inflammatory protein 1) family, indicating an acute inflammatory host response at sites of injury mainly by recruiting pro-inflammatory cells, such as T-cell, NK cell, neutrophil and eosinophil.[22] Tumor necrosis factor alpha (TNFα) and interferon gamma were well known as one of the proinflammatory cytokines, also demonstrating to play a critical role in the liver injury. They formed a pro-inflammatory cytokine network in this study. Moreover, Cxcl2 (MIP-2) which was induced by TNFα was involved in the recruitment of neutrophils in the development of massive hepatocellular apoptosis and necrosis.[23] These findings implied the gene expressions in the spleen had a negative impact on the remnant liver after Hx in terms of inducing inflammatory response.

Second, "transcription factors," containing one or more DNA-binding domains, which are attached to specific sequences of DNA adjacent to the genes that they regulated including EGR1, EGR2, FOS, JUNB, and ATF3, were upregulated. JUNB and FOS were reported as transcription factors on oxidative stress pathways.[24] They were primary immediate-early genes expressed by many cell types in response to cellular stress, and DNA binding of their heterodimer was modulated by reduction-oxidation of a single conserved cysteine residue in the DNA-binding domains of the two proteins.[25] EGR1 was one of the zinc-finger transcription factors and upregulated at 3 h and 6 h in the current study. It was reported as a master switch coordinating upregulation of divergent gene families related to liver ischemia-reperfusion injury including IL-1β, Cxcl2 (MIP-2), tissue factor, and plasminogen-activating inhibitor 1.[26] It was also reported that EGR1 had crucial roles expressed in small-for-size grafts, which had a small remnant of the liver after liver transplantation and was similar to massive Hx.[27]

We concluded that in the spleen numerous gene expressions would occur under the massive Hx, which was similar to the small-for-size graft. Based on these results, the spleen could take a harmful role and provide a negative impact in this situation due to inducing chemokine and transcription factor including GRO1 and EGR1. However, the precise mechanism is not clear. We did not measure the level of cytokine and transcription factor in the portal flow. In this microarray analysis, the samples in each group were mixed together, therefore; we could not put SD. So, we should interpret these data very carefully. As the number of samples in this study was also relatively small, a large sample size is needed in the further study.

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ORIGINAL ARTICLE

Homing effect of adipose-derived stem cells to the injured liver: the shift of stromal cell-derived factor 1 expressions

Yu Saito · Mitsuo Shimada · Tohru Utsunomiya · Tetsuya Ikemoto · Shinichiro Yamada · Yuji Morine · Satoru Imura · Hiroki Mori · Yusuke Arakawa · Mami Kanamoto · Shuichi Iwahashi · Chie Takasu

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Abstract
Background Whether systemically transplanted human adipose-derived stem cells (ADSCs) homed to the injured liver in nude mice under stress with subsequent hepatectomy (Hx) and ischemia-reperfusion (I/R) was investigated in the present study. The types of cells in the liver that were involved in the homing of ADSCs were clarified, with focus on the stromal-derived factor-1 (SDF-1)/C-X-C chemokine receptor type 4 (CXCR-4) axis.
Methods Adipose-derived stem cells were transplanted intravenously immediately after 70% Hx and I/R. ADSCs were traced by in vivo imaging for 24 h after transplantation and ADSCs were histologically detected in the liver. SDF-1 and CXCR-4 expressions in the liver were evaluated by real time RT-PCR. The immunohistochemical analysis of SDF-1 was also performed to identify SDF-1 expressing cells in the liver.
Results Adipose-derived stem cells were found in various organs immediately following transplantation and almost accumulated in remnant liver or spleen at 6 h after transplantation. ADSCs were also histologically revealed in the harvested liver. Hx and I/R injury significantly enhanced SDF-1 expressions regardless of ADSCs transplantation, and only ADSC transplantation increased CXCR-4 expressions. The predominant SDF-1 positive cells in the liver were equally identified in parenchymal and non-parenchymal cells at 6 h, but shifted to non-parenchymal cells at 24 h after transplantation.
Conclusions Systemically transplanted ADSCs homed to the injured liver after transplantation, possibly based on the mechanisms of SDF-1/CXCR-4 axis. Therefore, systemic transplantation might be an effective and practical route for the transplantation of ADSCs.

Keywords Adipose derived stem cells · C-X-C chemokine receptor type 4 · Hepatic ischemia-reperfusion · Homing · Stromal derived factor-1

Y. Saito (✉) · M. Shimada · T. Utsunomiya · T. Ikemoto · S. Yamada · Y. Morine · S. Imura · H. Mori · Y. Arakawa · M. Kanamoto · S. Iwahashi · C. Takasu
Department of Surgery, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan
e-mail: saito@clin.med.tokushima-u.ac.jp

C. Takasu
Molecular and Environmental Pathology, Institute of Health Bioscience, The University of Tokushima Graduate School, Tokushima, Japan

Introduction

Regenerative medicine using mesenchymal stem cells (MSCs) has been developed in recent years. In particular, adipose tissue-derived mesenchymal stem cells (ADSCs) are attractive sources for regenerative medicine because they are more easily accessible through minimally invasive methods than other sources such as bone marrow, umbilical cord blood, amniotic fluid, scalp tissue, placenta and so on [1]. In the fields of liver surgery and liver transplantation, the beneficial effects of MSCs including ADSCs for ischemia-reperfusion (I/R) injury through anti-inflammatory, anti-oxidative and anti-apoptotic abilities have been reported using rodent models [2–4]. We also reported that ADSCs were able to ameliorate liver injury and stimulate liver regeneration in subsequent hepatectomy (Hx) and I/R injured model mice through the trophic molecules of ADSCs [5].

Meanwhile, we focus on the homing ability of MSCs among their various effects because this ability is crucial to determine their transplantation routes. Homing is the

process in which cells migrate, engraft, and exert some functional effects in the target tissues [6]. If MSCs, injected into peripheral veins, home to the injured liver, it is not necessary to transplant them into the portal vein. Indeed, it was reported that systemically infused human MSCs homed to injured livers that had been treated with carbon tetrachloride in mice [7]. This result indicated that the injured liver produced several key regulatory factors, which were necessary for the homing of MSCs to injured sites. It has also been shown that C-X-C chemokine receptor type 4 (CXCR-4) and its ligand stromal derived factor-1 (SDF-1) play a key role in the homing effect of MSCs [8–11]. SDF-1 is a well-known chemokine that is capable of activating, mobilizing, homing and retaining hematopoietic stem cells (HSCs, specifically CD 34^+ cells) [12]. During injury, cells from the injured organ express high levels of SDF-1, which causes an elevation of localized SDF-1 levels. This leads to the recruitment and retention of circulating HSCs at the injury site via chemotactic attraction toward a gradient of SDF-1 [13]. CXCR-4 is thought to be the sole receptor of SDF-1 since it plays a unique role in physiological processes [14].

Concerning the homing of exogenous MSCs to injured sites, some reports have mentioned the importance of SDF-1/CXCR-4 axis. Freshly isolated MSCs expressed CXCR-4 on their surface and this was suggested to be important for the homing of MSCs [15]. It was further supported by the observation that enforced surface expression of CXCR-4 led to MSC migration and functional recovery after acute myocardial infarction (AMI) [16–18]. SDF-1 expressions had been upregulated mainly in damaged vascular endothelial cells immediately following AMI, and exogenous MSCs migrated towards the endothelial cells where SDF-1 was overexpressed [19]. In addition, systemically injected MSCs homed to the inflamed myocardium and produced a reduction in infarct size at 3 h after AMI [20], and approximately 3% of injected MSCs were found engrafted at 24 h after AMI [21]. In a hepatic ischemia-reperfusion (I/R) injury model, systemically transplanted MSCs were reported to affect the injured liver at 6–24 h after reperfusion as well [2–4]. In our previous report, ADSCs protected liver functions at 6 h after operation in subsequent Hx and I/R model mice [5]. Taken together, systemically injected MSCs homed to and protected the injured sites especially in the acute phase after transplantation. However, it remains to be clarified which types of cells in injured sites were involved in SDF-1/CXCR-4 axis in such conditions.

Therefore, the aim of this study was to investigate whether systemically transplanted human ADSCs homed to the injured liver in mice under stress with subsequent Hx and I/R focusing on the acute phase within 24 h after transplantation, and to clarify which types of cells in the liver were involved in SDF-1/CXCR-4 axis.

Methods

Animals

Six-week-old female BALB/c nu-nu mice were obtained from Charles River, Tokyo, Japan. All mice were provided with water and a standard laboratory diet for at least 7 days before use. Throughout the experiment, all mice were maintained behind barriers under controlled conditions and had free access to tap water and food before and after the operation. The present study was conducted in compliance with the requirements of the Division for Animal Research Resources, Institute of Health Biosciences, University of Tokushima. The experiments and procedures were approved by the Animal Care and Use Committee of the University of Tokushima.

Isolation and culturing of ADSCs

STEMPRO human ADSCs were purchased from Life Technologies, Tokyo, Japan [22]. The ADSCs were isolated from human adipose tissues collected during liposuction procedures and cryopreserved from primary cultures. Before cryopreservation, the ADSCs were expanded for one passage in MesenPRO RS medium (Life Technologies). Cells were tested for purity by flow cytometry and for their ability to differentiate into osteogenic, chondrogenic, and adipogenic lineages. These cells were positive for CD29, CD44, CD73, CD90, CD105, and CD166 (> 95%), and negative for CD14, CD31, CD45, and Lin1 (< 2%). Thawing of the cells and initiation of the culture process were performed according to the manufacturer's instructions. ADSCs were plated in tissue culture flasks and cultured with MesenPRO RS basal medium (Life Technologies) containing 2% fetal bovine serum (FBS), MesenPRO RS growth supplement (Life Technologies), 2 mM of L-glutamine at 37°C, 5% CO_2 and 90% humidity. When cells were attached to the growth surface, the medium was replaced with an equal volume of fresh complete MesenPRO RS medium. Cells were used for experimentation between passages 2 and 5. At the time of ADSC transplantation, cells were harvested with 0.05% trypsin-EDTA (Invitrogen, Tokyo, Japan) and washed twice with phosphate buffered saline (PBS). The cells were then transplanted in the PBS.

Surgical procedures and ADSC transplantation

Mice were anesthetized with isoflurane. After upper-abdominal midline laparotomy, the hepatoduodenal ligament was clamped for 15 min with a vascular clip (BEAR Medic Corporation, Chiba, Japan). During the ischemic time, 70% Hx was performed using the modified technique

of Higgins and Anderson [23]. After 15 min of ischemic time, the vascular clip was removed, the remnant liver was reperfused and ADSCs (1.0 × 10 [5] cells/mouse) in a volume of 100 μl or PBS were injected from the tail vein. Mice were killed at 6 or 24 h after transplantation. Whole livers were removed and one part of the caudate lobe was later put in RNA and stored at –80°C until RNA extraction for real-time reverse transcription-polymerase chain reaction (RT-PCR). The remnant part of the liver was fixed in 10% formaldehyde or frozen by liquid nitrogen and stored at –80°C for histological analysis.

Experimental protocol

All mice were divided into three groups: Simple laparotomy (Sham group, $n = 4$), 70% Hx following IR with PBS (Hx I/R group, $n = 8$), and 70% Hx following IR with ADSCs (Hx I/R ADSC group, $n = 8$). In order to confirm their homing effect in the acute phase after transplantation, we traced transplanted ADSCs during the 24 h after transplantation using an in vivo imaging system and detected them histologically using fluorescent microscopy at 6 h after transplantation in the Hx I/R ADSC group. We evaluated expression levels of SDF-1 and CXCR-4 mRNA in the remnant liver at 6 h after transplantation by real time RT-PCR in all three groups. We also evaluated the expressions of SDF-1 proteins at 6 and 24 h after transplantation by immunohistochemistry and compared the difference in the sites of SDF-1 expressing liver cells between 6 and 24 h after transplantation in the Hx I/R group.

In vivo imaging of transplanted ADSCs

Carbocyanine lipophilic NIR fluorescent membrane dye, DiR (Invitrogen, Carlsbad, CA, USA) was used for labeling the ADSCs [24]. ADSCs were incubated with DiR (1.0 × 10 [7] cells in 10 ml phosphate buffered saline [PBS] containing 3.5μg/ml dye and 0.5% ethanol) for 30 min at 37°C. Thereafter, ADSCs were washed twice with PBS and then transplanted intravenously from the tail vein into the mice in the Hx I/R ADSC group (1.0 × 10 [5] cells/mouse). In addition, the group, "Sham with ADSCs transplantation (Sham ADSC)" was also made as a control group. Mice were examined at 0, 6, 12 and 24 h after transplantation using the imaging system, IVIS 200 (Alameda, CA, USA). Mice were left for 5 min while being anesthetized in a chamber with isofluorane and then imaged using a 20-cm field of view and an exposure time of 3 min. The excitation and emission filter set in the IVIS was 710 to 760 nm and 810 to 860 nm, respectively.

Detection of ADSCs in the remnant liver

Fluorescent staining was used to detect transplanted ADSCs in the remnant liver histologically. ADSCs were stained with NEO-STEM (Biterials, Seoul, Korea) [25]. ADSCs were incubated with NEO-STEM (1.0 × 10 [7] cells in 10 ml MesenPRO RS medium containing 0.2 mg/ml dye) for 24 h at 37°C. Thereafter, ADSCs were washed three times with PBS and then transplanted intravenously from the tail vein into the mice in the Hx I/R ADSC and Sham ADSC group (1.0 × 10 [5] cells/mouse). The mice were killed at 6 h after transplantation. Not only the remnant livers, but also hearts, lungs and spleens were harvested and frozen with liquid nitrogen and stored at –80°C, then sectioned at a thickness of 10 μm to be observed under a fluorescent microscope (IX71, OLYMPUS).

Real time RT-PCR of SDF-1 and CXCR-4

Harvested livers (parts of the caudate lobes) were homogenized with a multi-beads shocker (Yasui-Kikai, Osaka, Japan). The RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA, USA). The RNA was reverse-transcribed with a high capacity cDNA reverse transcription kit (Applied Biosystems, Tokyo, Japan). Quantitative real-time RT-PCR was performed using the Applied Biosystems 7500 real-time PCR system, TaqMan gene expression assays-on-demand, and Taq-Man universal master mix (Applied Biosystems). The following assays (assay identification number) were used: SDF-1 (Mm00445553_m1) and CXCR-4 (Hs00607978_s1). GAPDH gene (4352338E) was used as an endogenous expression control (Applied Biosystems). The thermal cycler conditions were as follows: 2 min at 50°C, 10 min at 95°C, then 40 cycles of 15 s at 95°C and 1 min at 60°C. Amplification data were analyzed with an Applied Biosystems Prism 7500 Sequence Detection System ver. 1.3.1 (Applied Biosystems).

Immunohistochemistry of SDF-1

Harvested liver specimens were fixed in 10% formaldehyde and embedded in paraffin. 4 μm-thick sections were cut from archival formalin-fixed paraffin-embedded tissue blocks. The samples were deparaffinized and dehydrated using a graded series of ethanol solutions. Endogenous peroxidase activity was halted through the administration of 0.3% hydrogen peroxidase and methanol for 20 min. After having been rinsed in phosphate-buffered saline (PBS), the tissue sections were processed in a 0.01 M citrate buffer (pH 6.0) inside a heat-resistant plastic container. The sections were then irradiated in a domestic microwave oven for 20 min. After microwave irradiation, the slides were allowed

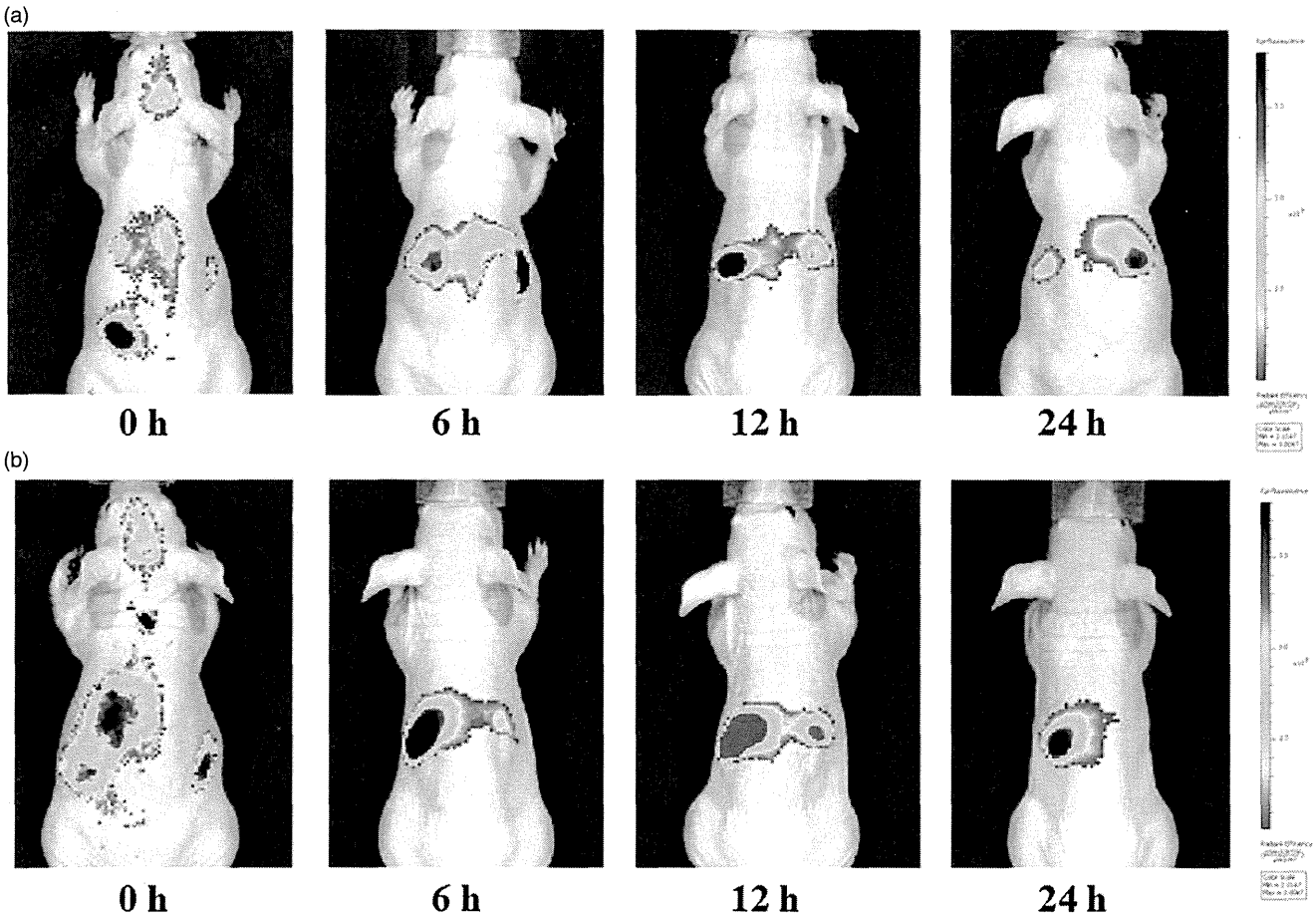

Fig. 1 *In vivo* imaging after adipose-derived stem cells (ADSCs) transplantation. (**a**) HxI/R ADSC; ADSCs were found in various organs immediately following transplantation and gradually accumulated in the central area of whole bodies. (**b**) Sham ADSC; similar with HxI/R ADSC group, ADSCs were gradually accumulated in the central area of whole bodies

to cool at room temperature. The sections required a primary rabbit polyclonal antibody against SDF-1α (ab25117, Abcam, Cambridge, UK). The antibody was diluted at 1:100. After 2 h incubation at 4°C with the primary antibody, indirect immunoperoxidase staining with the avidin-biotin complex (Dako, Glostrup, Denmark) and DAB Tablet (Wako Pure Chemical Industries, Osaka, Japan) were applied for the visualization of antigens. Finally, nuclear counterstaining was undertaken using Mayer's hematoxylin solution. All cell counts were performed using a Nikon DIGITAL CAMERA DXM 1200F photomicroscope at a magnification of ×200 (×20 objectives and ×10 eyepiece) and five fields were randomly chosen to determine the expression of SDF-1α. The assessment of SDF-1α staining was undertaken by a pathologist in our facility.

Statistical analysis

All results were presented as mean ± SD. Multiple group comparisons were performed by one-way analyses of variance followed by the Scheffe procedure for comparison of means. Comparisons between the two groups were performed using the Mann–Whitney *U*-test using statistical software (JMP 8.0.1., SAS Institute, Cary, NC, USA). A *P*-value of less than 0.05 was considered statistically significant.

Results

Ability of ADSCs to home to the remnant liver after Hx and I/R

In both Hx I/R ADSC and Sham ADSC group, ADSCs were found in various organs immediately following transplantation and gradually accumulated in the central area of whole bodies (Fig. 1a,b). Among the harvested tissues (hearts, lungs, livers and spleens) at 6 h after transplantation, ADSCs were almost accumulated in the livers or spleens and slightly trapped in the lungs in Hx I/R ADSC group (Fig. 2ai). ADSCs were also histologically detected in the harvested tissue sections (Fig. 2aii). On the other hand, much more ADSCs trapped in the lungs were found in Sham

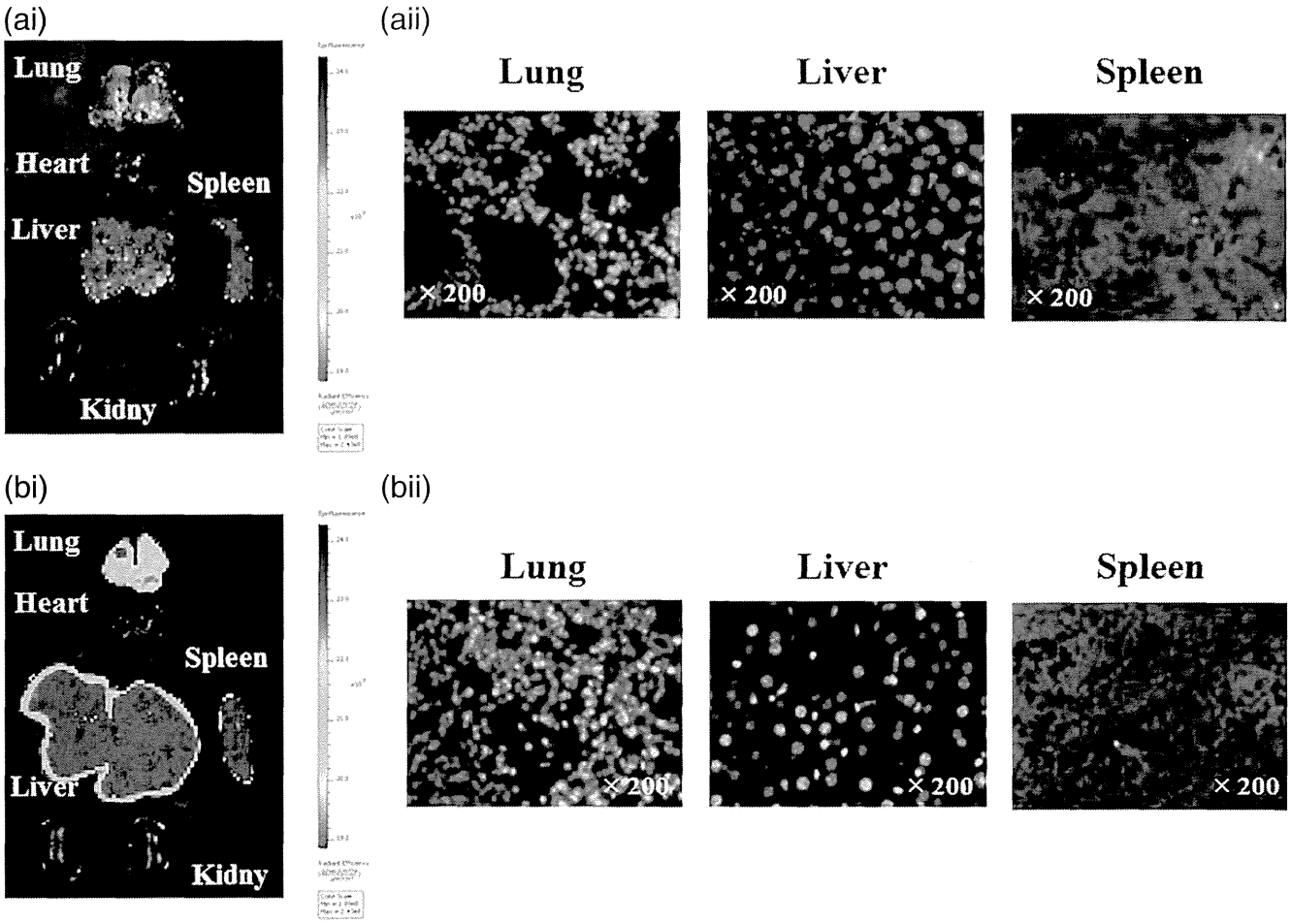

Fig. 2 The detection of transplanted adipose-derived stem cells (ADSCs). (**a**) HxI/R ADSC: (i) ADSCs were almost accumulated in the livers or spleens and slightly trapped in the lungs; (ii) ADSCs were histologically detected in the harvested tissues. (**b**) Sham ADSC: (i) ADSCs were accumulated in the livers or spleens and trapped in the lungs much more than HxI/R ADSC group; (ii) Accumulated ADSCs in the livers were less than HxI/R ADSC group

ADSC group (Fig. 2bi). ADSCs accumulated in livers were less than HxI/R ADSC group (Fig. 2bii).

Expression levels of SDF-1 and CXCR-4 mRNA in the remnant liver

Hx and I/R injury significantly upregulated SDF-1 levels in the liver at 6 h after transplantation and ADSC transplantation did not affect the SDF-1 levels (Fig. 3a).

Meanwhile, CXCR-4 levels in the liver at 6 h after transplantation were significantly upregulated only in the Hx I/R ADSC group (Fig. 3b). The homing of ADSCs expressing CXCR-4 to the remnant liver has been suggested as a possibility.

Identification of SDF-1 expressing liver cells

Immunohistochemical analysis revealed that the expressions of SDF-1 at 6 h after Hx and I/R were equally detected in both the liver parenchymal cells (PCs) and non-PCs. There were no statistically significant differences (Fig. 4a). On the other hand, the expressions of SDF-1 at 24 h after Hx and I/R were almost all detected in non-PCs. The average number of SDF-1 positive non-PCs was significantly larger at 24 h after Hx and I/R (Fig. 4b). It was revealed that the dominant SDF-1 positive cells had changed from PCs to non-PCs over time.

Discussion

Recent studies [4, 26] have suggested that systemically transplanted MSCs homed to the injured liver in Hx I/R or I/R injury rodent models and exerted beneficial effects by inactivating the MEK/ERK signal pathway or promoting hepatic regeneration. However, there have been no reports describing in detail how exogenous MSCs home to the injured liver. Herein, we demonstrated the following findings: (1) systemically transplanted human ADSCs homed to

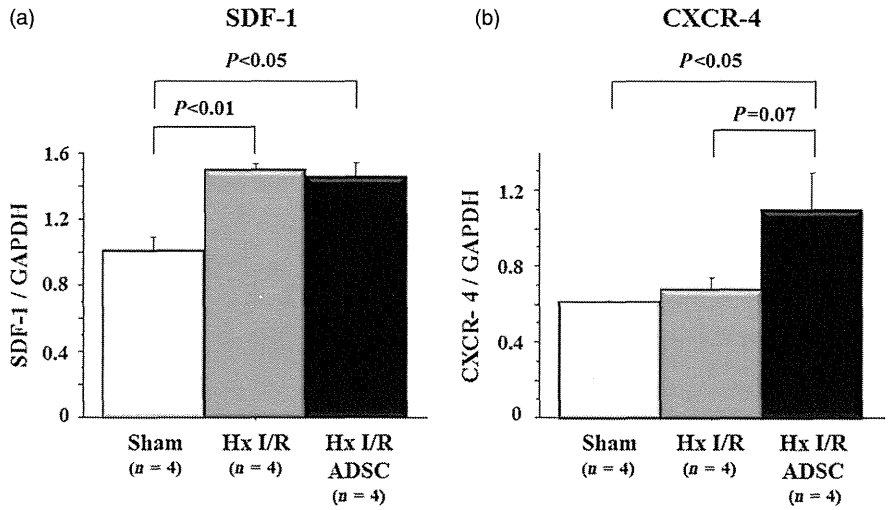

Fig. 3 Expression levels of stromal-derived factor-1 (SDF-1) and C-X-C chemokine receptor type 4 (CXCR-4) mRNA in the remnant liver. (**a**) Hx and I/R injury significantly up-regulated SDF-1 levels in the liver at 6 hours after transplantation and adipose-derived stem cell (ADSC) transplantation did not affect the SDF-1 expressions. (**b**) CXCR-4 levels in the liver at 6 hours after transplantation were significantly up-regulated in the Hx I/R ADSC group

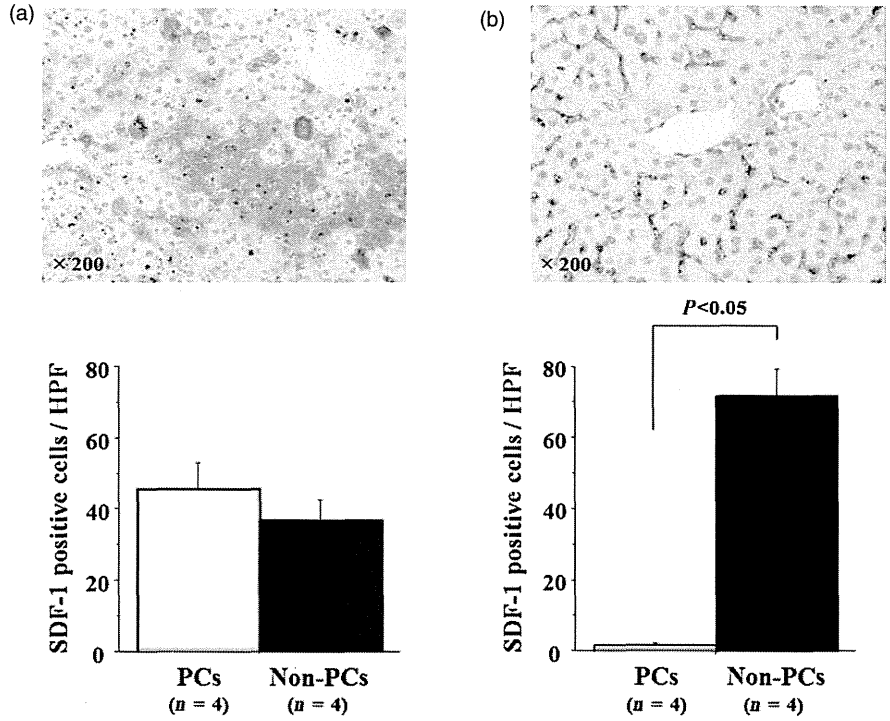

Fig. 4 Identification of stromal-derived factor-1 (SDF-1) expressing liver cells. (**a**) The expressions of SDF-1 at 6 hours after Hx and I/R were equally detected in both the liver parenchymal cells (PCs) and non-PCs. There were no statistically significant differences. (**b**) The expressions of SDF-1 at 24 hours after Hx and I/R were almost all identified in non-PCs. The average number of SDF-1 positive non-PCs was significantly larger

the injured liver under stress with subsequent Hx and I/R during the 24 h after transplantation in mice; (2) expression levels of CXCR-4 mRNA in the livers were upregulated in the Hx I/R ADSC group and CXCR-4 expressing ADSCs might home to the injured liver; and (3) Hx and I/R injury enhanced the expression levels of SDF-1 mRNA in the injured liver and the predominant SDF-1 positive cells shifted from PCs to non-PCs over time.

The safety of systemic injection of ADSCs remains controversial. It has already been reported that an intravenous transplantation of a large amount of ADSCs induced pulmonary embolisms in mice [27, 28]. On the other hand, Ra et al. [29] reported that the systemic transplantation of ADSCs appeared to be safe and did not induce pulmonary embolisms or other adverse events, not only in rodents but also humans. In our study, all ADSC-transplanted mice survived with no symptoms and had no pulmonary embolisms as determined by *in vivo* imaging. The unsolved issues, such as cell dose, numbers and combination therapy with anticoagulant [27] should be resolved before clinical use.

Regarding the CXCR-4 expressions in MSCs, it has already been reported that freshly isolated MSCs expressed CXCR-4 on their surface, however, culture-expanded MSCs progressively downregulate CXCR4 expression and lose their ability to migrate toward the SDF-1 gradient [15, 30, 31] and that the overexpression of CXCR-4 increased the

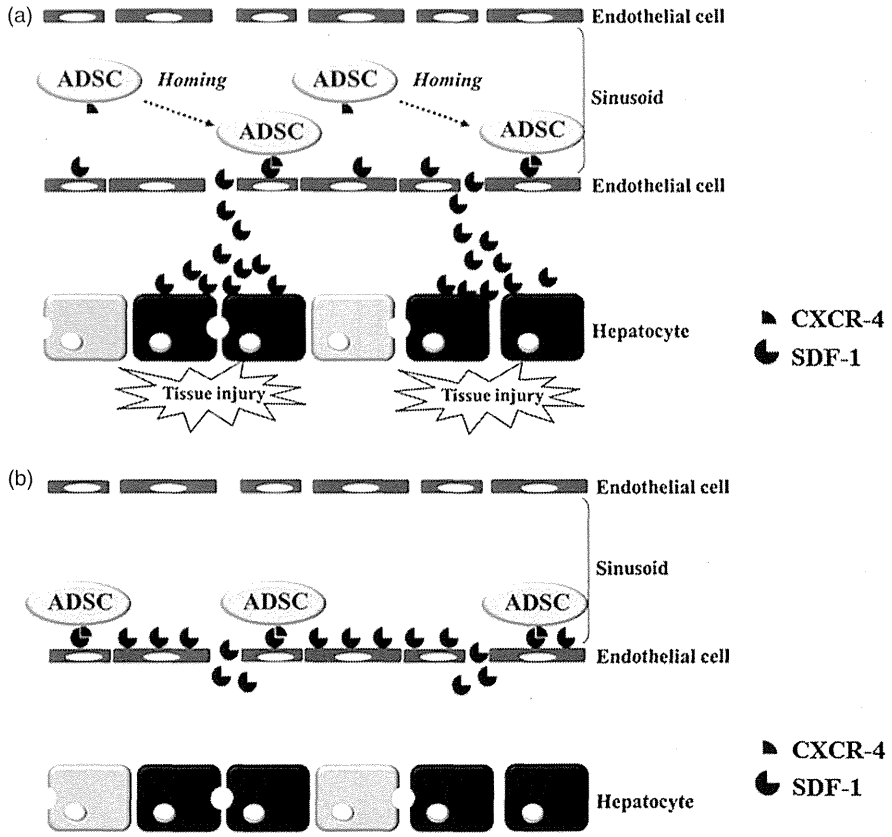

Fig. 5 The hypothesis of the mechanism of adipose-derived stem cells' (ADSCs') homing to the injured liver. ADSCs expressing C-X-C chemokine receptor type 4 (CXCR-4) on the cell surfaces home to sinusoidal endothelial cells expressing stromal-derived factor-1 (SDF-1). (**a**) Hepatic parenchymal cells (PCs) contribute to enhance the SDF-1 expressions at early postoperative period in Hx and I/R injury state. (**b**) Predominant cells, which generate SDF-1, shift from PCs to non-PCs

MSCs that homed to the injured tissues [8, 16–18]. ADSCs were also reported to express CXCR-4 on their cell surfaces [32]. Though we did not determine the location of CXCR-4 expressions in transplanted ADSCs, it was suggested that exogenous ADSCs were stimulated by chemokines under stress with Hx I/R injury and increased CXCR-4 expressions of cell surfaces led to ADSCs' homing to the injured liver in our study.

It was shown that SDF-1 gene expression was regulated by the transcription factor, hypoxia-inducible factor-1, in ischemic tissue [33]. Further, Lai et al. demonstrated that microvasculature endothelial cells were potential contributors to the generation of SDF-1 in the ischemic retina using rodent models of retinal ischemia-reperfusion injury [34]. Indeed, in the present study, Hx and I/R injury enhanced the SDF-1 expressions in the injured liver and the expressions of SDF-1 at 6 h after Hx and I/R were equally detected in both the liver PCs and non-PCs. Thereafter, the SDF-1 positive cells shifted from PCs to non-PCs over time, including sinusoidal endothelial cells. Taken together, we hypothesized that hepatic PCs contribute to enhance the SDF-1 expressions during the early postoperative period in the state of Hx and I/R injury (Fig. 5a). Then, ADSCs expressing CXCR-4 on the cell surfaces home to the sinusoidal endothelial cells expressing SDF-1, because the predominant cells, which generate SDF-1, shift from PCs to non-PCs (Fig. 5b).

In conclusion, we demonstrated that systemically transplanted human ADSCs homed to the injured liver under stress with Hx and I/R in the acute phase within 24 h after transplantation in nude mice. Though the safety of the systemic transplantation of ADSCs should be confirmed before clinical use, systemic transplantation might become an effective and practical route for ADSC transplantation.

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Conflict of interest None declared.

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A steroid minimization immunosuppression protocol using basiliximab in adult living donor liver transplantation for hepatitis C virus-related cirrhosis

Authors: Taizo Hibi[1], Masahiro Shinoda[1,*], Osamu Itano[1], Hideaki Obara[1], Minoru Kitago[1], Yuta Abe[1], Hiroshi Yagi[1], Masayuki Tanaka[1], Ken Hoshino[1], Akihiro Fujino[1], Tatsuo Kuroda[1], Shigeyuki Kawachi[2], Minoru Tanabe[3], Motohide Shimazu[2], Yuko Kitagawa[1]

[1]Department of Surgery, Keio University School of Medicine

[2]Division of Digestive Surgery and Transplantation Surgery, Tokyo Medical University Hachioji Medical Center

[3]Department of Hepatobiliary and Pancreatic Surgery, Tokyo Medical and Dental University

Running title: Steroid minimization in LDLT for HCV cirrhosis

*Corresponding author contact information:

Masahiro Shinoda, M.D., Ph.D.

35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

Phone: +81-3-5363-3802

Fax: +81-3-3355-4707

E-mail address: masa02114@yahoo.co.jp

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Abstract

Aim: Recent randomized trials have failed to prove the benefit of steroid-free immunosuppression in liver transplantation for hepatitis C (HCV)-related cirrhosis. Furthermore, there is a lack of data on the use of basiliximab in living donor liver transplantation (LDLT). This pilot study evaluated the safety and efficacy of a steroid minimization protocol using basiliximab compared with standard immunosuppression.

Methods: A single-center, prospective cohort analysis was conducted to compare 2 immunosuppression regimens: calcineurin inhibitor/mizoribine/basiliximab (the St− group) and calcineurin inhibitor/mizoribine/steroid (the St+ group), in adult recipients who underwent LDLT for HCV since 2004. Study endpoints were rejection rates, recurrent HCV, patient survival, and other adverse events up to 2 years after transplantation.

Results: A total of 27 consecutive patients were enrolled. Transplantation characteristics were similar between the 2 groups (14 St− and 13 St+) except ABO incompatible cases being more common in the St+ group. Rejection rates, recurrent HCV, patient survival, fibrosis stage, and new-onset diabetes mellitus at 2 years were comparable between the 2 groups. ABO incompatibility did not affect short- and long-term outcomes. Nine St− and 7 St+ recipients underwent interferon and ribavirin therapy for recurrent HCV, with a sustained virological response rate of 33% and 29%, respectively.

Conclusion: A steroid minimization protocol with basiliximab in adult LDLT for HCV is safe and affords equivalent rejection rates compared with standard immunosuppression. However, no significant differences are observed with respect to recurrent HCV, patient survival, and metabolic complications.

Keywords: immunosuppression, steroids, basiliximab, living donors, liver transplantation, hepatitis C virus

INTRODUCTION

Steroid-free immunosuppression in liver transplantation has been advocated to have potential benefits, such as decreasing infection rates, hypertension, metabolic complications, rejection rates, and recurrent hepatitis C (HCV) (1-3). However, recent randomized clinical trials have reported contradictory results (4-8) and whether maintenance of immunosuppression with steroids has a deleterious impact on disease progression after liver transplantation for HCV-related cirrhosis is yet to be determined. Likewise, although the use of basiliximab (a murine/human chimeric monoclonal antibody to the alpha chain of the high affinity IL-2 receptor complex) in living donor liver transplantation (LDLT) has been described as a safe option (9-11), its pros and cons remain controversial. To date, there is only one multicenter trial that compared standard immunosuppression with a steroid-free regimen using basiliximab in adult LDLT for HCV (12).

We hypothesized that substituting steroids with basiliximab may decrease recurrent HCV and other adverse events related to long-term use of steroids without increasing the risk of rejection. This pilot study evaluated the safety and efficacy of a steroid minimization protocol (a calcineurin inhibitor, mizoribine, and basiliximab) compared with standard immunosuppression using steroids in adult LDLT for HCV.

METHODS

This single-center, non-randomized, prospective cohort analysis conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was conducted with approval from the Institutional Review Board of Keio University School of Medicine. Eligible patients were 18 years of age or older who were scheduled for LDLT since 2004 for

HCV-related cirrhosis with positive HCV-RNA load at the time of transplantation. Patients with a history of previous transplantation were excluded from the study. The presence of extrahepatic metastasis or macroscopic vascular invasion on preoperative imaging precluded transplantation in patients with hepatocellular carcinoma.

Potential advantages and disadvantages associated with basiliximab as an induction immunosuppression were fully explained to all ABO identical/compatible transplant candidates with HCV. Only patients who consented to its use were assigned to the steroid minimization protocol (the St− group). These patients were administered two 20 mg intravenous doses of basiliximab on the day of transplantation and day 4 after transplantation. The remaining patients who refused to use basiliximab as well as ABO incompatible cases where steroids were universally administered through an intraportal infusion catheter (13) were allocated to the standard immunosuppression protocol (the St+ group). Methylprednisolone was administered to all recipients as a 500 mg intravenous bolus at the time of graft reperfusion and the St− group received no subsequent doses of steroids. In the St+ group, methylprednisolone was then administered at 2 mg/kg/day from day 1 to day 3, at 1 mg/kg/day until day 6, and at 0.5 mg/kg/day thereafter. Steroids were slowly discontinued around 6 months after transplantation.

In both groups, the selection of calcineurin inhibitors, i.e., cyclosporine A (CyA) or tacrolimus (Tac), was dependent on the surgeon's preference. CyA was initiated from day 0 at the dose of 1.6 mg/kg/day using a 4-hour intravenous infusion protocol (14). Intravenous CyA was converted around day 14 to oral microemulsion formulation that was administered twice daily. The dose was adjusted to maintain target trough and peak levels of 300–400 ng/ml and 700–1000 ng/ml, respectively, during the first month, 150–

300 ng/ml and 500–700 ng/ml, respectively, until month 3, and 80–150 ng/ml and 300–500 ng/ml, respectively, thereafter. Tac was administered orally twice daily at the initial dose of 1 mg/body maintaining trough levels of 10–12 ng/ml until week 2, 7–10 ng/ml until month 1, 5–7 ng/ml until month 3, and around 5 ng/ml thereafter. In the event of drug toxicity, CyA and Tac were crossed over. Mizoribine, 100–200 mg/day, was administered primarily with few exceptions in critically ill patients (15). Conversion to mycophenolate mofetil (up to 1000 mg daily) was decided at the surgeon's discretion, if rejection or drug-induced hepatotoxicity was clinically suspected.

Liver function tests were routinely monitored and any unexplainable elevation in the enzymes was followed up by a biopsy. Acute cellular rejection was confirmed with liver biopsy according to the Banff criteria (16). In principle, rejection was treated by either increasing or switching the ongoing calcineurin inhibitor with addition of mycophenolate mofetil. If subsequent liver function tests showed no improvement, steroid pulse therapy (intravenous methylprednisolone bolus injection of 500 mg/day for 3 consecutive days followed by tapering) was initiated as a last resort. The diagnosis of recurrent HCV was made on the basis of abnormal liver enzymes and histological findings in the liver biopsy specimen according to the METAVIR score (17). Antiviral therapy using interferon with ribavirin was administered only to patients with histologically confirmed recurrent HCV. Splenectomy was performed at the time of transplantation when the platelet count was <50,000 cells/mm^3 and in all ABO incompatible cases.

Primary endpoints of the study were the incidence of biopsy-proven acute rejection (BPAR) and recurrent HCV within 2 years after transplantation. Secondary endpoints were overall patient survival at 2 years and adverse events, including metabolic

disorders (new-onset diabetes mellitus defined as requiring insulin or oral medications over 6 months to control fasting blood glucose <126 mg/dl and hyperlipidemia), hypertension, kidney dysfunction, and infection (bacterial, viral, and fungal etiologies requiring intravenous antibiotic treatment). In addition, we conducted subgroup analyses by excluding ABO incompatible cases from the St+ group to examine if the difference in immunosuppression protocol had any influence on short-term outcomes and safety profiles. Similarly, the patients who received anti-HCV therapy within 2 years post-transplant were removed from the analysis for HCV-RNA levels at each time point of the initiation of therapy.

Statistical methods

Analysis was performed on an intention-to-treat basis. Continuous data were depicted as means and standard errors or medians and interquartile ranges and compared using the Student t test or the Mann–Whitney U test as indicated. Categorical data were analyzed using the chi-square test or Fisher's exact test when appropriate. Cumulative survival curves were estimated using the Kaplan–Meier method and compared with the log-rank test. A P value of <0.05 was considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics 21™ statistical software (SPSS, Inc., Chicago, IL).

RESULTS

Study population

A total of 27 consecutive adult recipients underwent LDLT for HCV-related cirrhosis since 2004. Of these, the St− group comprised 14 recipients and the St− group

comprised 13 recipients. The recipient and donor characteristics are summarized in Table 1. There were 5 ABO incompatible cases in the St+ group but other profiles were similar between the 2 groups.

Immunosuppression and acute cellular rejection

Table 2 depicts the doses of immunosuppressive agents in each cohort. The median trough levels of CyA and Tac were equivalent throughout the study period. Steroid was given up to 1 year after transplantation in the St+ group. Five cases of moderate to severe BPAR occurred in the entire cohort, within the first 90 days after transplantation. Of these, 3 recipients (1 St− and 2 St+) required steroid pulse therapy and eventually died. One patient in the St− group with hepatocellular carcinoma died of disease recurrence at 23 months after transplantation. One patient in the St+ group succumbed to uncontrolled infection at 6 months secondary to perforated duodenal ulcer. The other patient in the St+ group who underwent ABO incompatible LDLT died of recurrent HCV at 17 months. Twenty-four recipients without steroid pulse therapy did not reach median survival ($P = 0.001$: Figure 1). The incidence of BPAR and steroid pulse therapy in each group was comparable (Table 3). Similar results were obtained when ABO incompatible cases were excluded from the St+ group.

Recurrent HCV and antiviral therapy

During the study period, the frequency of histologically confirmed recurrent HCV, cumulative recurrence rate, and progression to fibrosis stage ≥2 was similar in both groups (Table 3 and Figure 2). These findings were upheld even after ABO incompatible cases were removed from the St+ group. Postoperative HCV-RNA levels

were significantly lower at 3 months in the St− group compared with the St+ group (Figure 3). All 16 patients with recurrent HCV underwent antiviral therapy with interferon and ribavirin. In 5 (31%) patients, the treatment was discontinued (3 patients with kidney dysfunction, 1 with pancytopenia, and 1 with depression). The differences in the rate of sustained virological response were not statistically significant (Table 3).

Overall survival

The median follow-up period was 40 (18–79) months. The 2-year overall survival rates were similar between the 2 groups (St− group, 79% ± 11% *versus* St+ group, 60% ± 16%, $P = 0.41$). There were 3 deaths in the St− group (1 recurrent HCV, 1 malignant lymphoma, and 1 recurrent hepatocellular carcinoma) and 4 in the St+ group (2 recurrent HCV and 2 infections). ABO incompatibility had no detrimental effect on long-term outcomes. No recipients received a second graft.

Other Adverse events

The St+ group showed a tendency to preserved kidney function compared with the St− group. However, when ABO incompatible cases were excluded from the St+ group, the renal protective effect disappeared (Table 3). The incidence of new-onset diabetes mellitus, hyperlipidemia, and infection were comparable between the 2 groups (Table 3). ABO incompatibility had essentially no impact on short-term outcomes. None of the normotensive recipients at the time of transplantation developed hypertension thereafter.

DISCUSSION

Replacement of steroids with basiliximab did not significantly alter the incidence of

BPAR and overall patient survival. Likewise, adverse outcomes were comparable between the 2 groups and steroid minimization did not decrease the incidence of recurrent HCV. These results were consistent, regardless of ABO incompatibility, and were contradictory to a multicenter trial in Japan, which describes favorable side effect profiles (less new-onset diabetes mellitus and cytomegalovirus infection) and lower recurrent HCV after LDLT with a steroid-free regimen comprising a calcineurin inhibitor, MMF, and basiliximab (12). In that study, antiviral treatment post-transplant was not standardized, recurrent HCV was not always diagnosed by liver biopsy, and the administration of steroids differed among institutions. Therefore, the data precluded definite conclusion regarding the impact of immunosuppression on recurrent HCV. Other centers have reported conflicting results as well (18,19).

Although we demonstrated that using basiliximab as a substitute for steroids in adult LDLT is a safe alternative, our data did not support the reported advantages of steroid minimization in terms of metabolic complications and acute rejection rates (9,19-22). The discrepancies can potentially be attributed to the differences in immunosuppression, particularly the use of steroids before reperfusion and selection of calcineurin inhibitors as well as dual or triple immunosuppressive regimens. Of note, patients who received steroid pulse therapy suffered dismal prognoses regardless of the immunosuppression protocol and the causes of death were infection, recurrent hepatocellular carcinoma, and recurrent HCV. Although the causal relationship remains unclear, steroid treatment for acute rejection has been shown to be associated with increased mortality in HCV+ recipients (23); therefore, avoidance of steroid boluses is recommended (3). Our results also mimicked a recent randomized, multicenter trial from Japan that demonstrated significantly reduced overall survival rates for patients