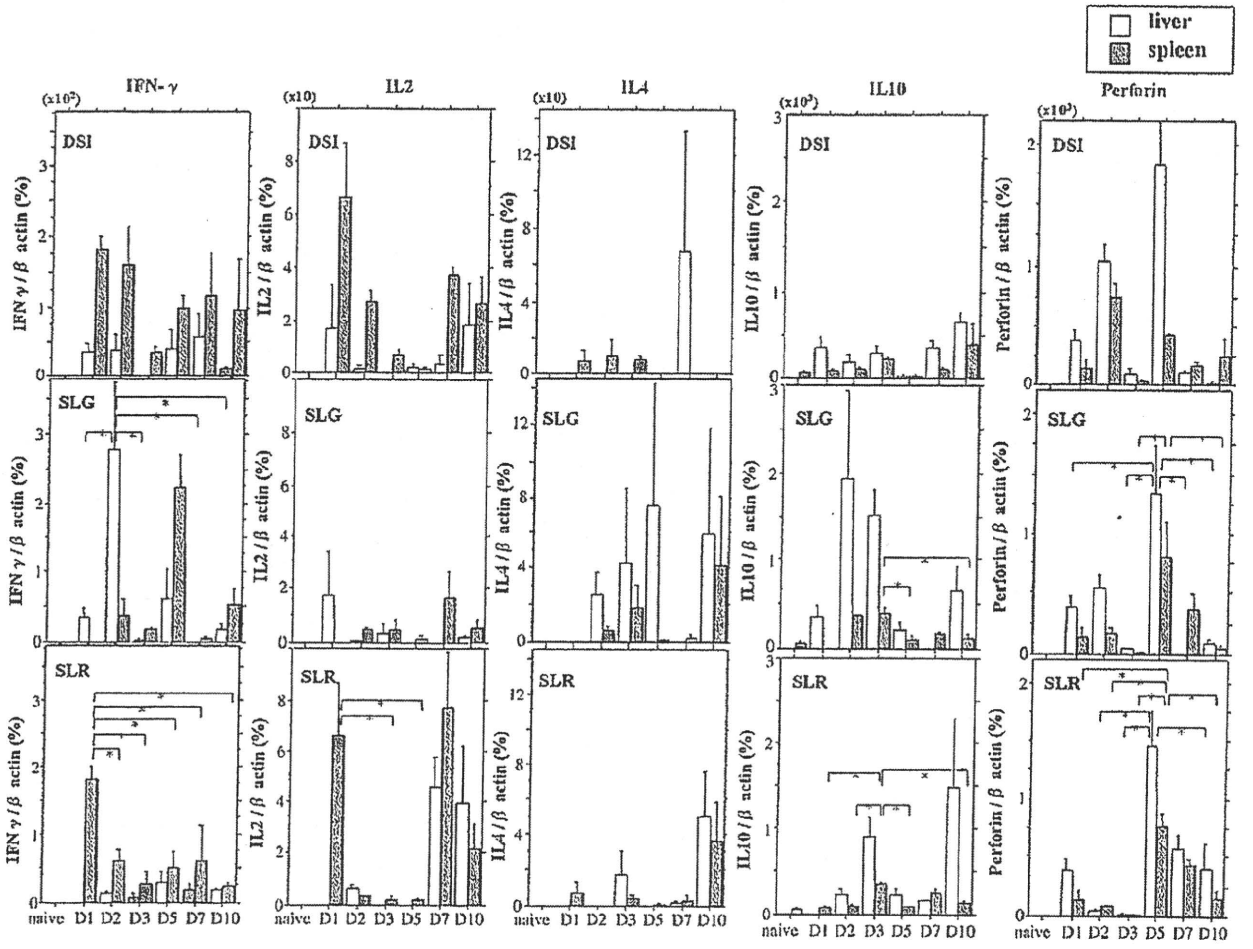

Fig. 2. Kinetics of various cytokines (IFN-γ, IL-2, IL-4, and IL-10) and cytotoxin (perforin) mRNA levels at 1, 2, 3, 5, 7, and 10 days after donor antigen administration in the liver and spleen. After alloantigen administration, in the liver of SL-Grafted, the highest level of IFN-γ was noted on day 2, followed by day 1, day 3, day 7, and day 10. In the spleen of SL-Removed, IFN-γ was rapidly upregulated reaching a peak level on day 1 but decreased thereafter. The expression of IL-2 in the spleen on day 1 was significantly higher than on day 3 ($p<0.005$), and day 5 ($p<0.005$). IL-4 was not different between groups. IL-10 levels in the liver and spleen in SL-Grafted on days 2 and 3 were higher than other days in the same group. In SL-Removed, IL-10 level in the spleen on day 3 was significantly higher than on day 1 ($p<0.0001$), day 2 ($p<0.0001$), day 5 ($p<0.0001$) and day 10 ($p<0.0001$). In DSI, SL-Grafted, and SL-Removed rats, perforin mRNA levels in the liver and spleen were the highest on day 5 compared to other days. DSI: donor-specific cell injection; SLG, SL-Grafted, sensitized liver grafted; SLR, SL-Removed, sensitized liver removed. Data represent mean ± SEM. *$p<0.05$.

4. Discussion

In this study, we indicated that the immune response to injected alloantigen varied according to its distribution inside or outside the liver. SL-Grafted rats with alloantigens limited to the liver rejected cardiac allografts in an accelerated fashion compared with either untreated animals or DSI animals with alloantigens distributed both inside and outside the liver. In contrast, SL-Removed rats with alloantigens distributed to other organs but not the liver rejected cardiac allografts in a delayed fashion [10,11]. These findings suggest that the immune reaction restricted by early distribution of the alloantigen inside the liver may be associated with allograft rejection, while the distribution outside the liver may be associated with a lack of immunoresponse.

To determine the mechanism of each outcome, we compared the expression levels of cytokine mRNAs in the liver, spleen, and allograft in each group. Previous studies reported a close temporal and physical correlation between upregulation of the early phase of IL-2 and IFN-γ in lymphoid tissues and subsequent rejection or tolerance of the transplanted organ [20,21]. In the SL-Grafted group, alloantigens were distributed solely in the liver after DSI and cardiac allografts were rejected in an accelerated fashion. In these animals, the expression level of IFN-γ mRNA was higher in the liver on day 1 after transplantation of liver grafts obtained from sensitized WS rats into naïve WS. In the SL-Removed group, where alloantigens were distributed extrahepatically and rejection was delayed, the mRNA expression levels of Th1 cytokines, IFN-γ and IL-2, were higher in the spleen and rapidly decreased. On the contrary, in DSI model, which has alloantigen inside and outside of the liver, the change of IFN-γ and IL-2 did not show remarkably in both the liver and spleen. According to this change of the cytokines compared with DSI model, it appears that the shorter graft survival of SL-Grafted is associated with early and major upregulation of IFN-γ in the liver, while longer graft survival of SL-Removed is associated with early upregulation and the following decrements of IFN-γ and IL-2 in the spleen. With regard to Th2 cytokines, IL-4 mRNA level was not significantly different among SL-Grafted, SL-Removed and DSI. On the other hand, in SL-Grafted rats, IL-10 mRNA levels in the liver and spleen were the highest on early phase and decreased thereafter; in SL-Removed rats, they were upregulated on day 3 and increased until day 10 in the liver and spleen. In DSI rats, these changes did not note. This should indicate that the late IL10 elevation in SL-Removed rats relates the late rejection of alloheart graft,

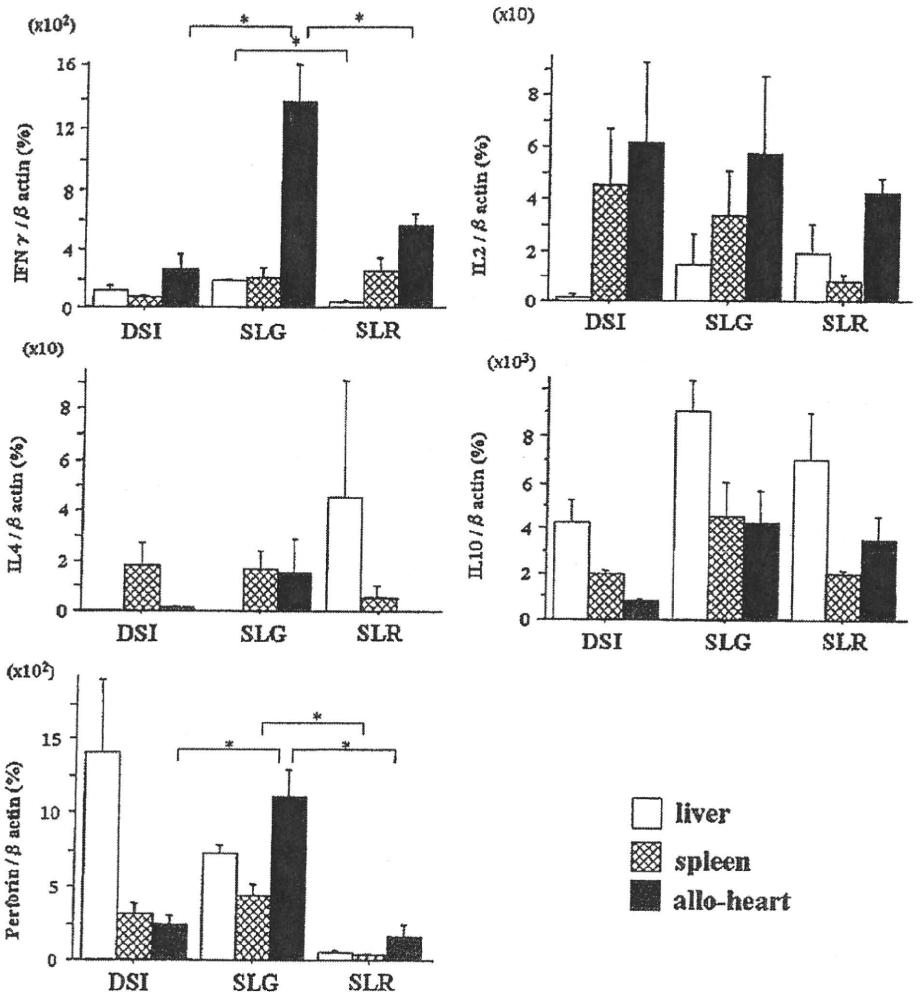

Fig. 3. Expression levels of IFN-γ and perforin mRNAs in liver, spleen and alloheart on day 14. IFN-γ mRNA levels were higher in SL-Grafted than in SL-Removed in the liver ($p<0.05$) and in the allograft ($p<0.05$). Perforin levels were higher in SL-Grafted than in SL-Removed in the spleen ($p<0.005$) and in the allograft ($p<0.001$). In SL-Grafted, IFN-γ ($p<0.005$) and perforin ($p<0.001$) were higher than in DSI in the allograft. Perforin mRNA levels in SL-Grafted were higher than in SL-Removed in liver ($p<0.05$). There were no significant differences between groups with regard to IL-2, IL-4, and IL-10 levels in the liver, spleen, and alloheart. DSI: donor-specific cell injection; SLG, SL-Grafted, sensitized liver grafted; SLR, SL-Removed, sensitized liver removed. Data represent mean ± SEM. *$p<0.05$.

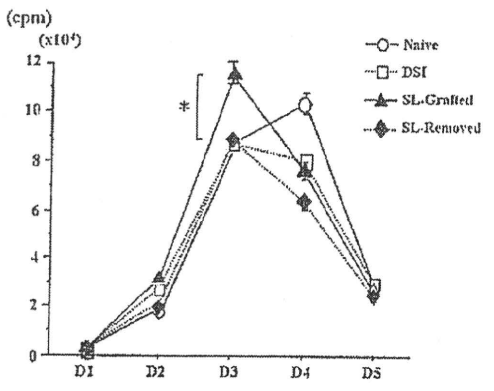

Fig. 4. MLR on day 10 after alloantigen administration. The peak of MLR proliferation in lymphocytes from SL-Grafted, SL-Removed, and DSI groups was one day earlier than in naive WS rats. MLR proliferation of WS responder cells toward donor DA stimulator cells in SL-Grafted was significantly higher than in SL-Removed ($p<0.0001$) and DSI ($p<0.005$). Data are mean ± SEM. *$p<0.05$. ($n\geq3$ per group).

in comparison with DSI model. The change of the cytokines in DSI model was not remarkable compared with the other models; the unique expression pattern in SL-Grafted and SL-Removed, but not in DSI, indicated that the change of the cytokines was essential for the immune reaction, but not the surgical influence; i.e. ischemic/reperfusion injury. Indeed, the correlation between IFN-γ and IL-10 was reported in the T-cell mediated graft rejection [22]. These results suggest that IFN-γ in early- and IL-10 in late-post-DSI phase may play a role in the control of allograft rejection in an accelerated or delayed fashion.

Another important observation in SL-Grafted rats was that when the recipient was challenged with a transplanted donor heart from the sensitizing DA strain, the expression of IFN-γ mRNA increased in both the allograft and liver. The high level of perforin mRNA in the allograft correlated well with IFN-γ upregulation in this tissue. These results support the role of Th1 cytokines [23] and the importance of increased IFN-γ expression in the enhancement of transformation of cytotoxic lymphocytes to effector cells [24]. Since the expression of perforin mRNA increased on day 14 in the spleen and allograft, SL-Grafted recipients may have strong activity of cytotoxic lymphocytes, inducing the accelerated rejection.

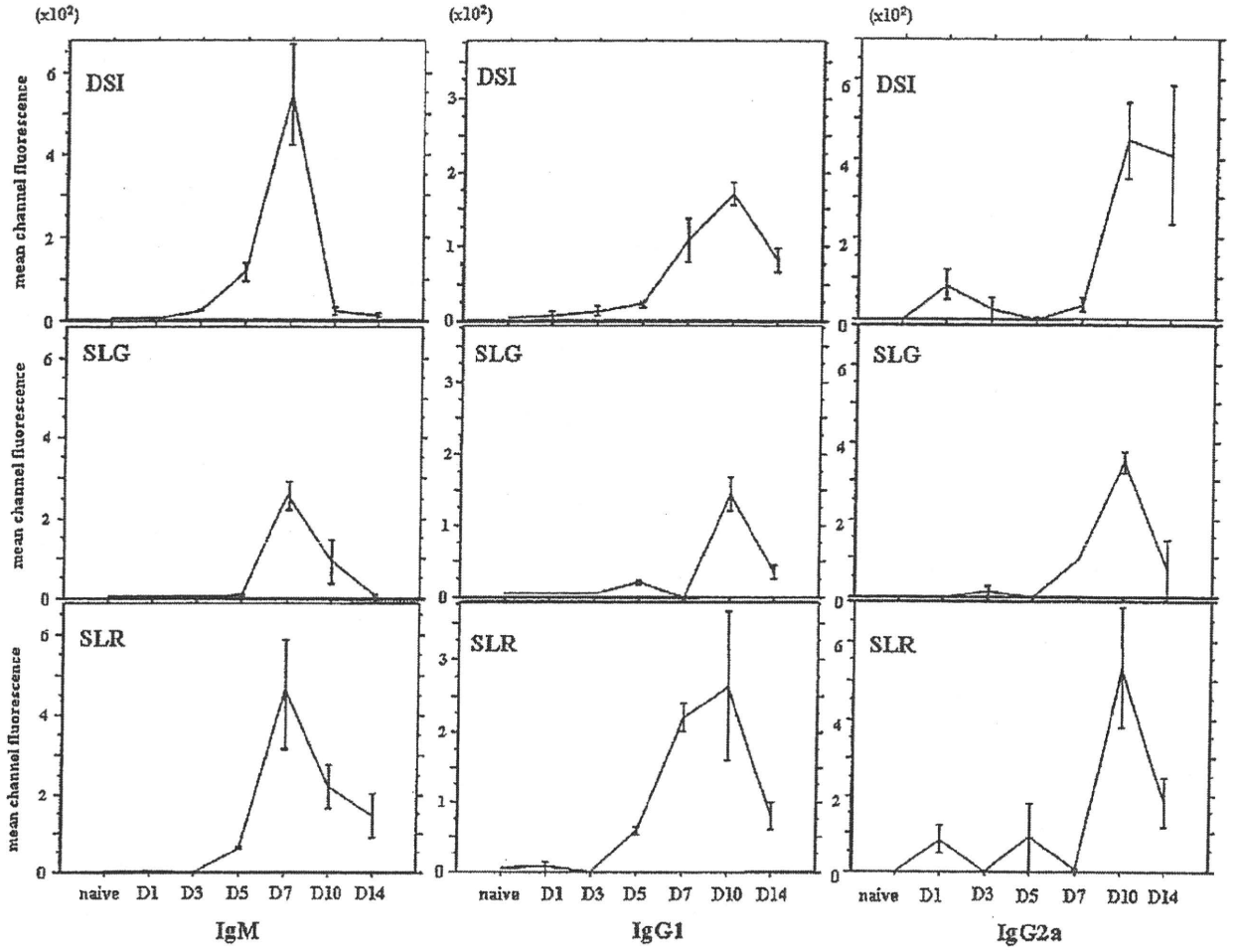

Fig. 5. Donor-specific alloantibody response. The response was tested by flow cytometry for IgM, IgG1, and IgG2a binding to mononuclear cells from DA lymph nodes. In SL-Grafted, SL-Removed, and DSI, each treatment after alloantigen administration elicited an IgM response that reached a peak level at day 7 and switched to an IgG1 and an IgG2a response that reached a peak level on day 10. There were no significant differences in the latency to peak switching from IgM to IgG1 and IgG2a responses among all groups. DSI: donor-specific cell injection; SLG, SL-Grafted, sensitized liver grafted; SLR, SL-Removed, sensitized liver removed. Data are mean ± SEM. $^{*}p<0.05$. ($n \geq 3$ per group).

Concerning this accelerated rejection, immunologic memory is the term used to describe the rapid and enhanced immune responses to antigens that have been encountered in the past. There is general agreement that IL-2 production by naive cells is greater than by memory T cells. On the other hand, IFN-γ is synthesized preferentially by memory T cells rather than naive cells [25]. Our results showed that IFN-γ mRNA levels were high in the liver and alloheart at day 14 in SL-Grafted but not in SL-Removed and DSI. In SL-Grafted rats, alloantigens were limited to the liver at day 1. This finding suggests that the development of immune response within the liver may induce memory T cells following DSI.

It has been reported that Th1 cytokines correlate with allogeneic influence of T-cell function while Th2 cytokines correlate with B cell function [26,27]. To determine whether the allogeneic response is directly due to the influence of T or B cell function alone or in combination, we examined pretransplant one-way MLR and measured the levels of IgM, IgG1 and IgG2a alloantibodies. In the allogeneic MLR response, the peak of MLR proliferation occurred one day earlier in SL-Grafted compared with SL-Removed and DSI. Increased levels of Th1 cytokines in the liver would be thought to lead to increase allogeneic MLR and T-cell clonal activation, which correlates with pretransplant production of memory T cells. In comparison, the response in alloantibody in SL-Grafted, SL-Removed and DSI, elicited an IgM response that reached a peak level on the same day (day 7) and switched to an IgG1 and IgG2a responses that reached peak levels on the same day (day 10). The latencies to peak switching from an IgM to an IgG1 and IgG2a responses were not significantly different among the groups; i.e., the alloantibodies produced by B cells were not significantly different. We have reported that accelerated graft failure in the SL-Grafted group was due to both antibody and cell-mediated rejection after re-challenge with donor type grafts [10,11]. In humans, previous studies reported accelerated graft rejection in sensitized recipients with mixed humoral and cellular rejection after transplantation [28]. However, before HTx as a re-challenge, the results suggest the significance of allogeneic influence of T-cell function, but not that of B cells, for the accelerated rejection in SL-Grafted.

Based on the sequestration theory, Sprent et al. [29–31] showed accumulation and activation of alloantigen reactive T cells in the spleen (as measured by disappearance of allospecific T cells from thoracic duct lymph), where intravenously injected antigens accumulated at day 1 after inoculation. According to this observation, sequestration and activation of alloreactive T cells might also occur in the liver at day 1 after allogenic spleen cell injection, as observed in our study (accumulation of about 18% of the injected donor spleen cells in the liver). At day 1 after injection, the liver was transplanted to naive second host or removed from sensitized host; thus, adoptive transfer of activated allospecific T cells or their elimination is a possible explanation of the striking

difference noted in our study in subsequent graft survival between SL-Grafted and SL-Removed. Sprent et al. [29–31] also observed that the activated T cells spread in the periphery and reappeared in the circulation after 3 days. Our results are consistent with these data; the graft survival period in both SL-Grafted and SL-Removed could not be observed when the timing of syngeneic liver transplantation was delayed to day 2 or later [25]. Based on these considerations, our results point to a unique immune response in the liver that occurs at a very early period after antigen inoculation. Our finding that vigorous antidonor immune response occurred in the liver does not contradict the tolerogenic effect of intraportal antigen inoculation. This is because tolerance to donor antigen after intraportal inoculation of the antigen usually takes 7–14 days to be established after antigen administration [6–8,17], whereas we analyzed the immune responses in the liver shortly after antigen inoculation. Furthermore, our SL-Grafted model lacked late IL-10 elevation, compared with liver specific antigen expressing model [32].

On the contrary, from the aspect of tolerance, our SL-Removed model produced IL-10 in liver lately, like the other tolerant model [32]. In DSI model, there was no remarkable change of IL-10 in both the liver and spleen. These would suggest that late IL-10 regulation, which is considered to be contributed by the accumulation of regulatory T cells to the liver, may influence the immune tolerance. The discrepancy between our SL-Grafted/Removed models and liver specific antigen expressing model in the recent paper [32] might be caused by the disconnection of the circulating cellular immune response after the liver transplantation. In SL-Removed model, late IL-10 elevation would be caused by the lack of the regulator for IL-10 production or producing cells in the naïve liver, and in DSI models the lack of IL-10 elevation would be caused by the regulator in the sensitized liver.

In conclusion, our *in vitro* results clearly indicated that two compartments of the immune system (liver and other organs) behave differently to alloantigen, suggesting an early Th1 reaction in the liver and spleen. The liver may play an important role in determining the alloimmune response following DSI.

Acknowledgment

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Effects of Preceding Interferon Therapy on Outcome After Surgery for Hepatitis C Virus-Related Hepatocellular Carcinoma

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Background and Objectives: Interferon (IFN) can eradicate hepatitis C virus (HCV)-RNA from serum and hepatic tissue, and suppress the development of hepatocellular carcinoma (HCC). Despite such effectiveness, HCC develops even in HCV patients successfully treated with IFN therapy.

Methods: HCV-related HCC patients who underwent curative hepatectomy for HCC were divided into three groups according to preceding IFN for HCV infection therapy and the therapeutic effect: responders group (n = 23), non-responders group (n = 46), and no-IFN group (n = 215). Postoperative outcome was retrospectively examined in the three groups.

Results: AST and ALT were significantly lower in responders group than non-responders group ($P < 0.001$, $P = 0.001$) and no-IFN group ($P = 0.001$, $P = 0.002$). Platelet count was significantly higher in responders group than other groups ($P = 0.008$, $P = 0.001$). The percentage of cirrhotic patients in responders group was significantly lower than other groups ($P = 0.017$, $P = 0.014$). Multivariate analysis identified preceding IFN therapy to be associated with disease-free survival at marginal significance ($P = 0.086$), and as a significant independent factor for overall survival ($P = 0.042$).

Conclusions: Preceding IFN therapy for HCV infection improves postoperative outcome in HCV-related HCC patients treated successfully with IFN.

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KEY WORDS: **hepatocellular carcinoma (HCC); interferon (IFN); hepatitis C virus (HCV); hepatic resection**

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Approximately 80% of Japanese HCC patients have a history of chronic infection with hepatitis C virus (HCV), which is a known cause of HCC [1,2]. Recent advances in imaging modalities and treatment have brought some improvement to the prognosis of patients with HCV-related HCC, but the outcome remains unsatisfactory. Even after curative hepatic resection for HCV-related HCC, the rate of tumor recurrence within 1 year is 20–40%, rising to about 80% by 5 years [3,4]. This high recurrence rate and the progression of the underlying hepatic damage due to HCV-related chronic hepatitis (CH) or cirrhosis result in unfavorable postoperative outcome in patients with HCV-related HCC.

Interferon (IFN) is the only agent known to be effective against HCV infection [5–10]. It can eradicate HCV-RNA from peripheral blood and hepatic tissue, prevent deterioration of liver dysfunction in patients with HCV infection, and suppress the development of HCC. HCV-infected patients treated with IFN, especially those who develop sustained virological response (SVR), defined as negative HCV-RNA polymerase chain reaction at 6 months after the end of treatment, enjoy the benefits of such treatment [11,12]. However, despite such effectiveness of IFN therapy, there have been recently some reports of development of HCC even in HCV patients who had gained SVR following IFN therapy [13,14]. With regard to the HCC development in patients treated successfully with IFN, HCV-related HCC patients can be divided into three groups according to the clinical background of preceding IFN therapy: successfully treated, unsuccessfully treated, or not treated with IFN. However, to date, there have been few studies on the correlation between clinical background of previous IFN therapy for HCV infection and surgical outcome of HCV-related HCC [15,16].

In the present retrospective study, we reviewed HCV-related HCC patients who had undergone surgery in our hospital. We analyzed the factors that affected postoperative outcome including history of previous IFN therapy and the effect of such therapy.

MATERIALS AND METHODS

The present study included 284 patients with HCC who had undergone curative hepatic resection at the Department of Surgery, Osaka University Hospital between January 1990 and December 2008. Patients with HCC grade Vp3, Vp4, Vv2, and Vv3, defined according to the classification system of the Liver Cancer Study Group of Japan, were excluded from this study [17]. Curative resection was defined as complete removal of all macroscopically evident tumors. Patients who had undergone surgery for recurrent HCC were also excluded from this

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study. Among the 284 patients, 215 patients were not treated with IFN (no IFN group). The remaining 69 patients received IFN therapy for HCV infection. In the latter group, HCC had not been detected at the IFN therapy, and was detected after the IFN therapy. The IFN therapy was performed not for HCC, but for HCV-related hepatitis. The administration of IFN therapy was determined based on the informed consent between each physician and patient. The response to IFN therapy was assessed retrospectively based on changes in HCV-RNA. Based on the response, patients were divided into the responders group and non-responders group; 23 patients whose HCV-RNA disappeared after IFN therapy were categorized as the responders group, and 46 patients whose HCV-RNA did not disappear after IFN therapy in non-responders group. Figure 1 summarizes the classification of the enrolled patients. The type, dosage, and duration of IFN administration before surgery varied, though all patients received IFN-α.

Hospital records were retrospectively reviewed for clinical factors including previous history of IFN therapy, tumor- and surgery-related factors. The surgical procedure was selected based on the extent of the tumor and residual liver function. The HCC staging was performed according to the classification system of the Liver Cancer Study Group of Japan [17]. The histological grade of differentiation of HCC was determined according to the Edmondson–Steiner classification, and was based on the areas of the tumor with the highest grade [18]. Non-cancerous lesion of the liver was divided histopathologically into chronic hepatitis CH and liver cirrhosis (LC).

Patients were followed up after hepatic resection at regular intervals of 3–4 months with physical examination, tumor markers including alpha-fetoprotein (AFP), and protein induced by vitamin K absence or antagonists-II (PIVKA-II), liver biochemical tests, abdominal ultrasonography, and abdominal computed tomography (CT) to check for intrahepatic recurrence, and chest radiography and bone scintigraphy for extra-hepatic recurrence. The median duration of clinical follow-up after the initial hepatectomy was 51.2 months.

Data were expressed as mean ± standard deviation. Differences between groups were assessed by the chi-square test, Fisher's exact test, or the Mann–Whitney U test. Survival rates were calculated according to the Kaplan and Meier method and compared using the log-rank test. Statistical analysis was performed using StatView (version 5.0, SAS Institute Inc., Cary, NC). A P-value <0.05 was considered statistically significant. The study protocol was approved by the Human Ethics Review Committee of Osaka University Hospital and a signed consent form was obtained from each patient.

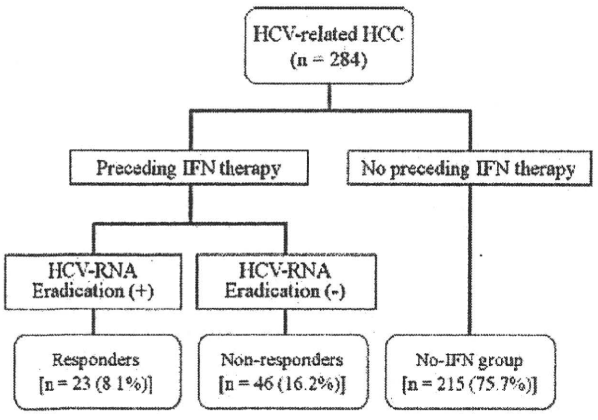

Fig. 1. Distribution of patients enrolled in this study according to the clinical background of preceding IFN therapy. HCV, hepatic C virus; HCC, hepatocellular carcinoma; IFN, interferon.

RESULTS

The study group comprised 222 (78.2%) men and 62 (21.8%) women, with a mean age of 65 (range, 39–79). Table I summarizes the clinicopathological characteristics of the responders group, the non-responders group, and the no-IFN group. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were significantly lower in the responders group than the non-responders group ($P < 0.001$, $P = 0.001$) and no-IFN group ($P = 0.001$, $P = 0.002$). There were no significant differences in the levels of AST and ALT between the non-responders group and the no-IFN group. Platelet count was significantly higher in the responders than that in the non-responders ($P = 0.008$) and that in the no-IFN group ($P = 0.001$). In the responders group, histopathological status of the non-cancerous liver tissue obtained at surgery was CH in 16 patients (69.6%) and LC in seven patients (30.4%). The percentage of patients of the responders group with LC was significantly lower than that of the non-responders group (28/46, 60.9%; $P = 0.017$) and that of the no-IFN group (123/215, 57.2%; $P = 0.014$). Liver function assessed by Child–Pugh classification was not different among the three groups. Other clinical factors listed in Table I were also not different among the three groups, including tumor- and surgical-related factors. Adjuvant therapy of IFN was administered in a small number of patients (n = 14, 4.9%), and the frequency of such patients was not different among the three groups.

For all the 284 patients, the 1-, 3-, and 5-year disease-free survival (DFS) rates were 70.8%, 36.7%, and 22.8%, respectively. There was no significant difference in DFS between the IFN group (the responders group and the non-responders group) and the no-IFN group ($P = 0.396$). However, the DFS of the responders group (1 year: 89.2%, 3 years: 59.4%, 5 years: 59.4%) was significantly better than that of the no-IFN group (1 year: 70.8%, 3 years: 35.7%, 5 years: 21.6%; $P = 0.039$), and tended to be better than that of the non-responders group (1 -year: 60.4%, 3 years: 32.3%, 5 years: 16.9%; $P = 0.051$; Fig. 2). However, there was no significant difference in DFS between the non-responders group and the no-IFN group ($P = 0.673$). The 1-, 3-, and 5-year overall survival rates for all patients were 94.5%, 80.4%, and 66.9%, respectively. The overall survival rates of the IFN group (responders group and non-responders group) tended to be higher than those of the no-IFN group ($P = 0.093$). The 1-, 3-, and 5-year overall survival rates for the responders group were 100%, 100%, and 100%, respectively, and were significantly higher than the non-responders group (1-year: 94.4%, 3 years: 78.6%, 5 years: 55.4%; $P = 0.026$) and the no-IFN group (1 year: 94.0%, 3 years: 79.0%, 5 years: 66.1%; $P = 0.009$; Fig. 3). There was no significant difference in overall survival between the non-responders group and the no-IFN group ($P = 0.904$).

Univariate analysis was performed between DFS and various clinicopathological factors (Table II). Microscopic vascular invasion (negative vs. positive), tumor stage (I, II vs. III, IV), number of nodules (single vs. multiple), the diameter of largest tumor nodules (<5 cm vs. ≥5 cm), AFP level (<5 ng/m vs. ≥5 ng/m), and preceding IFN therapy (responders vs. non-responders, no-IFN) were significant factors ($P < 0.001$, $P = 0.006$, $P = 0.008$, $P = 0.021$, $P = 0.017$, $P = 0.037$). Multivariate analysis for DFS using the above six factors identified the number of nodules and microscopic vascular invasion as significant independent factors ($P = 0.014$, $P = 0.041$; Table III). In the same analysis, preceding IFN therapy showed a borderline significance with DFS ($P = 0.086$). The diameter of the largest tumor nodules and AFP level also tended to be associated with DFS ($P = 0.090$, $P = 0.098$).

Univariate analysis for overall survival using various clinicopathological factors demonstrated that microscopic vascular invasion (negative vs. positive), preceding IFN therapy (responders vs. non-responders, no-IFN), number of nodules (single vs. multiple), diameter of largest nodules (<5 cm vs. ≥5 cm), and AFP level (<5 ng/m vs. ≥5 ng/m) were significant factors ($P = 0.004$, $P = 0.009$, $P = 0.015$,

TABLE I. Clinicopathological Characteristics of Patients With HCV-Related HCC

	IFN group			*P*-value		
	Responders (n = 23)	Non-responders (n = 46)	No-IFN (n = 215)	Responders versus non- responders	Responders versus no-IFN	Non- responders versus no-IFN
Clinical factors						
Gender (male/female)	19/4	33/13	170/45	0.323	0.793	0.278
Age (years)	66 ± 7	64 ± 7	65 ± 7	0.355	0.653	0.424
Alcohol abuse (+/−)	14/9	27/19	132/83	0.862	0.961	0.733
HCV serotype (1/2/unknown)	19/4/0	35/5/6	166/29/20	>0.999	0.795	0.969
HBs Ag (+/−)	1/22	1/45	7/208	>0.999	0.562	>0.999
AST (IU/L)	28 ± 13	49 ± 27	46 ± 21	<0.001	0.001	0.184
ALT (IU/L)	26 ± 16	52 ± 36	47 ± 29	0.001	0.002	0.233
Platelet count ($\times 10^4/\mu l$)	16.4 ± 3.3	13.0 ± 5.3	13.2 ± 4.5	0.008	0.001	0.867
Albumin (g/dl)	4.0 ± 0.4	3.8 ± 0.6	3.8 ± 0.5	0.142	0.175	0.975
Total bilirubin (mg/dl)	0.6 ± 0.2	0.7 ± 0.2	0.7 ± 0.3	0.114	0.104	0.362
Prothrombin time (%)	77 ± 10	77 ± 8	76 ± 9	0.719	0.888	0.442
Hepaplastin test (%)	81 ± 13	78 ± 12	77 ± 12	0.244	0.217	0.834
Child-Pugh (A/B)	22/1	37/9	187/28	0.148	0.326	0.265
Non-cancerous lesion (CH/LC)	16/7	18/28	92/123	0.017	0.014	0.648
Tumor-related factors						
AFP (ng/ml)	1,791 ± 6,654	545 ± 1,444	851 ± 4,004	0.226	0.332	0.610
PIVKA-II (mAU/ml)	1,773 ± 5,433	1,418 ± 4,733	2,006 ± 4,879	0.786	0.837	0.459
Preoperative TAE (+/−)	10/13	23/23	119/96	0.609	0.278	0.509
Postoperative IFN (+/−)	1/22	1/45	12/203	>0.999	>0.999	0.476
Number of nodules (single/multiple)	17/6	33/13	152/63	0.849	0.747	0.888
Tumor diameter (cm)	3.5 ± 1.9	3.2 ± 2.0	3.6 ± 2.6	0.287	0.725	0.171
Vascular invasion (+/−)	2/21	2/44	18/197	0.596	>0.999	0.543
Stage (I/II/III/IV)	5/12/4/2	12/24/7/3	45/109/49/12	0.967	0.890	0.671
Edmondson-Steiner grade (I, II/III, IV/unknown)	13/10/0	26/16/4	117/89/9	0.672	0.980	0.541
Surgery-related factors						
Hr (0/S/1/2)	13/3/3/4	29/5/7/5	116/41/37/21	0.869	0.615	0.543
Volume of resection (g)	152 ± 118	137 ± 151	165 ± 162	0.393	0.682	0.186
Blood loss (ml)	1,022 ± 1,583	996 ± 702	1,167 ± 1,217	0.770	0.571	0.197
Operation time (min)	253 ± 128	236 ± 99	236 ± 99	0.337	0.940	0.174
Transfusion (+/−)	4/19	7/39	52/163	>0.999	0.465	0.187

Data are expressed as mean ± standard deviation.

IFN, interferon; HCV, hepatic C virus; HBs Ag, hepatitis B surface antigen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CH, chronic hepatitis; LC, liver cirrhosis; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; TAE, transcatheter arterial chemoembolization; Hr, hepatic resection; 0: partial resection; S, subsegmentectomy; 1, one segmentectomy; 2, two segmentectomies.

$P = 0.034$, $P = 0.045$; Table II). Multivariate analysis for overall survival using the above five factors identified number of nodules, microscopic vascular invasion, and preceding IFN therapy as significant independent factors ($P = 0.025$, $P = 0.037$, $P = 0.042$; Table III).

HCC recurred postoperatively in nine (39.1%) patients of the responders group, 29 (63.0%) of the non-responders group, and in 157 (73.0%) of the no-IFN group. Table IV summarizes the clinical characteristics of patients with recurrent HCC at diagnosis of the recurrence. AST and ALT levels in the responders group were

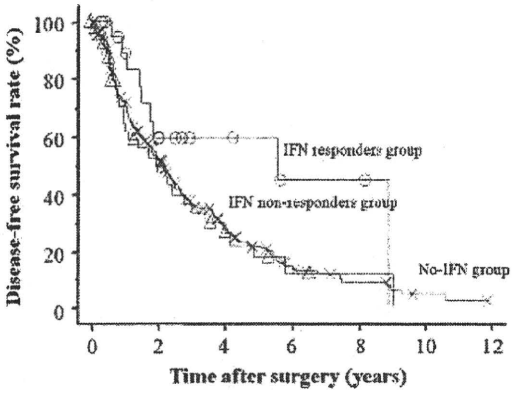

Fig. 2. Disease-free survival after initial surgery for HCC in the responders group, the non-responders group, and the no-IFN group. Open circles: responders (n = 23), open triangles: non-responders (n = 46), crosses: no-IFN (n = 215). IFN: interferon.

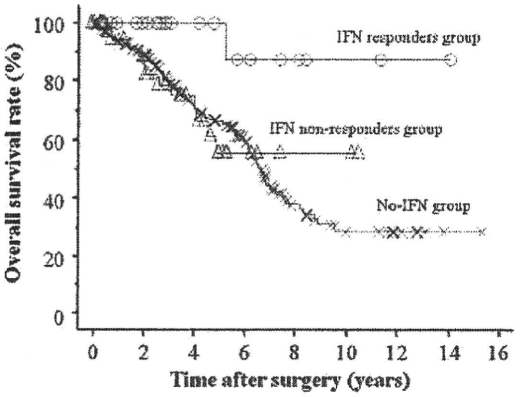

Fig. 3. Overall survival calculated from the initial surgery for HCC for the responders group, the non-responders group, and the no-IFN group. Open circles: responders (n = 23), open triangles: non-responders (n = 46), crosses: no-IFN (n = 215). IFN: interferon.

TABLE II. Univariate Analysis of Disease-Free Survival and Overall Survival of Patients With HCV-Related HCC

	Number of patients	Disease-free survival	Overall survival
Clinical factors			
Gender (male/female)	222/62	0.732	0.789
Age, years (<66/≥67)	143/141	0.682	0.842
Alcohol abuse (+/−)	172/112	0.955	0.572
HCV genotype (1/2/unknown)	220/40/25	0.612	0.427
AST (IU/L) (<40/≥40)	126/158	0.496	0.547
ALT (IU/L) (<40/≥40)	122/162	0.216	0.301
Total bilirubin (mg/dl) (<1.0/≥1.0)	252/32	0.890	0.587
Albumin (g/dl) (<3.5/≥3.5)	114/170	0.174	0.171
Prothrombin time (%) (<70/≥70)	77/207	0.693	0.875
Hepaplastin test (%) (<70/≥70)	75/209	0.427	0.398
Platelet count ($\times 10^4/\mu l$) (<10/≥10)	83/201	0.176	0.123
Child-Pugh (A/B)	246/38	0.866	0.594
Non-cancerous lesion (CH/LC)	126/158	0.247	0.177
Tumor-related factors			
AFP (ng/ml) (<5/≥5)	54/230	0.021	0.045
PIVKA-II (mAU/ml) (<400/≥400)	190/83	0.130	0.142
Preceding IFN (responders/non-responders, no-IFN)	23/261	0.037	0.009
Preoperative TAE (+/−)	152/132	0.863	0.562
Postoperative IFN (+/−)	14/270	0.222	0.253
Number of nodules (single/multiple)	202/82	0.008	0.015
Tumor diameter (cm) (<5/≥5)	232/52	0.017	0.034
Vascular invasion (+/−)	22/262	<0.001	0.004
Stage (I, II/III, IV)	207/77	0.006	0.197
Edmondson-Steiner grade (I, II/III, IV)	156/115	0.328	0.265
Surgery-related factors			
Hr (0/S, 1, 2)	158/126	0.313	0.893
Intraoperative blood loss (L) (<1/≥1)	151/133	0.289	0.270
Operation time (min) (<240/≥240)	141/143	0.221	0.493
Transfusion (+/−)	63/221	0.756	0.180

IFN, interferon; HCV, hepatic C virus; HBs Ag, hepatitis B surface antigen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CH, chronic hepatitis; LC, liver cirrhosis; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; TAE, transcatheter arterial chemoembolization; Hr, hepatic resection; 0: partial resection; S, subsegmentectomy; 1, one segmentectomy; 2, two segmentectomies.

significantly lower than those in the non-responders group ($P = 0.047$, $P = 0.045$) and those in the no-IFN group ($P = 0.028$ and $P = 0.034$). There were no significant differences in AST and ALT levels between the non-responders and no-IFN groups. Platelet count was significantly higher in the responders group than that in the no-IFN group ($P = 0.029$) and tended to be higher than that in the non-responders group ($P = 0.079$). Figure 4A shows the distribution of interval between initial hepatectomy and recurrence. In most patients, HCC recurred within 2 years in the three groups, and the distribution of the interval was not different among the three groups. In all groups, the

TABLE III. Multivariate Analysis of Disease-Free Survival and Overall Survival of Patients With HCV-Related HCC

	OR	95% CI	*P*-value
Disease-free survival			
AFP (ng/ml) (<5/≥5)	1.427	0.937–2.174	0.098
Preceding IFN (responders/non-responders, no-IFN)	1.809	0.919–3.561	0.086
Number of nodules (single/multiple)	1.707	1.022–2.850	0.041
Tumor diameter (cm) (<5/≥5)	1.391	0.951–2.037	0.090
Vascular invasion (−/+)	2.331	1.186–4.587	0.014
Stage (I, II/III, IV)	1.287	0.715–2.315	0.401
Overall survival			
AFP (ng/ml) (<5/≥5)	1.689	0.847–3.367	0.137
Preceding IFN (responders/non-responders, no-IFN)	7.750	1.076–55.798	0.042
Number of nodules (single/multiple)	1.622	1.062–2.476	0.025
Tumor diameter (cm) (<5/≥5)	1.381	0.842–2.268	0.200
Vascular invasion (−/+)	2.247	1.049–4.808	0.037

IFN, interferon; HCV, hepatic C virus; HBs Ag, hepatitis B surface antigen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CH, chronic hepatitis; LC, liver cirrhosis; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; TAE, transcatheter arterial chemoembolization; Hr, hepatic resection; 0: partial resection; S, subsegmentectomy; 1, one segmentectomy; 2, two segmentectomies. OR, odds ratio, 95% CI, 95% confidence interval.

TABLE IV. Clinicopathological Characteristics of Patients With Recurrent HCC in the Responders Group, the Non-Responders Group, and the No-IFN Group

	IFN group			*P*-value		
	Responders (n = 9)	Non-responders (n = 29)	No-IFN (n = 157)	Responders versus non-responders	Responders versus no-IFN	Non-responders versus no-IFN
Clinical factors						
Gender (male/female)	9/0	22/7	126/31	0.164	0.212	0.590
Age (years)	67 ± 7	66 ± 6	67 ± 7	0.641	0.971	0.378
AST (IU/L)	30 ± 25	50 ± 28	55 ± 28	0.047	0.028	0.786
ALT (IU/L)	32 ± 26	53 ± 33	54 ± 34	0.045	0.034	0.902
Platelet count ($\times 10^4/\mu l$)	14.8 ± 3.3	12.2 ± 3.7	11.8 ± 3.5	0.079	0.029	0.720
Albumin (g/dl)	3.9 ± 0.3	3.7 ± 0.4	3.6 ± 0.4	0.122	0.085	0.782
Total bilirubin (mg/dl)	0.7 ± 0.2	0.7 ± 0.2	0.8 ± 0.3	0.216	0.242	0.757
Prothrombin time (%)	76 ± 8	75 ± 12	75 ± 11	0.894	0.942	0.918
Hepaplastin test (%)	75 ± 11	74 ± 11	73 ± 13	0.872	0.817	0.907
Child-Pugh (A/B)	8/1	25/4	130/27	>0.999	>0.999	0.791
Tumor-related factor						
AFP (ng/ml)	51 ± 112	60 ± 98	81 ± 305	0.983	0.848	0.757
PIVKA-II (mAU/ml)	90 ± 83	258 ± 712	200 ± 696	0.491	0.640	0.744
Latency to recurrence (years)	2.6 ± 2.8	2.0 ± 2.0	2.2 ± 2.1	0.497	0.561	0.707
Recurrence site (intrahepatic/extrahepatic)	8/1	29/0	150/7			
Intrahepatic recurrence (single/multiple)	6/2	11/18	57/93			

Data are expressed as mean ± standard deviation.
IFN, interferon; HCV, hepatic C virus; HBs Ag, hepatitis B surface antigen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CH, chronic hepatitis; LC, liver cirrhosis; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; TAE, transcatheter arterial chemoembolization; Hr, hepatic resection; 0: partial resection; S, subsegmentectomy; 1, one segmentectomy; 2, two segmentectomies.

majority of first recurrence sites were residual liver [responders group: 89% (8/9), non-responders group: 100% (29/29), no-IFN group: 94% (150/157)] (Fig. 4B). In the responders group, among eight patients with intrahepatic recurrence, solitary recurrence was seen in six patients (75.0%). On the other hand, the percentage of solitary intrahepatic recurrence was 37.9% (11/29) in the non-responders group and 38.0% (57/150) in the no-IFN group. In the responders group, surgery, percutaneous therapy, and transarterial chemoembolization (TACE) was selected in three, four, and two patients for treatment of recurrence, respectively (Fig. 4C). The proportion of patients in whom surgery or percutaneous therapy was selected for treatment in the responders group (7/9, 77.8%) was significantly higher than that of the non-responders group (7/29, 24.1%, $P = 0.006$) and the no-IFN group (28/157, 17.8%, $P < 0.001$).

Figure 5 shows the overall survival from diagnosis of the first HCC recurrence in the three groups. The overall survival rate of the

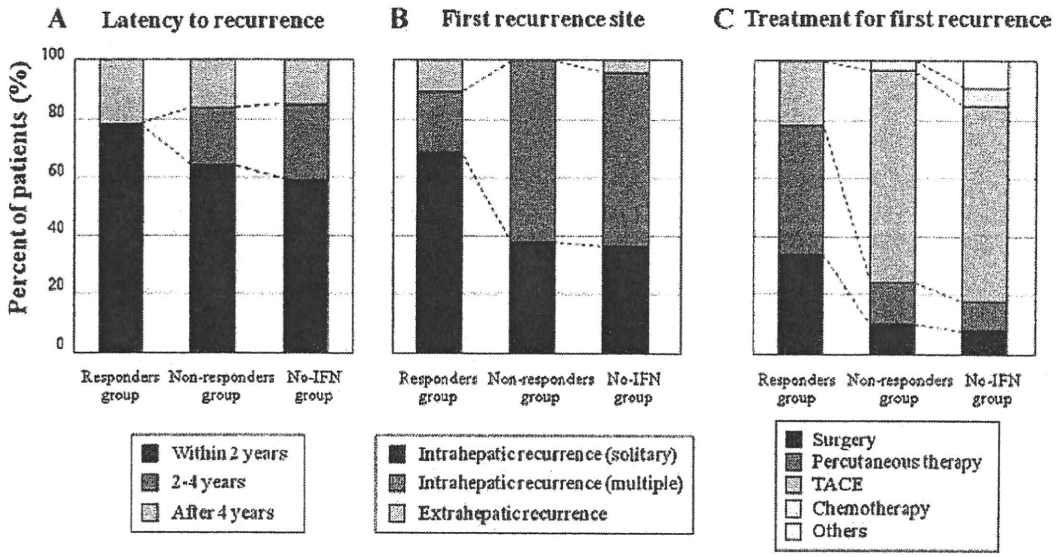

Fig. 4. A: Distribution of the latency from the initial hepatectomy to HCC recurrence for the responders, the non-responders, and the no-IFN group. B: Distribution of the first recurrence site in patients with HCC recurrence of the responders, the non-responders, and the no-IFN group. C: Distribution of selected treatment for first HCC recurrence in the responders, the non-responders, and the no-IFN group. IFN: interferon, TACE: transcatheter arterial chemoembolization.

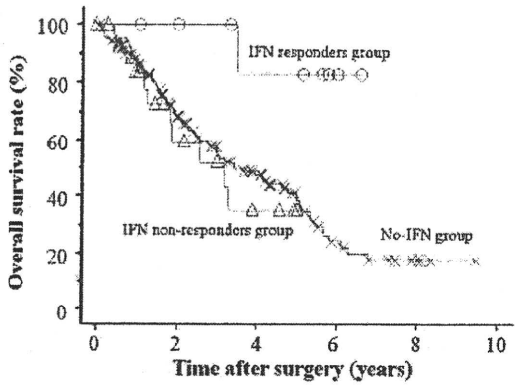

Fig. 5. Overall survival rates calculated from the diagnosis of first HCC recurrence in the responders group, the non-responders group, and the no-IFN group. Open circles: responders (n = 9), open triangles: non-responders (n = 29), crosses: no-IFN group (n = 157). IFN: interferon.

responders group was significantly higher than that of the non-responders group ($P = 0.012$) and that of no-IFN group ($P = 0.011$).

DISCUSSION

The present study demonstrated that a significantly better DFS from the initial hepatectomy in the responders group than the other two groups. This result was similar to that reported previously by Uenishi et al. [16]. Based on the pattern of the DFS curve of the responders group in this study, the recurrence rate appeared to decrease mainly in 2 years later. We have reported that DFS curves for postoperative HCC patients in the early (within 2 years) and late (4 years after surgery) represented both intrahepatic metastasis and multicentric carcinogenesis, respectively [19]. Based on this viewpoint, the decrease in recurrence in the responders group was probably mainly due to the suppression of new multicentric carcinogenesis. A number of investigators have reported that suppression of increased liver inflammation, as assessed by AST and ALT, contributes to inhibition of hepatocarcinogenesis and postoperative intrahepatic recurrence after HCC surgery, which is more likely to originate from multicentric carcinogenesis [20,21]. IFN has been reported also to be effective in eradication of HCV-RNA from the serum and hepatic tissue and prevention of deterioration of liver dysfunction in patients with HCV infection [5–8,10]. It is possible that the suppression of new multicentric carcinogenesis seen in the IFN responders group of this study was due to these effects of IFN therapy. This speculation is supported by the findings of the present study that the levels of aminotransferases and platelet count in the responders group were significantly lower and higher, respectively, than those of the other groups, at the initial hepatectomy and first recurrence, and that the frequency of LC in the responders group was significantly lower than that of the other groups.

On the other hand, IFN has been reported to have anti-tumor effects [22–24]. These anti-tumor effects of IFN had been actually verified also in IFN-alpha/5-fluorouracil combination therapy for advanced HCC in a series of studies by our group [25–32]. Additionally, in a previous report by Uenishi et al. [16], only one patient developed postoperative recurrence about 5 years after the initial surgery among 11 patients of the responders group, and the recurrence pattern of the responders group was also suggestive of the inhibitory effect of IFN on metastasis originating from the primary HCC. Taken together, also in the present study, the decrease of recurrence might be potentially derived from the suppression of intrahepatic metastasis by IFN.

In the present study, overall survival from the initial hepatectomy in the responders group was also significantly better than those of the other two groups. This improvement of overall survival was caused by the aforementioned decrease of HCC recurrence rate in the responders group. In addition, in the responder group, the percentage of patients who underwent selective surgery or percutaneous therapy for the treatment of recurrent HCC was higher than other groups. In general, the treatment for the postoperative HCC recurrence is frequently restricted for the residual liver function, which is one of the reasons for the unfavorable postoperative outcome [3,4]. Considering such restriction of the treatment, the improved liver function by IFN therapy was also speculated to contribute to the better overall survival. Finally, it could be argued that IFN therapy was the main reason for the improvement in both DFS and overall survival rates in the responders group.

To date, several studies examined the impact of IFN therapy after curative loco-regional treatment for HCC [33–37]. For example, in a randomized controlled trial, Ikeda et al. [33] reported that IFN therapy suppressed tumor recurrence after surgery or ethanol injection for HCV-related HCC. Kubo et al. [36] also reported that postoperative IFN therapy significantly decreased recurrence after resection of HCV-related HCC in a randomized controlled trial. That several randomized controlled trials indicated improved posttreamtemt outcome in patients with HCV-related HCC who received postoperative IFN therapy, adds support to our conclusion of the effectiveness of preceding IFN therapy.

Since the present study is retrospective in nature, few details of IFN therapy are unavailable. For example, the duration of HCV-RNA clearance was not clear in several patients treated with IFN. Therefore, in this study, we could not divide patients of the responders group into those with SVR or not. In order to examine more strictly the effectiveness of preceding IFN therapy for surgical outcome, is prospectively designed study is necessary.

CONCLUSIONS

The present study demonstrated the effectiveness of IFN therapy for HCV infection administered before HCC resection as assessed by evaluating the disease-free and overall survival. IFN therapy for HCV might be essential not only for the treatment of HCV infection but also for improvement of prognosis of patients who are susceptible to the development of HCC.

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Combined Transplantation of Pancreatic Islets and Adipose Tissue-Derived Stem Cells Enhances the Survival and Insulin Function of Islet Grafts in Diabetic Mice

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Background. Overcoming significant loss of transplanted islet mass is important for successful islet transplantation. Adipose tissue-derived stem cells (ADSCs) seem to have angiogenic potential and antiinflammatory properties. We hypothesized that the inclusion of ADSCs with islet transplantation should enhance the survival and insulin function of the islet graft.

Methods. Syngeneic ADSCs and allogeneic islets were transplanted simultaneously under the kidney capsules of diabetic C57BL/6J mice. Rejection of the graft was examined by measurement of blood glucose level. Revascularization and inflammatory cell infiltration were examined by immunohistochemistry.

Results. Transplantation of 400 islets only achieved normoglycemia with graft survival of 13.6±1.67 days (mean±standard deviation), whereas that of 100 or 200 allogeneic islets never reversed diabetes. Transplantation of 200 islets with 2×10^5 ADSCs reversed diabetes and significantly prolonged graft survival (13.0±5.48 days). Results of glucose tolerance tests performed on day 7 were significantly better in islets-ADSCs than islets-alone recipients. Immunohistochemical analysis confirmed the presence of insulin-stained islet grafts with well-preserved structure in islets-ADSCs transplant group. Significant revascularization (larger number of von Willebrand factor-positive cells) and marked inhibition of inflammatory cell infiltration, including $CD4^+$ and $CD8^+$ T cells and macrophages, were noted in the islets-ADSCs transplant group than islets-alone transplant group.

Conclusions. Our results indicated that cotransplantation of ADSCs with islet graft promoted survival and insulin function of the graft and reduced the islet mass required for reversal of diabetes. This innovative protocol may allow "one donor to one recipient" islet transplantation.

Keywords: Type 1 diabetes, Islet transplantation, ADSCs, Revascularization.

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Pancreatic islet transplantation is a promising new treatment for patients with type 1 diabetes mellitus (*1*, *2*). However, this has been hindered by the inability to obtain a sufficient number of islets from a single donor pancreas (*3*). Sufficient numbers of islets derived from two or more donor organs are usually required to achieve insulin independence, because a substantial number of transplanted islets fail to engraft into the recipient liver because of apoptosis, inflammation, and ischemia (*4*). Native islets are richly supplied with blood; however, the procedure used to isolate the islets results in the destruction of various cellular and noncellular components, such as the vasculature, which are probably important for islet survival, and full revascularization of the transplanted islet graft takes sev-

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Y.O. designed the study, conducted experiments, performed data analysis, and drafted the article; M.T. provided the study design and the working hypothesis and completed the article; N.K. performed the immunohistochemical studies; T.M., T.T., and T.D. assisted in selecting the methods for islet transplantation, ADSCs culture, and FACS analysis; H.W., S.K., S.M., H.E., and Y.T. provided scientific advice; N.M. provided advice on immunohistochemical analysis; and T.I., H.N., Y.D., and M.M. provided guidance throughout the study.

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eral weeks (5, 6). Furthermore, islet transplantation exposes cells to a variety of stressful stimuli, notably ischemia and proinflammatory cytokines that enhance β-cell death and lead to graft failure (7). Therefore, new strategies must be developed to maintain the high quality of transplanted islets and prevent β-cell death. Moreover, the shortage of available donor organs spurs research into alternative means of generating β-cells from stem cells, including embryonic stem cells (8), adult stem cells (9), bone marrow-derived mesenchymal stem cells (BMSCs) (10, 11), and adipose tissue-derived stem cells (ADSCs) (12).

The ADSCs are usually isolated from the stromal vascular fraction of adipose tissues and bear a strong resemblance to BMSCs (13). However, unlike BMSCs, ADSCs can be obtained in large quantities at low risk. In addition to being more abundant and easily accessible, the adipose tissue yields far more stem cells than bone marrow on a per gram basis (5000 vs. 100–1000) (14). Therefore, it is conceivable that ADSCs may become the preferred choice of ADSCs in future clinical replacement of β-cells. ADSCs seem to have a great regenerative angiogenic potential because of their ability to differentiate into endothelial lineages and secrete angiogenic and antiapoptotic factors (15, 16), probably when both islet grafts and ADSCs, designated here "hybrid islet transplant," are transplanted simultaneously. Moreover, few ADSCs sometimes differentiate into spontaneous insulin-producing cells as demonstrated in an in vivo hybrid islet transplant model (17–19). ADSCs also share some of the immunomodulatory properties of BMSCs, including inhibition of T-cell proliferation, β-cell function, and dendritic cell maturation (20–22). Thus, the hybrid islet transplant-ADSCs can potentially inhibit the inflammatory response against transplanted islets and promote islet graft survival. These properties may allow the transplantation of a smaller number of islets for the treatment of type 1 diabetes. This study was designed to determine the effects of hybrid islet transplantation with ADSCs on the survival and function of allogeneic islet graft in diabetic mice.

RESULTS

Morphology and Cell Surface Expression Profile of ADSCs

The isolated ADSCs from donor animals expanded rapidly in the culture medium. Although a few viable ADSCs were detected at culture day 1 (Fig. 1A), the viable cells, representing fibroblast-like ADSCs, were spindle-like

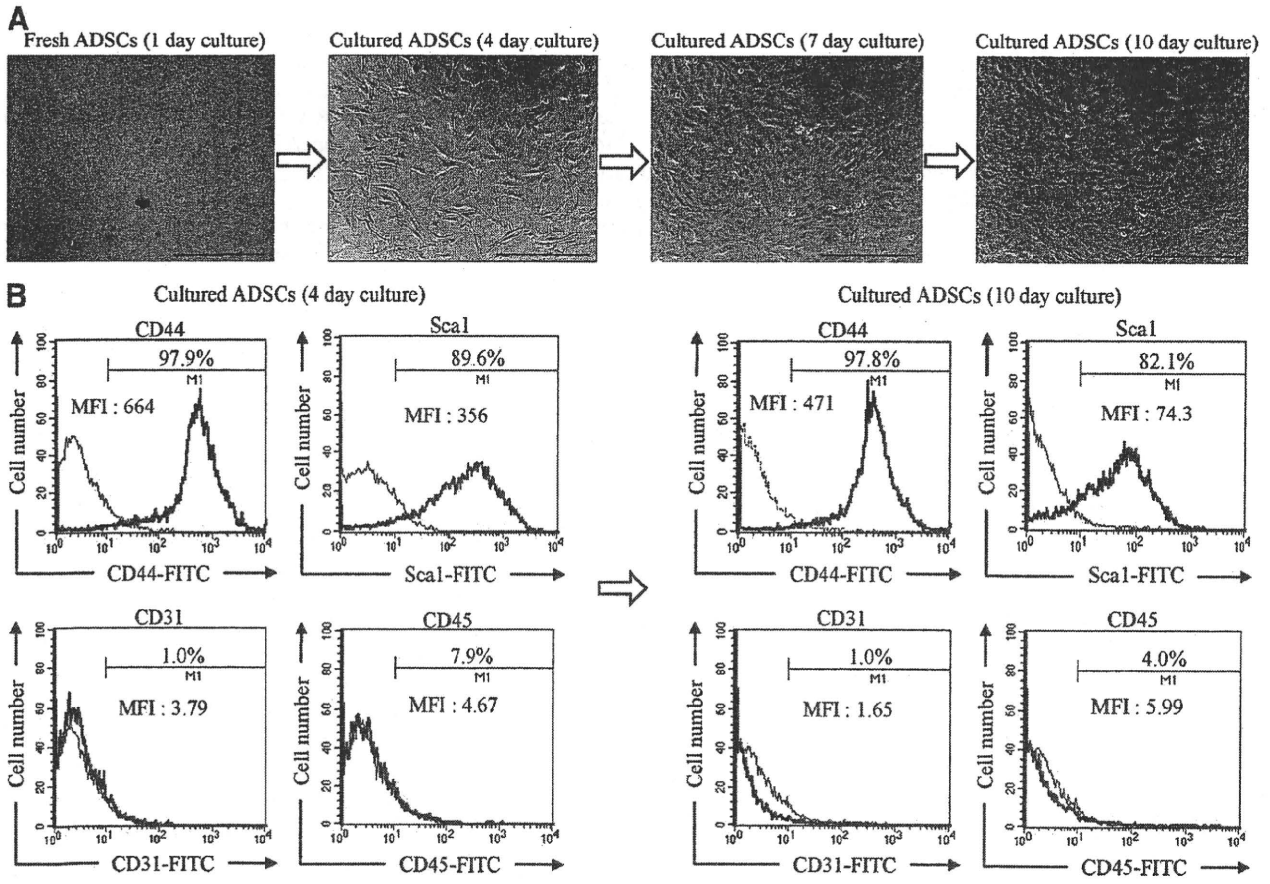

FIGURE 1. Morphologic changes and expression of cell surface antigens on adipose tissue-derived stem cells (ADSCs). (A) The presence of only a few spindle-like viable ADSCs at culture day 1. However, their number gradually increased with uniform size and shape until approximately culture day 10. Calibration bars: 100 μm. (B) The expression levels of CD44 and Sca1 were persistently high, whereas that of Sca1 at day 10 was slightly lower than at day 4. In contrast, the expression of both CD31 and CD45 remained consistently low. Each flow cytometry analysis was measured in triplicate, and representative data are shown. FITC, fluorescein isothiocyanate; MFI, mean fluorescence intensity.

A

ITx Groups	Islets (counts)	ADSCs (cells)	Graft survival (n=5) (days)
A : Simple ITx	100	—	0, 0, 0, 0, 0
B : Simple ITx	200	—	1, 1, 1, 1, 2
C : Simple ITx	400	—	11, 13, 14, 15, 15
D : Hybrid ITx	100	1×10^5	0, 0, 0, 0, 0
E : Hybrid ITx	200	1×10^5	6, 7, 7, 8, 10
F : Hybrid ITx	200	2×10^5	8, 10, 11, 14, 22
G : Hybrid ITx	200	4×10^5	7, 10, 12, 12, 22
H : Separated ITx	200	2×10^5	1, 1, 1, 2, 2
I : ADSCs alone Tx	—	2×10^5	0, 0, 0, 0, 0

B

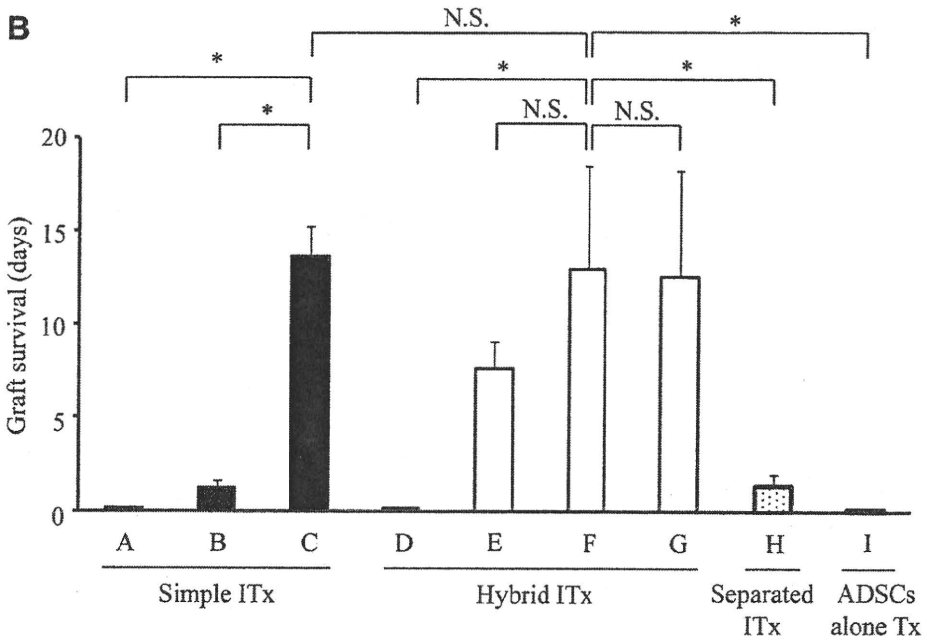

FIGURE 2. Hybrid islet transplantation (ITx) with marginal islet mass and syngeneic adipose tissue-derived stem cells (ADSCs) promotes long-term islet graft survival and sustained normoglycemia. (A) Data are graft survival of five mice per group. (B) The graft survival was significantly longer in group F than group B ($P<0.01$). The graft survival tended to be longer in group F than group E ($P=0.066$). *P less than 0.01.

cells that grew rapidly by day 4. The number of viable ADSCs gradually increased with continued culture, and ADSCs with uniform size and shape were noted up to day 10 (Fig. 1A). These viable cells were used for hybrid islet transplantation.

To assess the changes in cell surface marker profiles of ADSCs, fluorescence-activated cell sorter analysis of ADSCs was performed at days 4 and 10 (Fig. 1B). The expression level of CD44 was persistently high throughout the culture period, whereas that of Sca1 at day 10 was slightly suppressed compared with that at day 4 (Fig. 1B). Flow cytometry showed that 95.4% to 97.8% of cultured ADSCs expressed CD44, 76.4% to 82.1% expressed Sca1 at day 10, and the percentages of CD44- and Sca1-positive cells were similar at days 4 and 10. In contrast, the expression of both CD31 and CD45 remained low throughout the culture period. Taken together, the profiles of cell surface markers on cultured ADSCs at day 10, which were used for hybrid islet transplantation, were identical to those at day 4.

Hybrid Islet Transplantation With Marginal Islet Mass and Syngeneic ADSCs Promotes Long-Term Islet Graft Survival and Sustained Normoglycemia

First, we assessed the outcome of allogeneic islet transplantation using a marginal mass of ADSCs. For this purpose, the animals were divided into nine groups (n=5/group, Fig. 2A) to receive allogeneic islets or syngeneic ADSCs. Allogeneic islet grafts were transplanted under the kidney capsule as described in the *Materials and Methods*. Simple transplantation of 400 allogeneic islets was associated with graft survival of 13.6±1.67 days (Fig. 2A and B) and induction of sustained normoglycemia in islet-recipient mice (Fig. 3A). Interestingly, neither grafting of 200 nor 100 allogeneic islets promoted islets graft survival (200 islets: 1.20±0.45 days; $P<0.01$, group A or B vs. group C), and especially, islet graft of 100 islets never induced normoglycemia (Figs. 2A and B and 3A). Importantly, hy-

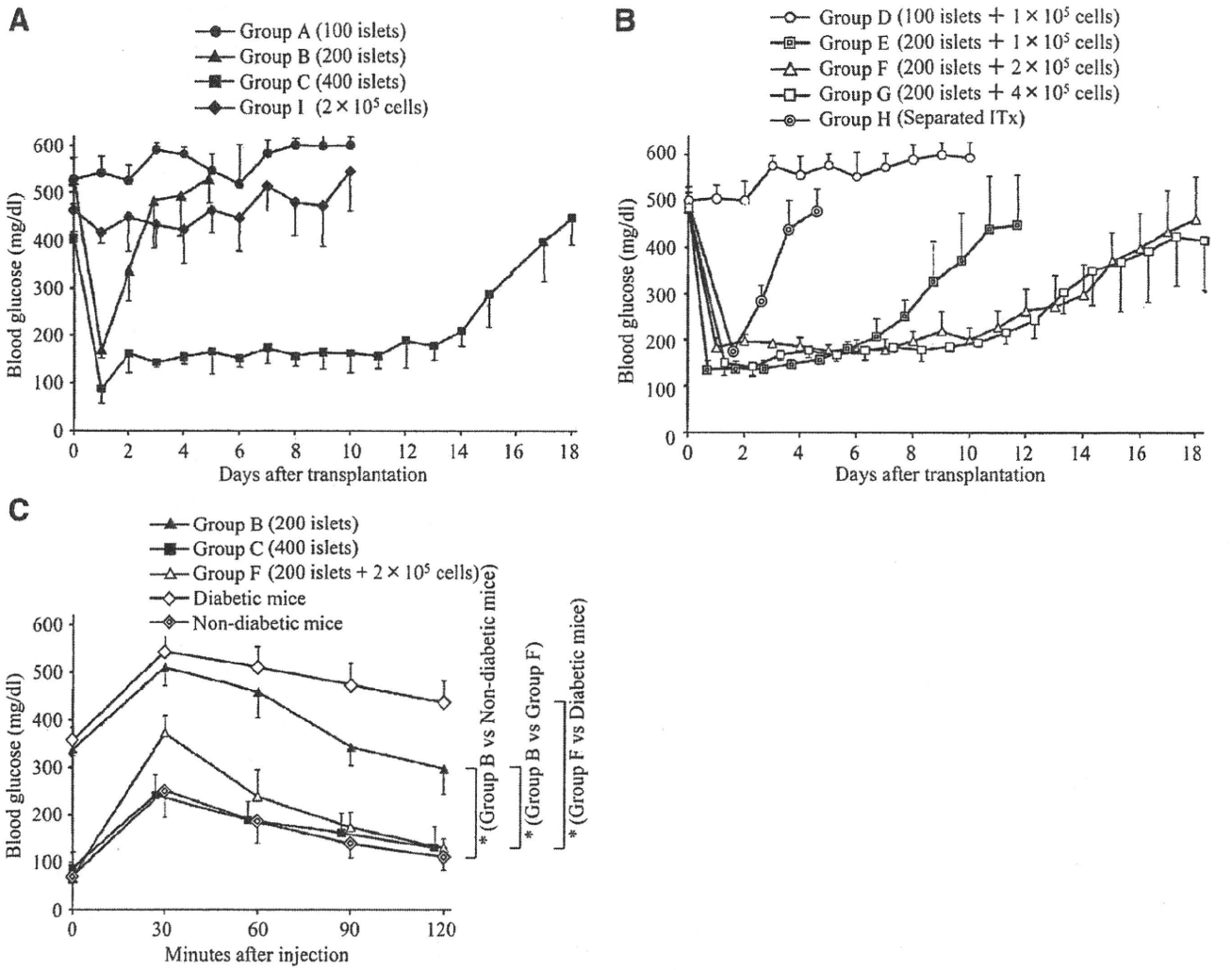

FIGURE 3. Nonfasting blood glucose level after transplantation and glucose tolerance test at day 10. (A) Data are presented as mean±standard deviation of five mice in group A to C and adipose tissue-derived stem cells only transplantation group. (B) Survival of grafted islets was prolonged in groups F and G but markedly reduced in group E. (C) There were no significant differences in blood glucose levels at 120 min between mice of groups C and F. A similar pattern of blood glucose level was observed in nondiabetic mice. *P less than 0.01.

brid transplantation of 200 islets combined with 2×10^5 cells of syngeneic ADSCs elicited significant prolongation of graft survival, compared with 200 islets of islet transplantation alone (13.0±5.48 days, group F vs. group B, $P<0.01$). The graft survival of hybrid islet transplant alone was similar to that of 400 islets of simple islet transplantation, representing the critical number for amelioration of diabetes (Figs. 2A and B and 3A and B).

Various conditions were also tested to determine the optimum condition for hybrid islet transplantation. In group E, transplantation of a marginal islet mass (200 islets) with 1×10^5 cells of syngeneic ADSCs, representing half the number of group F, tended to be associated with shorter islet graft survival (7.6±1.52 days, $P=0.066$, group E vs. group F; Figs. 2A and B and 3B). In contrast, no significant prolongation of graft survival was observed in hybrid islet transplantation with 200 islets and 4×10^5 cells of syngeneic ADSCs, that is, twofold that of group F (12.6±5.64 days, $P=0.912$, Figs. 2A and B and 3B). These findings indicate that the optimum condition of hybrid islet transplantation associated with the longest graft survival is the combined transplantation of 200 islets and 2×10^5 ADSCs, which are equal pellet volume of each cells. Transplantation of islets and ADSCs separately in each kidney was each ineffective in term of islet graft survival (Figs. 2A and B and 3B). In addition, transplantation of 2×10^5 cells of ADSCs alone never induced normoglycemia (Figs. 2A and B and 3A).

To examine islet graft function in vivo, intraperitoneal glucose tolerance test was performed at day 7 after transplantation. In nondiabetic mice, injection of glucose induced hyperglycemia with the peak recorded at 30 min later, but blood glucose level returned to normal at 120 min after the injection (Fig. 3C). A similar pattern was observed in mice transplanted with 400 islet graft. Conversely, blood glucose levels of mice transplanted with 200 islets or untreated diabetic mice were significantly higher than those of control nondiabetic mice before injection and at 30, 60, 90, and 120 min after injection of glucose. The blood glucose level of mice hybrid trans-

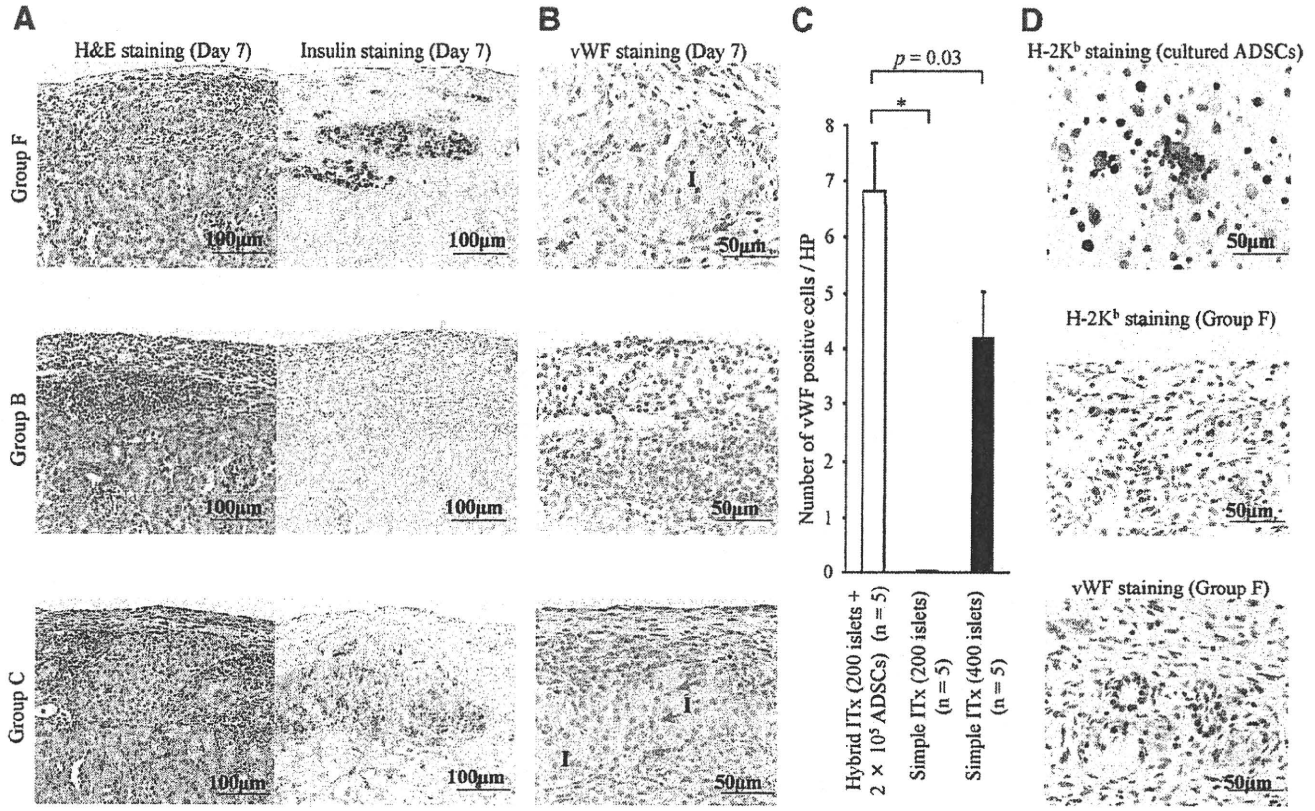

FIGURE 4. Hybrid transplantation of adipose tissue-derived stem cells (ADSCs) promotes revascularization of transplanted islet grafts. Each transplantation experiment was performed five times, and representative data are shown. (A) Survival of islets grafted under the kidney capsule was evident in group F, and only few inflammatory cells were detected around the grafts in this group. Calibration bars: 100 μm. (B) Revascularization in groups F and C. Calibration bars: 50 μm; I, islet. (C) The number of von Willebrand Factor (vWF)-stained cells was significantly higher in group F than groups B and C. ITx, islet transplantation (D) Staining with anti-mouse H-2K^b monoclonal antibody (mAb) and anti-vWF mAb. Cultured ADSCs were H-2K^b positive, and cells costained for H-2K^b and vWF were detected in group F. Calibration bars: 50 μm.

planted with 200 islets and 2×10^5 cells of ADSCs were significantly lower than those of untreated diabetic mice, and the pattern of blood glucose after injection in hybrid transplanted mice was similar to those of control nondiabetic mice and mice transplanted with 400 islets (Fig. 3C).

Hybrid Transplantation of ADSCs Promotes Revascularization of Transplanted Islet Grafts

Immunohistochemical staining for insulin demonstrated that hybrid transplantation of islets and ADSCs prolonged islet graft survival. No islet grafts were detected when the 200 islets-alone graft was rejected (i.e., group B), whereas insulin-positive islets grafts with well-preserved islet structure were found in the islets-ADSCs hybrid transplant (Fig. 4A).

Moreover, these grafts were surrounded by von Willebrand factor (vWF)-stained endothelial cells. The vWF-positive cell count, a marker of graft revascularization, was significantly higher in islets-ADSCs hybrid grafts than in 200 and 400 islets-alone grafts (Fig. 4B and C). These findings indicate that ADSCs promote islet revascularization. Moreover, to clarify the origin of vascular components in the grafted islets, the sections were immunostained with anti-vWF monoclonal antibody (mAb) or anti-mouse H-2K^b mAb. Cultured ADSCs stained strongly with anti-mouse H-2K^b mAb (Fig. 4D). In each section, approximately half of the cells were positive for both vWF and H-2K^b, suggesting that the double-positive cells, which are differentiated into endothelial cells, originated from the grafted ADSCs.

Next, transdifferentiation or changes in ADSCs was examined by Sca1 staining. Sca1-stained cells were found at posttransplant day 1 but not at days 4 or 7. These findings suggest that transplanted ADSCs start to change immediately after transplantation.

Hybrid Transplantation of ADSCs Inhibits Inflammatory Response Against Transplanted Islet Grafts

Finally, we assessed the antiinflammatory properties of ADSCs, the phenotype of infiltrated lymphocytes, including CD4$^+$ and CD8$^+$ T cells and macrophages. A large number of inflammatory cells were found around the transplanted islet grafts in 200 and 400 islets-alone grafts at posttransplant day 7 (Fig. 4A, hematoxylin-eosin staining). In comparison, fewer inflammatory cells were detected around the grafts in islets-ADSCs hybrid grafts (Fig. 4A). Only a few CD4$^+$/CD8$^+$ T cells and CD68$^+$ macrophages were detected in the islets-ADSCs hybrid grafts, compared with large numbers of CD4$^+$ T cells and CD68$^+$ macrophages in the islets-alone grafts (Fig. 5B). These results suggest that prolongation of islet graft sur-

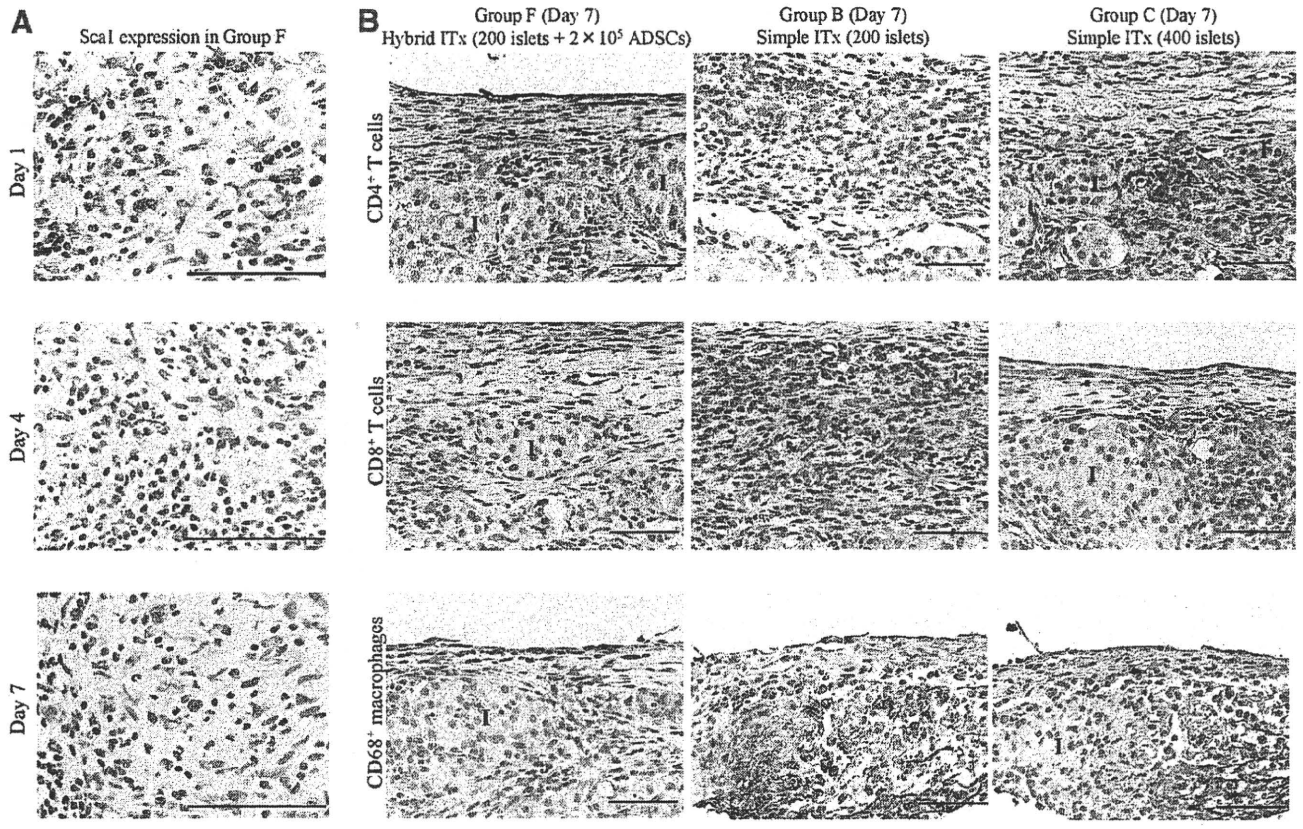

FIGURE 5. Hybrid transplantation of adipose tissue-derived stem cells (ADSCs) suppresses inflammatory response toward transplanted islet grafts. Each experiment was performed five times, and representative data are shown. (A) Sca1-stained cells appeared at posttransplantation day 1, but fewer at day 4, and disappeared at day 7. (B) Few $CD4^+$ and $CD8^+$ T cells and $CD68^+$ macrophages were present in islets-ADSCs hybrid grafts. Calibration bars: 50 μm; I, islet. ITx, islet transplantation.

vival in islets-ADSCs hybrid grafts is because of the antiinflammatory properties of ADSCs.

DISCUSSION

We demonstrated in this study, ADSCs mediated improvement of islet engraftment and function. Specifically, 200 allogeneic islets plus 2×10^5 cells of ADSCs implanted under the kidney capsule resulted in the disappearance of diabetes in our mice, whereas none of the recipient mice with islet transplantation alone showed normoglycemia. Moreover, 200 islet cotransplanted with 1×10^5 cells of ADSCs (representing 50% of the above dose) elicited normoglycemia, although islet graft survival was markedly shorter (Figs. 2A and B and 3B). Thus, the use of ADSCs adds value to islet engraftment. Further studies are needed to confirm the clinical effectiveness of allogeneic islets-syngeneic ADSCs hybrid transplantation in islets transplantation, because this could have an impact on the number of transplanted islets and hence allowing "one donor to one recipient or to multirecipients."

We also explored the potential uses of ADSCs. Our experiments on revascularization showed abundant vWF-positive cells in the vicinity of the islet graft under the kidney capsule after hybrid transplantation of islets grafts-ADSCs, compared with only a few such cells in islets transplantation alone (Fig. 4B) and none when ADSCs only were transplanted (data not shown) or even when these cells were transplanted separately from the islets graft under the kidney capsule (Fig. 2A and B). These results indicate that cotransplantation of ADSCs in areas close to the graft is essential for revascularization and functionality of the grafted tissue. Although this study did not investigate the exact mechanism of the effects of ADSCs on graft survival, it is possible that these effects involve, at least in part, the induction of revascularization in a paracrine manner. In support of this notion, ADSCs are known to secrete high levels of various angiogenic factors such as vascular endothelial growth factor, transforming growth factor-β, and basic fibroblast growth factor (*23*), which together can induce the growth and differentiation of endothelial cells. It is also possible that the increased graft tissue blood flow associated with islet grafts-ADSCs implantation/revascularization rescues or activates silent/inactivated stem cells derived from ADSCs, which then divide and differentiate into insulin-producing cells. To confirm such scenario and the long-term therapeutic effects of ADSCs in islet transplantation and any potential side effects, further studies are needed, including the use of syngeneic islet transplant model. Furthermore, the safety of ADSCs with regard to the possible growth of teratomas also needs to be verified through long-term follow-up studies, although these ADSCs have already been used in the treatment of other conditions such as cardiac infarction (*24*), breast reconstruction (*25*), and anal fistula associated with Crohn's disease (*26*).

The enhanced survival of islet graft in mice coimplanted with ADSCs could also be due to the immunomodulatory properties of ADSCs, especially the inhibition of any immune response against islet grafts. Several studies have reported that murine ADSCs down-regulate systemic and local inflammatory processes in various models of inflammatory conditions, such as arthritis, encephalomyelitis, graft-versus-host disease, and Crohn's disease (*27–30*). Although the specific molecular mechanisms involved in such process are not fully understood, the reported data (*24–30*) suggest the involvement of both cell-to-cell contact and soluble factors in the deactivation of macrophages and T cells by ADSCs. Especially, prostaglandin-E2 and interleukin-10 seem to play a critical role in this immunomodulatory effect (*31*). For example, prostaglandin-E2 inhibits allogeneic lymphocyte reaction in a cell-to-cell contact in independent manner (*32*). However, our results demonstrated that the separate transplantation of ADSCs had no beneficial effects on islets survival, thus arguing for the important role of cell-to-cell contact between islets and ADSCs in the beneficial immunomodulatory effect of the latter on islet transplantation.

For the clinical application to human islet transplantation, we plan to use autologous ADSCs obtained from the abdomen of the islet recipient. These ADSCs are isolated over several hours by the Cytori's proprietary cell processing device, the Celution system (Cytori Therapeutics, San Diego, CA) (*33*). Isolation of ADSCs is conducted simultaneously with that of islets from the donor pancreas.

In conclusion, hybrid transplantation of islets with ADSCs significantly reduced the number of islets required for transplantation, improved graft function, and cure diabetes by promoting graft revascularization. Further studies are required to determine whether similar outcome can be obtained by transplantation of islets combined with infusion of ADSCs into the portal vein.

MATERIALS AND METHODS

Islet Isolation

Donor male BALB/cA mice, 10- to 12-week old, weighing 20 to 30 g, were purchased from CLEA Japan, Inc. (Tokyo, Japan). All experiments were approved by the institutional ethics committee of Osaka University Medical School. The mice were anesthetized by intraperitoneal injection of tribromoethanol (Sigma-Aldrich, St. Louis, MO) and then underwent bile duct cannulation with pancreatic inflation after clamping the distal common bile duct using 3 mL of extracellular type trehalose containing Kyoto solution (ET-Kyoto, Otsuka Pharmaceutical, Tokyo), which contained 1 mg/mL of collagenase VIII (Sigma-Aldrich). The pancreas was excised; cleaned off lymph nodes, fat, and bile duct; and digested with collagenase VIII (1 mg/mL), without shaking, at 37°C for 15 min. The digested pancreas was washed by centrifugation (270*g* for 2 min at 4°C) and then purified with a discontinuous density gradient (1.111, 1.104, 1.097, and 1.072 g/mL) in a modified ET-Kyoto/OptiPrep (Axis-Shield, Oslo, Norway) solution. Washes after purification were performed at 1000*g* for 20 min (*34*).

Isolation and Culture of ADSCs

Mouse ADSCs were isolated as described previously (*35, 36*). Briefly, 12-week-old male C57BL/6J donor mice were purchased from CLEA Japan. Adipose tissue was obtained from inguinal fat pad and cut into fine pieces then placed in antibiotic-antimycotic-containing phosphate-buffered saline (PBS) (Sigma-Aldrich) and then in Dulbecco's modified Eagle medium (Sigma-Aldrich) containing 1 mg/mL collagenase II (Sigma-Aldrich) and antibiotic/antimycotic agents, at 37°C for 60 min with gentle agitation. The digested tissue was filtered through sterile 70-μm nylon mesh, centrifuged at 430*g* for 5 min, and resuspended. This process was repeated twice. ADSCs were seeded onto culture dishes with Dulbecco's modified Eagle complete medium containing 10% fetal bovine serum.

Flow Cytometry Analysis

Isolated ADSCs were treated with fluorescein isothiocyanate-labeled anti-CD44, -Sca1, -CD45, and -CD31 antibodies (BD Biosciences, Franklin Lakes, NJ). Cells were first stained with each antibody and then analyzed for antigen expression level by fluorescence-activated cell sorter Calibur flow cytometry (BD Immunocytometry, San Jose, CA). Triplicate measurements were performed for each antigen.

Islet Transplantation

The recipient 8- to 10-week old male C57BL/6J mice were divided at random into nine experimental groups (Fig. 2A, n=5/group) to receive allogeneic islets or syngeneic ADSCs. Seven days before transplantation, diabetes was induced in the recipient mice by a single 180 mg/kg intraperitoneal injection of streptozotocin (Nacalai tesque, Kyoto, Japan). Diabetes was defined as blood glucose level of more than 400 mg/dL detected twice consecutively after streptozotocin injection. The diabetic mice were anesthetized with tribromoethanol, and a 2-cm incision was made through the skin and muscle of the right flank to approach the right kidney. A small lateral cut was made in the kidney capsule, and then allogeneic islets and syngeneic ADSCs were dispensed beneath the capsule. In mice of groups A to C (simple islet transplantation), a pellet of 100, 200, or 400 islets was implanted under the right kidney capsule. In mice of groups D to G (hybrid islet transplantation), a pellet of marginal mass of islet was mixed with 1×10^5, 2×10^5, or 4×10^5 cells of syngeneic ADSCs and implanted under the right kidney capsule. In mice of group H (separate islet transplantation), 200 islets and 2×10^5 cells of ADSCs were implanted under the capsule of the right and left kidneys, respectively. In mice of group I (ADSCs alone transplantation), 2×10^5 cells of ADSCs alone were implanted under the right kidney capsule. After transplantation, nonfasting blood glucose level was monitored daily using samples obtained from the tail vein. Islet graft rejection was defined when two consecutive blood glucose levels exceeded 250 mg/dL after transplantation. At 1 week posttransplantation, the grafted kidneys were dissected out to assess revascularization or the immune response to islet grafts by immunohistochemistry.

Glucose Tolerance Test

Intraperitoneal glucose tolerance test was performed to determine the effect of islet transplantation on diabetes, using the method described previously (*37*). Mice were fasted for 6 hr and then injected intraperitoneally with 2 g glucose in saline/kg body weight. Blood glucose levels were measured for 2 hr at 30 min intervals.

Immunohistochemical Analysis

The engrafted kidneys were excised, fixed in formalin, and embedded in paraffin. Tissue sections (2-μm thick) were placed in 0.3% H_2O_2/methanol to quench endogenous peroxidase activity and incubated with 5% bovine serum albumin-PBS to block nonspecific reaction. The slides were incubated with rabbit antiinsulin polyclonal antibody (pAb, Santa Cruz Biotechnology, Santa Cruz, CA) to detect the transplanted islets. Sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Bethyl Laboratories Inc., Montgomery, TX), and then immunostaining was visualized with 0.02% diaminobenzidine (DAB, Sigma-Aldrich) as the chromogen. After washing, the sections were counterstained with hematoxylin. Control tissue sections were prepared in a similar fashion except no primary antibody was used.

To identify endothelial cells that differentiate from ADSCs, cells were stained with goat anti-vWF pAb (Millipore, Bedford, MA). After incubation with HRP-conjugated secondary antibody, immunostaining was developed with DAB as described earlier. The number of vWF-stained endothelial cells was counted by VH-analyzer software (Keyence, Woodcliff Lake, NJ). To further identify the phenotype of the infiltrated lymphocytes in allogeneic

islet grafts, cells were stained with the following mAbs: rat anti-mouse CD4 (RELIA Tech, Braunschweig, Germany) for $CD4^+$ T cells; rat anti-mouse CD8 (BioLegend, San Diego, CA) for $CD8^+$ T cells; and rat anti-mouse CD68 (Cedarlane, Burlington, ON, Canada) for $CD68^+$ macrophages. After incubation with HRP-conjugated secondary antibody, immunostaining was developed with DAB.

Kidney specimens were excised, embedded in optimum cutting temperature compound (Sakura Finetechnical Co., Tokyo), cut (7-μm thick) with a cryostat, treated with 0.3% H_2O_2/methanol to quench endogenous peroxidase activity, blocked with 5% bovine serum albumin-PBS, and incubated with rat anti-mouse Sca1 pAb (R&D System Inc., Minneapolis, MN) to detect the grafted ADSCs. The sections were then incubated with HRP-conjugated secondary antibody, and immunostaining was visualized with DAB.

To determine the origin of the vascular component in the grafted islets, frozen sections were incubated with both anti-vWF mAb and anti-mouse $H\text{-}2K^b$ mAb (BD Pharmingen, San Jose, CA). After incubation with the respective HRP-conjugated secondary antibody, immunostaining was developed with DAB.

Statistical Analysis

Data were expressed as mean±standard deviation. Differences between two groups were examined for significance using the Student's *t* test. Differences were considered significant at p less than 0.05.

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生体ドナーに必要な術前画像診断*

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Key Word 肝移植提供者 肝切除 術前US MD-CT

要旨

肝移植提供者に対する肝切除術における術前に必要な画像診断とその意義・有用性について概説する．術前画像診断としては，肝USに加えて全例にMD-CTを施行し，ドナー残肝容積率が35%以上，レシピエントのSLVの40%以上になるようなグラフトを選択する．また，肝動脈，門脈，肝静脈などの脈管系についてその構築を十分に把握することが重要である．さらに，DIC-CTによる胆管の分岐・走行や，さらには切離予定線まで術前に診断することが，安全な肝提供者手術(肝切除)においては肝要である．

肝胆膵画像 2010；12：25-31

はじめに

肝臓移植は，代替治療法のない末期肝不全患者にとって，現時点において唯一無二の治療法である．しかしながら本邦では，例えば脳死判定の問題などを含めた，本邦独自のいわゆる「倫理的」な問題点に加えて，また，欧米との「死後の価値観」の相違，臓器提供施設の制限などの種々の問題点がいまだ山積している．本年(2009年)7月に改正臓器移植法が衆・参両院で可決され成立し，来年7月には施行される予定であり，脳死肝移植に一筋の光が見えたとはいえ，いまだ現状では生体部分肝移植がその主流を占める．

この生体部分肝移植においては，健常者である移植肝提供者(以下，ドナー)の存在が不可欠である．そして生体部分肝移植手術においては何よりも，健常者である移植肝提供者の安全が最も優先されることはいうまでもない．その一方で肝臓の周辺には，下大静脈などの大血管が存在し，肝門部には肝動脈，門脈，胆管などの主要な脈管が存在し，その損傷の程度によっては術後の重篤な合併症を誘発する可能性も高い．また，正確な肝切除率の算定も重要なポイントの1つとなる．したがって，ドナー手術を安全に施行するためには，必要十分な肝容量の算出と肝内の脈管系の解剖の十分な把握など，術前，術中，術後の画像診断が極めて重要で，不可欠である．

本稿においては，生体部分肝移植ドナー手術における，術前評価において不可欠な画像診断とその意義について概説する．

術前画像診断(表1)

術前に必要な画像診断として，移植肝・切除後残肝の容量の算出と，肝動脈，門脈，肝静脈と胆管の

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* The pre-operative diagnostic imaging for the living-donor hepatic surgery

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