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Cross-priming of CD8$^+$ T cells in vivo by dendritic cells pulsed with autologous apoptotic leukemic cells in immunotherapy for elderly patients with acute myeloid leukemia

Toshio Kitawaki[a], Norimitsu Kadowaki[a], Keiko Fukunaga[a], Yasunari Kasai[b], Taira Maekawa[b], Katsuyuki Ohmori[c], Tatsuya Itoh[d], Akira Shimizu[d], Kiyotaka Kuzushima[e], Tadakazu Kondo[a], Takayuki Ishikawa[a], and Takashi Uchiyama[a]

[a]*Department of Hematology and Oncology;* [b]*Center for Cell and Molecular Therapy, Department of Transfusion Medicine and Cell Therapy;* [c]*Department of Clinical Laboratory Medicine;* [d]*Department of Experimental Therapeutics, Kyoto University Hospital, Kyoto, Japan;* [e]*Division of Immunology, Aichi Cancer Center Research Institute, Nagoya, Japan*

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Objective. **The prognosis for elderly patients with acute myeloid leukemia (AML) remains dismal. To explore the potential of immunotherapy for improving clinical outcomes for these patients, we performed a phase I clinical trial of dendritic cell (DC)–based immunotherapy for elderly patients with AML.**

Materials and Methods. **Autologus monocytes were obtained after reducing tumor burden by chemotherapy. Immature DCs induced with granulocyte-macrophage colony-stimulating factor and interleukin-4 were pulsed with autologous apoptotic leukemic cells as antigens. DCs were administered intradermally to four patients five times at 2-week intervals. To facilitate DC migration to lymph nodes, injection sites were pretreated with killed *Streptococcus pyogenes* OK-432 one day before. DCs were coinjected with OK-432 to induce maturation and interleukin-12 production in vivo.**

Results. **Antileukemic responses were observed by an interferon-γ enzyme-linked immunospot assay or a tetramer assay in two of four patients. In a human leukocyte antigen–A*2402-positive patient, induction of CD8$^+$ T-cell responses to WT1- and human telomerase reverse transcriptase–derived peptides were observed, indicating cross-priming in vivo. The two patients with antileukemic immunity showed longer periods of disease stabilization than the other two patients.**

Conclusions. **This study demonstrates the immunogenicity of autologous DCs that cross-present leukemia-associated antigens from autologous apoptotic leukemic cells in vivo in elderly patients with AML.**

Management of elderly patients with acute myeloid leukemia (AML) remains a challenge because of a high rate of therapy-related mortality and chemotherapy resistance [1]. Antigen-specific immunotherapy, which is less toxic and kills leukemic cells through different mechanisms than chemotherapy, has the potential capacity to improve the clinical outcomes of these patients. Recent identification of several leukemia-associated antigens prompted us to develop immunotherapy for elderly patients with AML [2].

Active immunization by peptide vaccines can induce antileukemic immunity and clinical responses in AML [3–6]. Clinical trials of dendritic cell (DC)–based immunotherapy for AML have also been reported [7–12]. However, the trial using leukemic cell–derived DCs showed that the generation of leukemic cell–derived DCs was feasible in only a limited number of patients, and even in vaccinated patients the treatment could not induce clinical responses [9]. This may be due to lower immunostimulatory activity of leukemic cell–derived DCs than monocyte-derived DCs (MoDCs) [13]. Recently, the efficient generation of MoDCs from patients with AML has been demonstrated in vitro [14], providing a rationale for the use of MoDCs in immunotherapy for AML.

Offprint requests to: Norimitsu Kadowaki, M.D., Ph.D., Department of Hematology and Oncology, Graduate School of Medicine, Kyoto University, 54 Shogoin Kawara-cho, Sakyo-ku, Kyoto 606-8507, Japan; E-mail: kadowaki@kuhp.kyoto-u.ac.jp

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There are several parameters to enhance the immunogenicity of MoDC vaccines. Whereas monocytes are cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 conventionally for 5 to 7 days to induce DCs, a shorter period of culture is sufficient to induce equivalently potent DCs [15]. Among DC maturation–inducing factors, microbial components that trigger the production of IL-12 are beneficial to induce effective adaptive immunity [16]. An extended period of stimulation with microbial components results in DC exhaustion in which DCs lose the capacity to produce IL-12 [17]. Thus, a short-term stimulation can generate optimal DCs that retain IL-12 production. Inflammation in the skin before DC injection facilitates DC migration to draining lymph nodes, leading to a stronger immune response [18,19]. Using apoptotic whole tumor cells as antigens may be instrumental in inducing multivalent immune responses [20].

We performed in vitro assays to optimize these parameters. Based on the results of these assays, we conducted a phase I clinical trial of immunotherapy for elderly patients with AML at the second or later remission setting, using DCs loaded with autologous apoptotic leukemic cells. The treatment was well-tolerated and safe and induced antileukemic immunity in two of four patients, which was associated with transient disease stabilization. Importantly, in one patient, cross-priming of leukemia antigen–specific $CD8^+$ T cells in vivo was explicitly demonstrated. This study indicates the safety and immunogenicity of immunotherapy using MoDCs that cross-present leukemic cell antigens in elderly patients with AML.

Materials and methods

Generation, maturation, and cryopreservation of DCs for in vitro assays

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers by density gradient centrifugation using Lympholyte H (Cedarlane, Ontario, Canada). Monocytes were purified using anti–CD14-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), or enriched by plastic adherence by incubating PBMCs at 37°C for 2 hours and removing nonadherent cells by pipetting. Monocytes were cultured with 800 IU/mL GM-CSF (Primmune, Kobe, Japan) and 500 IU/mL IL-4 (Primmune) in CellGro DC medium (CellGenix Technologie Transfer, Freiburg, Germany) for 3 days (3d-DCs) or 6 days (6d-DCs). In some experiments, 3d-DCs were frozen in CP-1 freezing medium (Kyokuto Pharmaceutical Industrial, Tokyo, Japan). CP-1 contains 12% hydroxymethyl starch and 10% dimethyl sulfoxide in normal saline and was mixed with 8% human serum albumin before use. DCs were matured with 0.1 KE/mL OK-432 (Picibanil; Chugai Pharmaceuticals, Tokyo, Japan), a penicillin-killed and lyophilized preparation of a low-virulence strain (Su) of *Streptococcus pyogenes* (group A) [21].

In vitro analysis of DC functions

Flow cytometric analysis, measurement of IL-12p70 production, T-cell–stimulatory capacity of DCs for allogeneic naive $CD4^+$ T cells, and the cytokine profile of $CD4^+$ T cells primed with DCs were analyzed as described previously [15,22].

Uptake of apoptotic cells by DCs and the cross-presenting capacity of DCs

Efficiency of uptake of apoptotic cells by DCs was assessed as described previously [23] using myeloid leukemia cell lines K562, OUN-1 [24] (Dr. Yasukawa, Ehime University, Japan), and a T-cell leukemia cell line MT2, which were killed by 120 Gy γ-irradiation and 48-hour serum-free culture in RPMI-1640 (Wako Pure Chemical Industries, Osaka, Japan). To examine the cross-presenting capacity of DCs, human leukocyte antigen (HLA)-A*2402–positive, immature 3d-DCs were pulsed with HLA-A*2402–negative, Epstein-Barr virus–transformed lymphoblastoid cell lines, which were killed as described here. DCs were matured with OK-432 (0.1 KE/mL) and prostaglandin E_2 (1 μg/mL) (MP Biomedicals, Solon, OH, USA) for 6 hours, and cocultured with autologous T cells at a DC-to-T cell ratio of 1:10. IL-2 (50 IU/mL; Teceleukin; Shionogi & Co., Ltd., Osaka, Japan) was added on the next day. For a positive control, DCs pulsed with HLA-A*2402–restricted EBNA3B peptide (TYSAGIVQI; KURABO Industries, Osaka, Japan) were used. Expansion of EBNA3A- and EBNA3B-specific $CD8^+$ T cells were evaluated by HLA tetramer staining [25].

Clinical trial protocol

The protocol was approved by the Ethics Committee, Graduate School and Faculty of Medicine, Kyoto University. Each patient gave written informed consent in accordance with the Declaration of Helsinki. The primary and secondary objectives were the assessment of safety and immunological and clinical responses, respectively.

Autologous leukemic cells were harvested before induction chemotherapy. Patients were required to be between 16 and 79 years of age and have a diagnosis of AML according to World Health Organization criteria [26,27]. Patients were excluded if they had another concurrent malignancy, an active autoimmune disease, positivity for blood-borne infectious agents, or a history of penicillin allergy (because OK-432 contains penicillin). Patients were enrolled if 5×10^7 or more leukemic cells were harvested. Thereafter, patients were treated with chemotherapy. More than 4 weeks after the last chemotherapy, patients proceeded to the DC vaccination if leukemic cells in bone marrow (BM) were <20%. In addition, to assess the clinical efficacy of DC vaccination, the presence of an evaluable lesion in BM, which was defined as 0.1% or more of leukemic cells by flow cytometry, was required. Furthermore, patients should have an Eastern Cooperative Oncology Group performance status of 0 to 2 and adequate vital organ functions. Patients were excluded if they had eligibility for hematopoietic stem cell transplantation or an uncontrollable infection. Concomitant chemotherapy and radiotherapy were prohibited.

DC vaccine generation

DC vaccines were generated from autologous monocytes under current Good Manufacturing Practice conditions. Autologous leukemic cells to be used as antigens were obtained as mononuclear cells (MNCs) by density gradient centrifugation over Ficoll-Hypaque (GE Healthcare, Buckinghamshire, UK) from BM and/or peripheral blood (PB) samples. MNCs were frozen in CP-1 freezing medium and stored at −150°C. Before added to DCs, MNCs were killed by 120 Gy

γ-irradiation and 48 hours serum starvation. Killing of MNCs was confirmed by the percentage of Annexin V–positive cells being 90% or more by flow cytometry and reduced uptake of [^{3}H]-thymidine to the baseline level.

Apheresis products, which were obtained with COBE Spectra (Caridian BCT, Lakewood, CO, USA) from 10 L blood, were processed by elutriation using Elutra (Caridian BCT) to enrich monocytes. At the time of apheresis, no leukemic cells were observed in the PB of the patients, as assessed by a routine clinical laboratory test. Monocytes were cultured with 800 U/mL GM-CSF and 500 U/mL IL-4 in CellGro DC medium in gas-permeable plastic bags (VueLife 118; CellGenix Technologie Transfer) at 37°C, 5% CO_2 to generate immature DCs. After 48 hours, DCs were pulsed with autologous apoptotic leukemic cells and 2 μg/mL keyhole-limpet hemocyanin (KLH; Biosyn Corporation, Carlsbad, CA, USA). The endotoxin level in the KLH preparation examined by the supplier was <0.1 IU/mg. After an additional 24 hours, DCs were frozen as immature DCs in CP-1 freezing medium and stored at −150°C.

Administration of the DC vaccine

A total of 1×10^7 DCs were intradermally injected at four sites in bilateral arms and thighs. Twenty-four hours before DC administration, the injection sites were pretreated by 0.2 KE/site OK-432. At the time of DC administration, DCs were thawed and mixed with 1 KE OK-432. Then, the mixture of DCs and OK-432 was injected. The DC administration was repeated at 2-week intervals for five administrations.

Monitoring of immunological and clinical responses

Antigen-specific immune responses were assessed at indicated time points. Immune responses to KLH and autologous leukemic cells were tested by skin delayed-type hypersensitivity tests and interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assays. In addition, in a HLA-A*2402–positive patient, immune responses to HLA-A*2402–restricted peptides derived from leukemia-associated antigens were examined by IFN-γ ELISPOT assay and HLA tetramer staining. The peptides used in the assays were the natural $WT1_{235-243}$ peptide (CMTWNQMNL) [24], the modified $WT1_{235-243}$ peptide (CYTWNQMNL) [28], the human telomerase reverse transcriptase $(hTERT)_{461-469}$ peptide (VYGFVRACL) [29], and the lower matrix 65-kd phosphoprotein (pp65) of cytomegalovirus (CMV) (amino acids 328–336; QYDPVAALF) [30]. All peptides were purchased from Multiple Peptide Systems (San Diego, CA, USA). Both PBMCs and BM mononuclear cells (BMMCs) were subjected to assays before and after 1-week in vitro stimulation with antigen- or peptide-pulsed DCs in the presence of 15 U/mL IL-2 (Teceleukin). To evaluate clinical responses, percentages of leukemic cells in BM were monitored by morphology and flow cytometry at indicated time points.

Skin delayed-type hypersensitivity test

The 4×10^5 antigen-pulsed DCs were intradermally injected in the forearm. Sizes of induration and erythema were measured 48 hours later. Erythema that was 1.5-fold or larger in diameter than the antigen-unpulsed control was considered positive.

IFN-γ ELISPOT assay

IFN-γ ELISPOT assays (Mabtech, Nacka Strand, Sweden) were performed using antigen-pulsed DCs and peptide-pulsed C1R-A*2402 (Dr. Masafumi Takiguchi, Kumamoto University, Kumamoto, Japan). Stimulator cells were plated at 2×10^4 cells/well. As responder cells, fresh and in vitro–stimulated MNCs from PB and BM were plated with fresh MNCs at 1 to 2×10^5 cells/well and in vitro–stimulated MNCs at 1 to 2×10^4 cells/well. After overnight incubation, spots were developed using 3-amino-9-ethylcarbazole (Sigma Chemical, St Louis, MO, USA) and counted by KS ELISPOT compact (Carl Zeiss MicroImaging, Tokyo, Japan). Numbers of specific spot-forming cells were calculated by subtracting the number of spots with unpulsed DCs from the number of spots with antigen-pulsed DCs.

HLA tetramer staining

Natural $WT1_{235-243}$ peptide/HLA-A*2402 tetramer was purchased from Medical & Biological Laboratories (Nagoya, Japan). Modified $WT1_{235-243}$ peptide/HLA-A*2402 tetramer and a peptide derived from the HIV envelope (env) protein/HLA-A*2402 tetramer were produced as described previously [30]. Fresh and in vitro–stimulated MNCs were stained with a tetramer and fluorescein isothiocyanate–conjugated anti-CD8 monoclonal antibody (BD Biosciences) and analyzed by flow cytometery (FACSCalibur; BD Biosciences) [30].

Results

In vitro assays to optimize generation of DCs

To optimize generation of DCs, we performed in vitro functional assays. We first compared DCs differentiated from monocytes in the presence of GM-CSF and IL-4 for 3 days with 6-day differentiated DCs conventionally used in clinical trials. After 24-hour exposure to OK-432, both 3d-DCs and 6d-DCs showed similar levels of surface molecule expressions, IL-12p70 production, and T-cell stimulatory capacity for allogeneic naïve $CD4^+$ T cells (Supplementary Figure E1; online only, available at www.exphem.org), indicating that 3d-DCs have functions comparable with 6d-DCs. Next, we examined the capacity of 3d-DCs to cross-present apoptotic cell–associated antigens. At the DC-to-apoptotic cell ratio of 1:1, 11% to 33% of immature 3d-DCs incorporated apoptotic leukemia cell lines (Fig. 1A). Moreover, HLA-A*2402–positive DCs pulsed with killed lymphoblastoid cell lines from an HLA-A*2402–negative donor induced expansion of $CD8^+$ T cells specific for the HLA-A*2402–restricted epitopes of EBNA3A and EBNA3B (Fig. 1B), indicating the capacity of DCs to cross-present apoptotic cell–derived antigens.

An extended period of exposure of DCs to lipopolysaccharide leads to DC exhaustion [17], as indicated by loss of IL-12–producing capacity by DCs. To examine whether OK-432 induces DC exhaustion, we analyzed the maturation kinetics of OK-432–stimulated 3d-DCs. Upregulation of the surface molecules (Fig. 2A) and IL-12p70 production (Fig. 2B) became evident 4 and 8 hours after OK-432 stimulation, respectively. Maximal levels of surface molecule expressions and IL-12p70 production were observed at 48 hours. Next, we examined how many hours of exposure to OK-432 is sufficient to elicit a maturation signal to DCs, using 3d-DCs that were cultured for a total of 48 hours with different

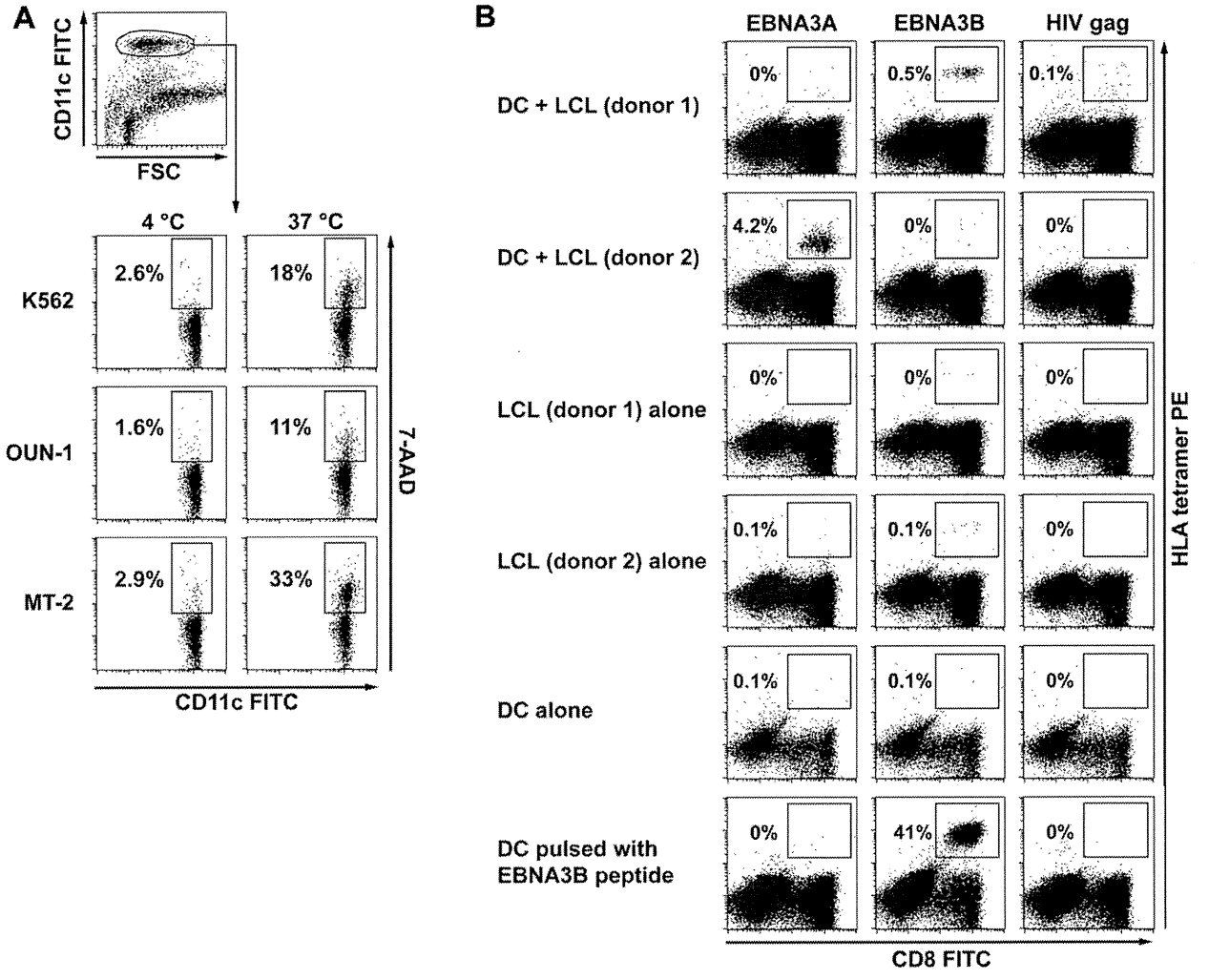

Figure 1. 3d-DCs incorporate apoptotic cells and cross-present cell-associated antigens. (**A**) Uptake of apoptotic cells by 3d-DCs. Apoptotic K562, OUN-1, and MT2 were labeled with 7-aminoactinomycin D (7-AAD) (20 μg/mL), and cocultured with immature 3d-DCs at a DC-to-apoptotic cell ratio of 1:1. After 4 hours of incubation at 4°C or 37°C, cells were stained with fluorescein isothiocyanate–conjugated anti-CD11c monoclonal antibody and analyzed by flow cytometry. Cells positive for both CD11c and 7-AAD were considered to be DCs that had phagocytosed apoptotic cells. (**B**) The cross-presenting capacity of DCs. Immature 3d-DCs from a HLA-A*2402–positive donor were pulsed with apoptotic HLA-A*2402–negative donor-derived lymphoblastoid cell lines (LCLs), matured with OK-432 and prostaglandin E_2, and cocultured with autologous T cells. For a positive control, DCs pulsed with the EBNA3B peptide were used as a stimulator. After 7 days, expansions of EBNA3A- and EBNA3B-specific $CD8^+$ T cells were evaluated by HLA tetramer staining. Dead cells are excluded by staining with propidium iodide. Numbers shown indicate percentages of tetramer-positive cells among $CD8^+$ cells. Representative data from two experiments are shown.

durations of exposure to OK-432 at the start of culture. As short as 2-hour exposure upregulated CD83 and CD86 (Fig. 2C) and induced IL-12p70 production (Fig. 2D) during the subsequent 46-hour culture without OK-432. Although at the time of 8-hour exposure, the induction of CD83, CD86 (Fig. 2A), and IL-12p70 (Fig. 2B) was low, 8-hour exposure was sufficient to induce maximal levels of CD83 and CD86 expression (Fig. 2C) and IL-12p70 production (Fig. 2D). Notably, although initial 24-hour exposure to OK-432 induced the maximal levels of CD83 and CD86 expression (Fig. 2C), DCs did not produce a detectable level of IL-12p70 during the last 24-hour culture (Fig. 2D). These data indicate that, like lipopolysaccharide [17], OK-432–induced IL-12p70 production was limited within the first 24 hours and most active between 8 and 24 hours after OK-432 stimulation. The functional significance of ongoing IL-12p70 production by DCs in priming naïve $CD4^+$ T cells was supported by the data that 3d-DCs matured with OK-432 for 6 hours showed a superior capacity to induce IFN-γ–producing T cells to those matured for 24 hours (Fig. 2E). Thus, extended stimulation with OK-432 induces DC exhaustion. To avoid it, we decided to administer immature DCs together with OK-432 to patients and to induce DC maturation in vivo.

It is convenient to prepare a large number of DCs from a single batch of apheresis and freeze them in aliquots. We assessed the effect of cryopreservation on DCs. Whereas cryopreserved immature 3d-DCs showed somewhat higher percentages of dead cells after 24-hour culture with or

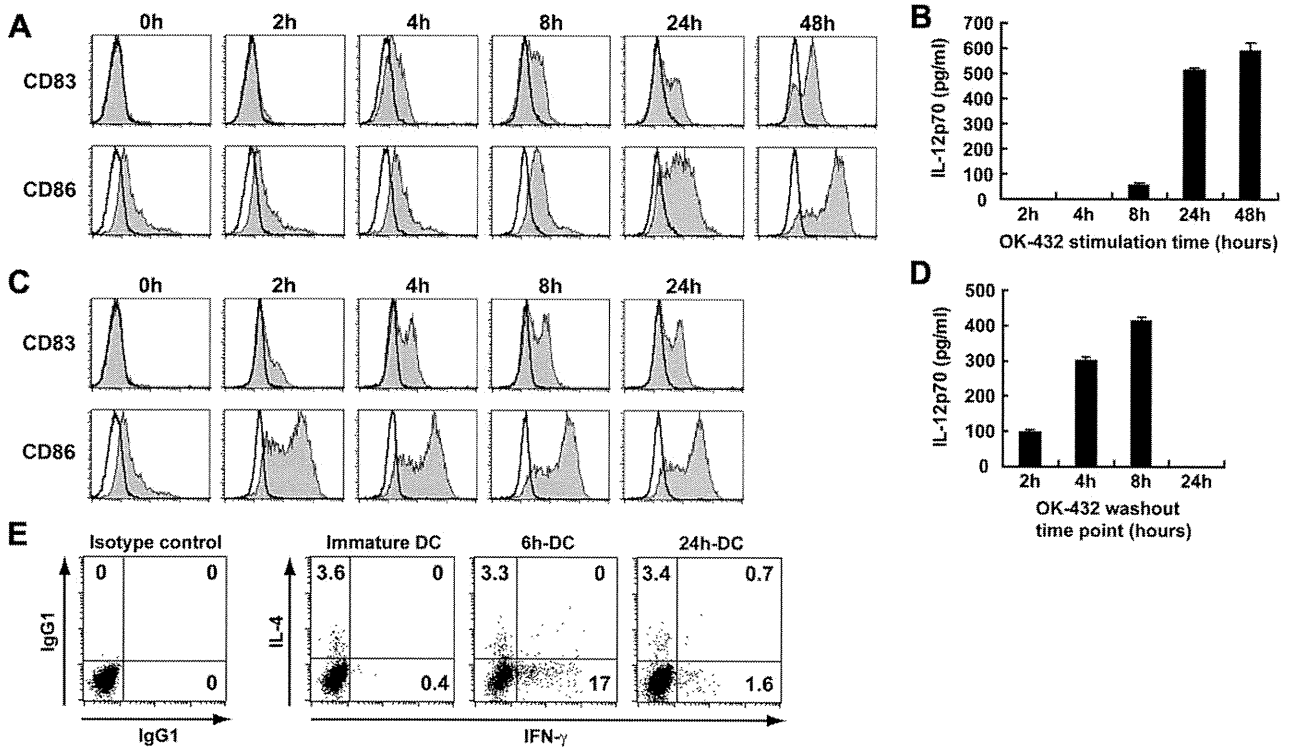

Figure 2. Short-term stimulation with OK-432 is optimal to generate Th1-inducing mature DCs. (**A**, **B**) Immature 3d-DCs were cultured in the presence of OK-432 (0.1 KE/mL) for indicated time periods, then harvested and analyzed. (**C**, **D**) Immature 3d-DCs were cultured in the presence of OK-432 for indicated time periods, washed, replated, and further cultured for a total of 48 hours. Cells and supernatants harvested at 48 hours were analyzed. (**A**, **C**) Expression of CD83 and CD86 was analyzed by flow cytometry. Dead cells were excluded by staining with propidium iodide. Open histograms indicate staining with isotype controls. (**B**, **D**) IL-12p70 production in culture supernatants of DCs (5×10^5 cells/mL) were measured by enzyme-linked immunosorbent assay. Error bars indicate the standard deviation of duplicate measurements. (**E**) Naïve $CD4^+$ T cell differentiation induced by DCs. Immature 3d-DCs were matured with OK-432 (0.1 KE/mL) for 6 or 24 hours and cocultured with allogeneic naïve $CD4^+$ T cells for 7 days. Cytokine profiles of T cells were analyzed by intracellular cytokine staining. Numbers indicate percentages of cells in each quadrant. Representative data from four experiments are shown.

without OK-432, and tended to produce a lower amount of IL-12p70 upon OK-432 stimulation as compared with non-cryopreserved DCs (Supplementary Figure E2A, C; online only, available at www.exphem.org), similar levels of CD83 and CD86 expression were induced by OK-432 in both DCs (Supplementary Figure E2B; online only, available at www.exphem.org). Thus, although cryopreservation of immature DCs impaired their function to some extent, cryopreserved DCs largely retained the viability and expression of immunostimulatory molecules. Considering the practical convenience to prepare a stock of DCs at one time, we decided to freeze DCs as immature DCs. Taken together, these data demonstrate that DCs generated in the present study are capable of inducing $CD8^+$ T-cell responses to apoptotic cell–derived antigens, and that immature DCs can be cryopreserved without critical loss of functions.

Patients, feasibility, and safety

Thirteen patients were recruited to the study for the leukemic-cell harvest at the onset of AML. After chemotherapy, four patients were eligible for DC vaccination (Table 1). In these patients, $>5 \times 10^7$ DCs for five vaccinations could be generated from a single apheresis. Autologous apoptotic leukemic cells were added to DCs as antigens at leukemic cell-to-DC ratios of 1:3.3 to 1:6.5, depending on the numbers of collected leukemic cells (Supplementary Table E1; online only, available at www.exphem.org). Status of PB and BM at the time of apheresis are shown in Supplementary Table E1 (online only, available at www.exphem.org). Representative data of surface molecule expressions on DCs are shown in Supplementary Figure E3 (online only, available at www.exphem.org).

All of the patients completed the five vaccinations safely (Table 1). In all the patients, grade 1 to 2 fever and grade 2 skin reactions at the injection sites were observed. The fever was resolved within 2 days after vaccination and most likely related to administration of OK-432. The skin reactions at the injection sites were transient and characterized by erythema, pruritus, and tenderness. No significant toxicities to vital organs or signs of autoimmunity were observed.

Induction of antigen-specific immune responses to KLH and leukemic cells

Induction of an immune response to KLH was detected by skin delayed-type hypersensitivity tests and/or IFN-γ ELISPOT assays in three patients, with the exception of patient no. 4

Table 1. Patient characteristics and results of the DC vaccination

Patient no.	Age/Sex	Diagnosis	DC vaccination was started		LC in BM at the first vaccination[a] (%)	Adverse effects[b]	Immune response		Clinical response	Died at (days after the last vaccination)
			After the last CT (d)	After diagnosis (d)			KLH	LC		
1	76/F	AML-MRC	82	93	1.8	Fever (1) Injection site reaction (2)	Yes	No	PD Died of sepsis with leukemia	186
2	75/M	AML-MRC	40	155	0.6	Fever (1) Injection site reaction (2)	Yes	Yes	Transient disease stabilization Died of leukemia	391
3	70/M	AML-MRC	44	344	2.9	Fever (2) Injection site reaction (2)	Yes	Yes	Transient disease stabilization Died of sepsis with leukemia	192
4	66/M	AML M2	67	144	0.2	Fever (1) Injection site reaction (2)	No	No	PD Died of leukemia	66

AML-MRC = acute myeloid leukemia with myelodysplasia-related changes; CT = chemotherapy; F = female; LC = leukemic cells; M = male; PD = progressive disease.

[a]Percentages of leukemic cells in bone marrow were determined by flow cytometry.

[b]Numbers in parentheses indicate grade of toxicity according to the National Cancer Institute-Common Terminology Criteria for Adverse Events version 3.0.

(Table 1 and data not shown). Two patients (patient nos. 2 and 3) showed induction of immune responses to leukemia-associated antigens. In patient no. 2, who was HLA-A*2402–negative, IFN-γ ELISPOT assays using autologous leukemic cell–pulsed DCs revealed the induction of antileukemic immunity in PBMCs and BMMCs without in vitro stimulation after the fourth vaccination (Fig. 3A). The antileukemic immune response was still detected 1 month after the fifth vaccination in in vitro–stimulated PBMCs and BMMCs (Fig. 3B), but was no longer detected without in vitro stimulation (Fig. 3A). We could not test antileukemic immunity at subsequent time points in this patient because the patient developed leukocytopenia, probably owing to progression of myelodysplastic syndrome.

In patient no. 3, who was HLA-A*2402–positive, HLA-A*2402–restricted peptides from WT1 and hTERT were used in immunological monitoring. $CMVpp65_{328-336}$ peptide was used as a positive control in ELISPOT assays (Fig. 4B). No responses to the leukemia-associated antigens were observed until the fourth vaccination. However, 2 months after the fifth vaccination, positive responses to the modified $WT1_{235-243}$ and the $hTERT_{461-469}$ peptides were detected in in vitro–stimulated PBMCs by HLA tetramer staining (Fig. 4A) and an IFN-γ ELISPOT assay (Fig. 4B), respectively. The PBMCs binding to the modified $WT1_{235-243}$ peptide/HLA-A*2402 tetramer also bound to the natural $WT1_{235-243}$ peptide/HLA-A*2402 tetramer (Fig. 4A), indicating that these cells were capable of recognizing the natural WT1 peptide presented on leukemic cells. These responses were short-lived and almost completely disappeared 3 months after the fifth vaccination. No responses were detected in PBMCs or BMMCs without in vitro stimulation (data not shown). Thus, the vaccinations induced HLA class I–restricted, antileukemic immunity, indicating that the DCs cross-presented leukemia-associated antigens in vivo. In addition, in patient no. 2, leukemic cell-reactive T cells were detected in BM (Fig. 3), the main tumor site in leukemia.

Clinical outcomes

The two patients with antileukemic immunity had longer periods of disease stabilization than the other two patients without antileukemic immunity (Fig. 5A). Notably, in patient no. 3, the percentages of leukemic cells in BM dropped from 11% to 5.2% during the second month after the fifth vaccination, when a positive antileukemic immunity was observed (Fig. 5B). Thus, these observations suggest that induction of antileukemic immunity was associated with extended the periods of disease stabilization in these patients.

Discussion

Novel therapies with less toxicity are necessary for intractable AML in elderly patients. In this study, we conducted a phase I clinical trial of immunotherapy for such patients using DCs pulsed with autologous apoptotic leukemic cells.

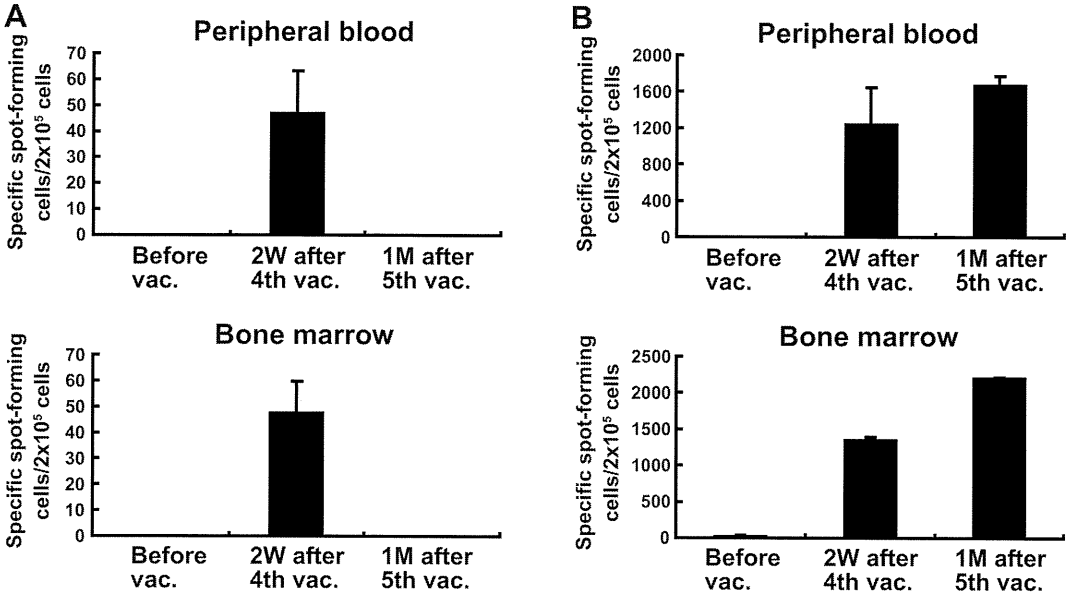

Figure 3. IFN-γ ELISPOT assay in patient no. 2. MNCs from PB and BM were obtained at indicated time points and subjected to IFN-γ ELISPOT assays directly after isolation (**A**) or after 1 week of stimulation with antigen-pulsed DCs (**B**). In IFN-γ ELISPOT assays, 2×10^5 MNCs (**A**) and 1×10^4 MNCs (**B**) were incubated with 1×10^4 leukemic cell–pulsed or unpulsed DCs. Numbers of specific spot-forming cells per 2×10^5 MNCs, calculated by subtracting numbers of spots with unpulsed DCs from numbers of spots with leukemic cell–pulsed DCs. Error bars indicate the standard deviation of duplicate measurements.

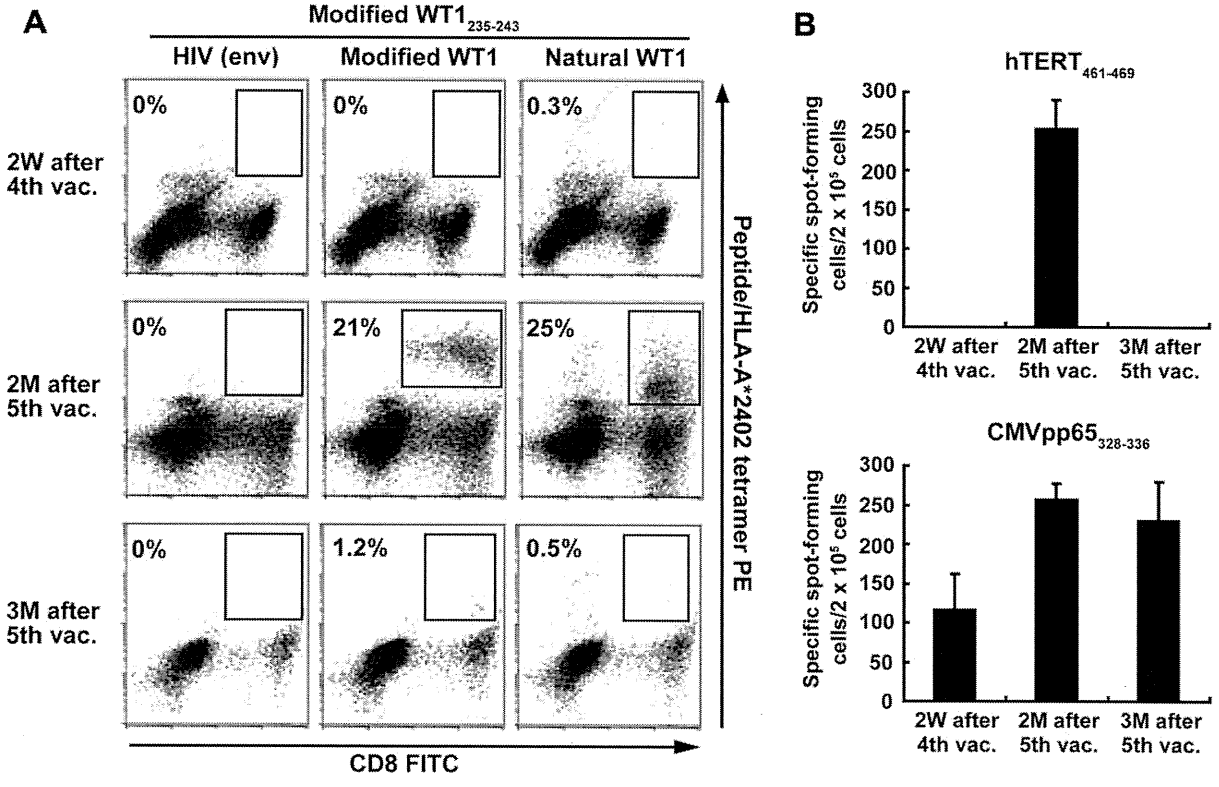

Figure 4. Immune responses in patient no. 3. (**A**) HLA tetramer staining. MNCs from PB were obtained at indicated time points, stimulated for 1 week with DCs pulsed with the modified $WT1_{235-243}$ peptide, stained with phycoerythrin-labeled peptide/HLA-A*2402 tetramers and fluorescein isothiocyanate–labeled anti-CD8 monoclonal antibody, and analyzed by flow cytometry. Dead cells were excluded by staining with propidium iodide. Numbers indicate percentages of tetramer-positive cells among $CD8^+$ cells. (**B**) IFN-γ ELISPOT assay. MNCs were stimulated for 1 week with DCs pulsed with the $hTERT_{461-469}$ or $CMVpp65_{328-336}$ peptide, and subjected to IFN-γ ELISPOT assays. In the assays, 2×10^4 MNCs were incubated with 2×10^4 C1R-A*2402 pulsed with or without the $hTERT_{461-469}$ or $CMVpp65_{328-336}$ peptide. Before vaccination, the assay was performed using DCs as a stimulator, which induced many nonspecific spots. Thus, the data before vaccination are not shown. Numbers of specific spot-forming cells per 2×10^5 MNCs, calculated by subtracting numbers of spots with unpulsed C1R-A*2402 from numbers of spots with antigen-pulsed C1R-A*2402, were depicted. Error bars indicate the standard deviation of duplicate measurements.

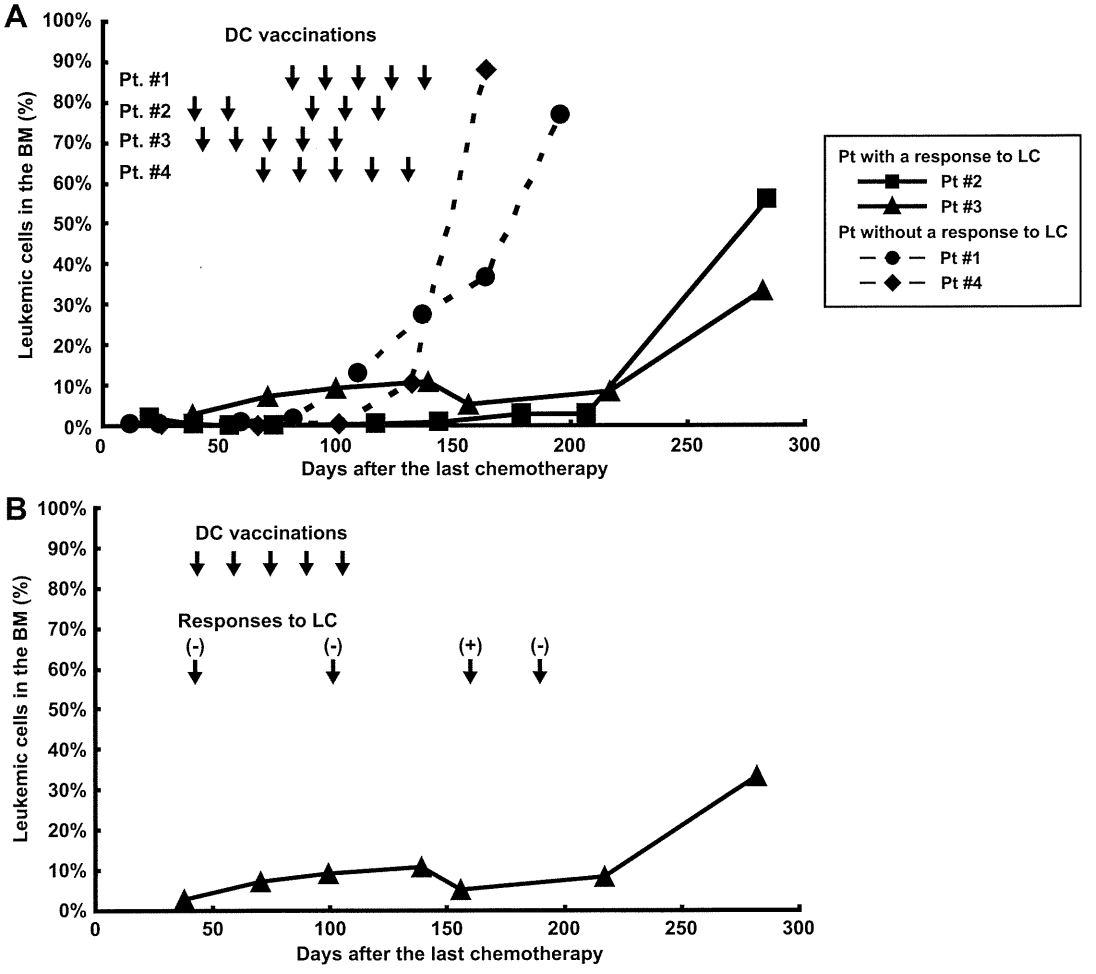

Figure 5. Clinical courses during the DC vaccination. (**A**) Percentages of leukemic cells in BM as determined by flow cytometry in four vaccinated patients are shown. Solid lines indicate patients with immune responses to leukemic cells (LCs) (patients 2 [■] and 3 [▲]). Dashed lines indicate patients without immune responses to LCs (patients 1 [●] and 4 [◆]). Arrows indicate time points when DC vaccines were administered to each patient. (**B**) The clinical course of patient no. 3. Arrows indicate time points when immunological monitoring was performed. Plus (+) or minus (−) signs indicates that immune responses to leukemic cells were detected or not detected at that time point, respectively.

Induction of antileukemic immunity was observed in two of four vaccinated patients. This is the first study that demonstrates cross-priming of $CD8^+$ T cells by DCs pulsed with apoptotic leukemic cells in vivo in humans, thus providing a proof of principle of this approach. The limited number of patients prevented us from drawing any definitive conclusion regarding clinical efficacy from the present trial. However, longer periods of disease stabilization observed in the two patients with antileukemic immunity compared to the other two patients without antileukemic immunity implied that induction of antileukemic immunity might have impacted on the clinical course of these patients.

There are several features in the method of DC vaccination in this trial: short-term 3-day culture to generate DCs in an attempt to reduce labor, cost, and time; use of whole leukemic cells as antigens to induce multivalent immune responses; use of the microbial adjuvant OK-432 as a maturation-inducing factor to generate Th1-inducing DCs; in vivo maturation of DCs to avoid DC exhaustion by extended stimulation in vitro with OK-432; and prior induction of inflammation at the injection sites to facilitate DC migration to draining lymph nodes.

We used autologous apoptotic leukemic cells as antigens because several studies have shown that apoptotic cells are more efficiently cross-presented by DCs to $CD8^+$ T cells than soluble antigens such as tumor lysate [31–34]. Furthermore, MoDCs has been shown to cross-present apoptotic leukemic cells to $CD8^+$ T cells in vitro [35]. Apoptotic cells as antigens also have advantages over peptides, in that the DCs have the ability to process multiple antigens from the apoptotic cells and present those antigens on their own HLA molecules. In this study, we clearly showed that MoDCs cross-presented leukemia-associated antigens WT1 and hTERT from apoptotic leukemic cells. Furthermore, T cells reactive to leukemic cells were detected in BM.

A murine study has shown that DC maturation not by inflammatory cytokines but by pathogen-derived components is crucial for DCs to acquire the capacity to differentiate naïve $CD4^+$ T cells into effector T cells [16]. We used OK-432, a preparation of killed *Streptococcus pyogenes* [21], which strongly triggers DC maturation through Toll-like receptor 4 [36–39]. We showed that, like lipopolysaccharide [17], longer stimulation with OK-432 induces DC exhaustion, resulting in the reduced capacity of DCs to induce Th1 responses. Several preclinical studies have shown that DCs briefly exposed to Toll-like receptor ligands are better inducers of Th1-type and cytotoxic T-cell responses [17,40,41]. Moreover, a clinical trial suggests superiority of briefly matured DCs in pediatric patients with cancer [42]. In this trial, we administered immature DCs together with OK-432 to avoid DC exhaustion before administration. The induction of IFN-γ detected by the ELISPOT assay implied IL-12 production by DCs in vivo.

Only a small proportion of intradermally administered DCs reach draining lymph nodes [43,44]. In a mouse model, pretreatment of administration sites with inflammatory cytokines enhance DC migration to regional lymph nodes [18]. Based on this finding, we pretreated administration sites with a low dose of OK-432. Because of unavailability of a cell-processing facility for cells labeled with indium-111 oxyquinoline [43,44], we could not evaluate the efficiency of DC migration to lymph nodes. Whether this administration procedure is superior to others should be evaluated in future studies.

In this study, multiple vaccinations were required to elicit antileukemic immunity, which rapidly declined after cessation of vaccination. Maintenance of antileukemic immunity might lead to improvement of clinical efficacy, and might be fulfilled by increasing the number of vaccinations, which was, however, impossible in this study because of the limited availability of autologous leukemic cells. Thus, if a peptide is available for the induced antileukemic $CD8^+$ T-cell response, peptide vaccination may be added after DC vaccination. Furthermore, blockade of immunosuppressive mechanisms may be combined.

In conclusion, we demonstrated the feasibility, safety, and immunogenicity of DC-based immunotherapy for elderly patients with AML. Cross-priming of $CD8^+$ T cells by DCs pulsed with autologous apoptotic leukemic cells was provoked in vivo. The results were promising, yet further intensification of vaccine potency is clearly required. This novel therapeutic approach may lead to improvement of clinical outcomes in elderly patients with AML, which has been difficult to achieve with other therapeutic approaches.

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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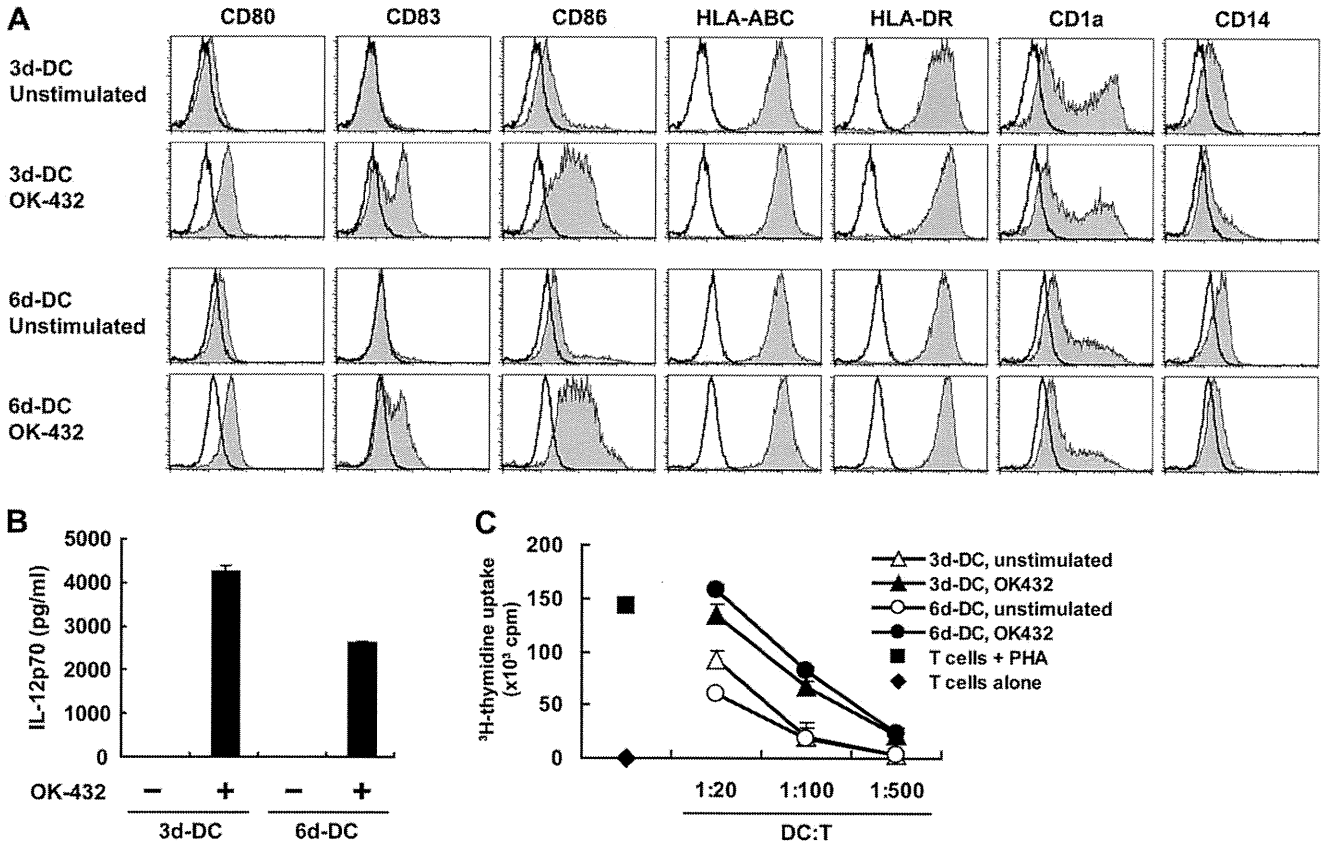
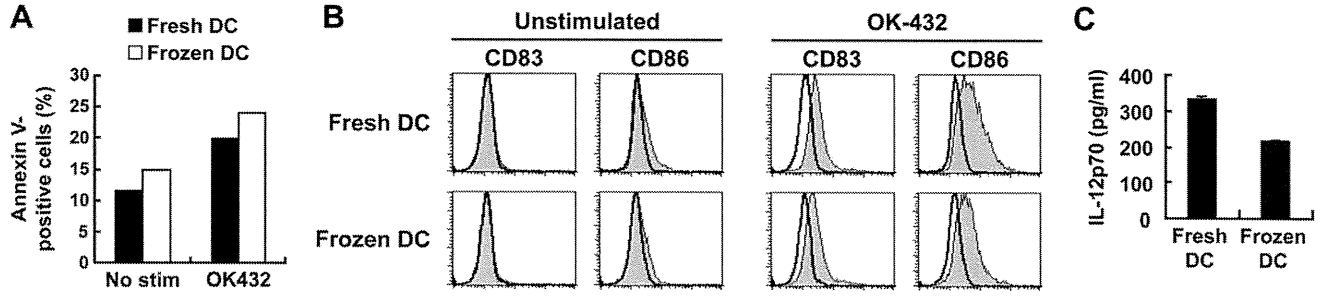
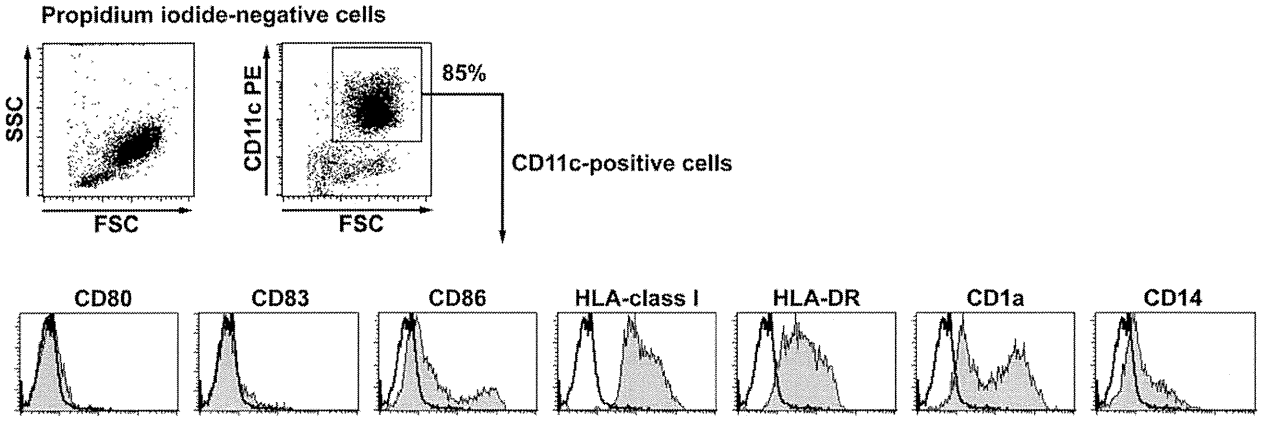


Supplementary Figure E1. 3d-DCs and 6d-DCs have comparable T-cell stimulatory capacity. (**A**) Expressions of surface molecules on DCs. Unstimulated or OK-432-stimulated DCs were analyzed by flow cytometry. Dead cells were excluded by staining with propidium iodide. Open histograms indicate staining with isotype controls. (**B**) IL-12p70 production by DCs (5×10^5 cells/mL) stimulated with OK-432 (0.1 KE/mL) for 24 hours was measured by enzyme-linked immunosorbent assay. Error bars indicate the standard deviation of duplicate measurements. (**C**) Proliferation of naive $CD4^+$ T cells stimulated with DCs. Allogeneic naive $CD4^+$ T cells were cocultured with DCs at indicated DC to T-cell ratios. On day 4, 1 Ci of [^{3}H]-thymidine was added. After 16 hours of further incubation, thymidine uptake was counted. Naive $CD4^+$ T cells were stimulated with 10 μg/mL phytohemagglutinin as a positive control. Representative data from three experiments are shown.



Supplementary Figure E2. Effects of cryopreservation on immature 3d-DCs. (**A**) Viability of fresh and frozen 3d-DCs after 24 hours of incubation with or without OK-432 (0.1 KE/mL) were evaluated by staining with Annexin-V. Percentages of Annexin-Vpositive cells are indicated. (**B**) Expression of surface molecules on fresh and frozen DCs after 24 hours of incubation with or without OK-432. (**C**) IL-12p70 production by fresh and frozen DCs (5×10^5 cells/mL) induced by 24-hour stimulation with OK-432 was measured by enzyme-linked immunosorbent assay. Error bars indicate the standard deviation of duplicate measurements. Representative data from four experiments are shown.



Supplementary Figure E3. Expression of surface molecules on DCs for vaccination. Cryopreserved DCs from patients were thawed, stained, and analyzed by flow cytometry. Dead cells were excluded by staining with propidium iodide. Numbers indicate percentages of cells in each quadrant. Representative data from patient no. 1 are shown.

Supplementary Table E1. DC vaccine generation

	At the time of apheresis				
Patient no.	Days after the last CT	PB WBC (/L)	PB Mo (%)	BM LC[a] (%)	Antigen dose (LC:DC)
1	74	4700	7	0.9	1:5
2	31	3000	9	2.0	1:6.5
3	43	3900	15	0[b]	1:6
4	46	4800	16	0.3	1:3.3

CT = chemotherapy; LC = leukemic cells; Mo = monocytes.
[a]Percentages of leukemic cells in bone marrow were determined by flow cytometry.
[b]Patient 3 was in complete remission at the time of apheresis. The patient subsequently relapsed and became eligible for DC vaccination.

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Safety and pharmacokinetics of recombinant human hepatocyte growth factor (rh-HGF) in patients with fulminant hepatitis: a phase I/II clinical trial, following preclinical studies to ensure safety

Akio Ido[1,2*], Akihiro Moriuchi[1,2], Masatsugu Numata[1,2], Toshinori Murayama[3], Satoshi Teramukai[4], Hiroyuki Marusawa[5], Naohisa Yamaji[1,2], Hitoshi Setoyama[1,2], Il-Deok Kim[1], Tsutomu Chiba[5], Shuji Higuchi[6], Masayuki Yokode[3], Masanori Fukushima[4], Akira Shimizu[7] and Hirohito Tsubouchi[1,2]

Abstract

Background: Hepatocyte growth factor (HGF) stimulates hepatocyte proliferation, and also acts as an anti-apoptotic factor. Therefore, HGF is a potential therapeutic agent for treatment of fatal liver diseases. We performed a translational medicine protocol with recombinant human HGF (rh-HGF), including a phase I/II study of patients with fulminant hepatitis (FH) or late-onset hepatic failure (LOHF), in order to examine the safety, pharmacokinetics, and clinical efficacy of this molecule.

Methods: Potential adverse effects identified through preclinical safety tests with rh-HGF include a decrease in blood pressure (BP) and an increase in urinary excretion of albumin. Therefore, we further investigated the effect of rh-HGF on circulatory status and renal toxicity in preclinical animal studies. In a clinical trial, 20 patients with FH or LOHF were evaluated for participation in this clinical trial, and four patients were enrolled. Subjects received rh-HGF (0.6 mg/m^2/day) intravenously for 12 to 14 days.

Results: We established an infusion method to avoid rapid BP reduction in miniature swine, and confirmed reversibility of renal toxicity in rats. Although administration of rh-HGF moderately decreased BP in the participating subjects, this BP reduction did not require cessation of rh-HGF or any vasopressor therapy; BP returned to resting levels after the completion of rh-HGF infusion. Repeated doses of rh-HGF did not induce renal toxicity, and severe adverse events were not observed. Two patients survived, however, there was no evidence that rh-HGF was effective for the treatment of FH or LOHF.

Conclusions: Intravenous rh-HGF at a dose of 0.6 mg/m^2 was well tolerated in patients with FH or LOHF; therefore, it is desirable to conduct further investigations to determine the efficacy of rh-HGF at an increased dose.

Background

Acute liver failure (ALF) is a rare but fatal clinical syndrome marked by the abrupt loss of hepatic cellular function, with the subsequent development of coagulopathy, jaundice and encephalopathy [1-3]. In Japan, ALF with the histological appearance of hepatitis, caused by viral infection, autoimmune hepatitis and drug allergy-induced liver injury, is classified as fulminant hepatitis (FH) or as the related disease late-onset hepatic failure (LOHF) [4]. FH is identified as hepatitis in which hepatic encephalopathy develops within 8 weeks after the onset of disease symptoms, with prothrombin time (PT) less than 40% of the standardized values. Also, FH is further classified into two subtypes: acute (FHA) and subacute type (FHSA) in which the encephalopathy occurs, respectively, within 10 days or after 11 days or more. Patients in whom the

* Correspondence: ido-akio@m2.kufm.kagoshima-u.ac.jp
[1]HGF Hepatic Regeneration Therapy Project, Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, Kyoto, Japan
Full list of author information is available at the end of the article

encephalopathy develops between 8 and 24 weeks after disease onset with PT less than 40% are diagnosed as having LOHF. This distinction is useful in guiding prognosis: the time to onset of encephalopathy is negatively correlated with outcome. The only effective therapy for FH is liver transplantation. Other therapies, including corticosteroids, have no demonstrable benefit [5], lamivudine for acute hepatitis B [6], and plasmapheresis [7]. Therefore, patients with FH who did not receive liver transplantation had extremely poor prognoses: the survival rates were 53.7% in FHA and 24.4% in FHSA, and 11.5% in LOHF in Japan [4].

Hepatocyte growth factor (HGF) was first purified as a potent mitogen for hepatocytes from the plasma of patients with FH [8,9]. HGF is one of the primary agents promoting the proliferation of mature hepatocytes [10-12]. The stimulatory effect of HGF on liver regeneration has been observed *in vivo* using normal and partially hepatectomized rats [11]. Additionally, HGF stimulates proliferation of hepatic progenitor cells, which appear following hepatic injury [13]. Furthermore, recent investigations using mice deficient in c-met, a specific receptor for HGF, demonstrated that the HGF/c-met signaling pathway is essential for efficient liver regeneration and repair [14,15]. Conversely, HGF exerts protective and anti-apoptotic functions toward hepatocytes *in vitro* [16-18] and *in vivo* [19-21], and is able to prevent Fas (CD95/APO-1)-triggered death of adult hepatocytes, leading to rescue from Fas-induced fulminant hepatic failure [20]. These results indicate that HGF has the potential to be a new therapeutic agent for ALF through its mitogenic and anti-apoptotic activities.

We have worked to develop translational medicine protocols for recombinant human HGF (rh-HGF), and have performed an investigator-initiated International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH)-Good Clinical Practice (GCP)-registered phase I/II clinical trial of rh-HGF. As this application is the first clinical trial to administer rh-HGF to humans, we performed additional preclinical studies to ensure minimization of the predicted side effects, and then treated four patients with repeated doses of rh-HGF in order to evaluate the safety, pharmacokinetics and clinical efficacy of FH therapy.

Methods

Animal experiments to ensure safety of rh-HGF administration

Animals

Female Crown miniature swine, six to seven months of age, and male Wistar rats, seven weeks of age, were obtained from Japan Farm (Kagoshima, Japan) and Charles River Laboratories Japan Inc. (Yokohama, Japan), respectively. The animals were maintained under constant room temperature (25°C), and given free access to water and the indicated diet throughout the study. The protocol for animal studies was approved by the ethics committee of the Graduate School of Medicine, Kyoto University (Kyoto, Japan). All animal experiments were performed after one to three weeks acclimation on a standard diet.

General pharmacological test

After Female Crown miniature swine were anesthetized by inhalation of sevoflurane, nitric dioxide and oxygen, catheters were inserted into one internal jugular vein (for injection of rh-HGF) and to one common carotid artery (to measure BP). One mg/kg of rh-HGF was injected through the internal jugular vein over the course of 20 min. HR was recorded by electrocardiographic monitoring, and cardiac function was measured via echocardiography. To evaluate the effect of stepwise infusion of rh-HGF on BP, 0.4 mg/kg of rh-HGF was injected over the course of three hours, with a stepwise increase in dose rate (10% of the total dose over the first 60 min, 30% over the next 60 min, and 60% over the last 60 min) through the catheter inserted into an internal jugular vein.

Evaluation of renal toxicity of repeat dose of rh-HGF

rh-HGF (0.4, 1.0 and 4.0 mg/kg) was administered to rats intravenously in a bolus for 14 days, followed by observation for 2 weeks. Urinary excretion of albumin and protein were measured periodically during and after rh-HGF administration. Animals were sacrificed at the ends of rh-HGF administration (day 14) and the observation period (day 28) to evaluate renal involvement, including serum creatinine and histological findings.

A phase I/II clinical trial for patients with acute liver failure

Overview

This single-arm, open-labeled, and dose-escalation study was conducted at Kyoto University Hospital, Kyoto, Japan. Study protocols were reviewed and approved by the Investigational Review Board and Ethics Committee governing Kyoto University Hospital before the commencement of patient enrollment. Studies were performed in accordance with principles of GCP, and conformed to ethical guidelines of the Declaration of Helsinki. All participating patients, or (when participants were not able to subscribe because of hepatic encephalopathy) their legal representatives provided written informed consent before being enrolled into the study.

Selection of patients

Consenting patients were prospectively screened from September 2005 to June 2008. Eligible patients with FHSA or LOHF, who were not able to receive liver transplantation, met at least one of the following four

parameters: (1) aged 45-year-old or above, (1) PT 10% or less of the standardized values, (3) total bilirubin (T-Bil) level of 18.0 mg/dL or more, or (4) direct/total bilirubin ratio less than 0.67. The following patients were not eligible: those under 16 years old; those treated with glucagon and insulin, or prostaglandin E1 48 hours before registration; those with presence or past-history of malignant tumors; those with heart failure; those with severe complication including pneumonia, sepsis, disseminated intravascular coagulation syndrome or gastrointestinal bleeding; and those with allergic reaction against rh-HGF. Pregnancy-aged women were also ineligible, because toxicity of rh-HGF to reproductive development in female animals has not been examined. Additionally, patients were also excluded on the grounds of renal involvement, including urinary excretion of ≥1 mg/mL protein, deformed red blood cells or RBC casts in sedimentary urine, a serum creatinine level of 2.0 mg/dL or more, or urine volume less than 400 mL/day.

Protocol therapy and observation after rh-HGF dosing period

rh-HGF was prepared as a GMP-grade material. The initial dose of rh-HGF was fixed at 0.6 mg/m^2/day, which ensured not only safety but also clinical efficacy, as determined by several preclinical animal studies. In this dose escalation study, dose of rh-HGF can be increased from the initial dose (0.6 mg/m^2) to 1.2, 1.8 or 2.4 mg/m^2. rh-HGF was administered intravenously with a stepwise increase during 3 hours for up to 14 days, followed by a 14-day observation period. All patients were followed in order to determine the outcomes after the study period (up to 28 days).

End points

The primary endpoint of interest was the safety of repeated doses of intravenous rh-HGF, which was evaluated on the basis of the occurrence, frequency, and severity of adverse events. All patients were treated in an intensive care unit. During the on-study period, patients were monitored for safety at regular intervals from the start of rh-HGF administration until 14 days after completion of study drug dosing. Safety assessments included physical examination, clinical laboratory test and adverse events. Adverse events were monitored throughout the duration of the study, and evaluated in terms of adverse events graded according to the Common Toxicity Criteria grading system. Causal association of adverse events with rh-HGF was determined by clinician's best judgment. All adverse events were treated appropriately regardless of the cause; where necessary, patients were withdrawn from the study. The incidence of adverse events was computed from the number of patients experiencing at least one adverse event from among those who received at least a single dose of rh-HGF.

The secondary endpoints were the pharmacokinetics of intravenously injected rh-HGF and clinical efficacy, including survival period and outcome. To examine pharmacokinetics of rh-HGF, blood samples were collected for analysis of rh-HGF at multiple time points on days 1, 3, 5, 8, and 11 for assessment. Serum concentrations of HGF were determined by enzyme-linked immunosorbent assay (ELISA) (Otsuka Co., Ltd., Tokushima, Japan) [22]. Laboratory data, including PT-international normalized ratio (PT-INR), T-Bil, serum albumin, alanine aminotransferase (ALT), and α-fetoprotein (AFP), were examined before plasma exchange or rh-HGF administration.

Statistical analysis

To evaluate survival benefits by administration of rh-HGF, the stratified proportional hazards model was used for analyzing matched datasets. All statistical analyses were done using SAS version 9.1 (SAS Institute, Inc., Cary, NC).

Results

Establishment of rh-HGF dosing method to respond to a decrease in blood pressure in miniature swine

In general pharmacological tests, intravenous rh-HGF (1.0 or 0.2 mg/kg) caused a rapid decrease in systolic blood pressure (BP) in miniature swine, whereas respiratory status was not affected (data not shown). Therefore, before starting the clinical trial, we further investigated the effect of rh-HGF on circulatory status in miniature swine under general anesthesia. When a total dose of rh-HGF of 1.0 mg/kg was administered over the course of 20 min, a decrease in systolic BP occurred promptly, and continued throughout rh-HGF administration (Figure 1A). Although heart rate (HR) gradually decreased, no electrocardiographic abnormalities, including arrhythmia and ischemic changes, were observed throughout the experimental period. Additionally, cardiac ultrasonography showed a decrease in left ventricular end-diastolic volume (LVEDV) as well as ejection fraction (EF), in parallel with a decrease in BP, but no abnormalities of left ventricular movement (Figure 1A). These results indicate that intravenous injection of rh-HGF reduced BP through dilatation of capacitance vessels.

Next, we tried to develop a method for rh-HGF administration that would avoid rapid BP reduction. We finally established a stepwise infusion method in which rh-HGF was administered with a stepwise increase over the course of three hours (10% dose for 60 min, 30% for next 60 min, and 60% for the last 60 min) (Figure 1B). We found that appropriate infusion effectively prevented the decrease in BP caused by intravenous rh-HGF administration (Figure 1C). The preventive effect of additional infusion also supports the idea that dilatation

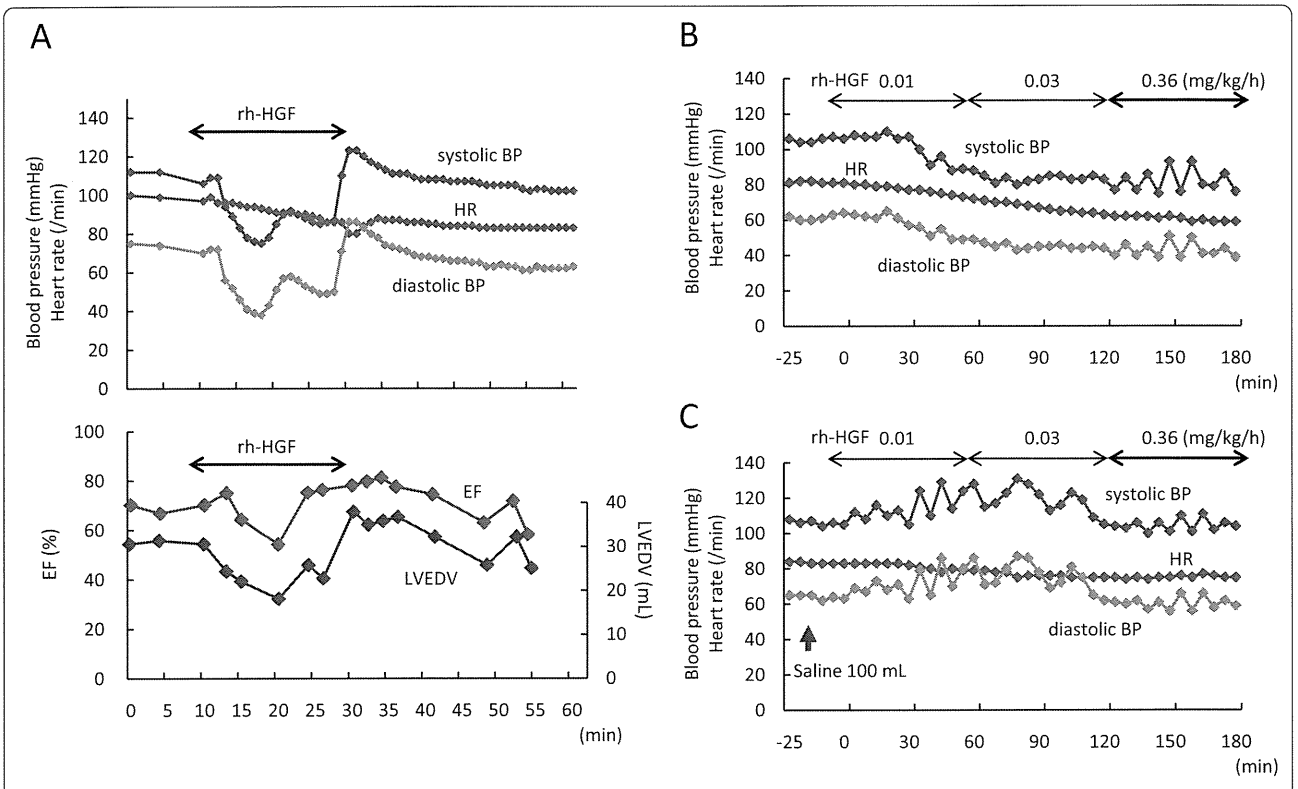

Figure 1 Intravenous injection of rh-HGF reduced blood pressure through capacitance vessels in miniature swine. Effect of intravenously administered rh-HGF on BP, HR, and cardiac function was examined in miniature swine under general anesthesia. (A) Intravenous injection of rh-HGF (1.0 mg/kg) rapidly reduced systolic and diastolic BP. Reduced BP was persistent during rh-HGF administration (for 20 min), and was immediately recovered after the rh-HGF injection. Echocardiography showed that ejection fraction (EF) and left ventricular end-diastolic volume (LVEDV) were reduced during rh-HGF administration. (B) rh-HGF (0.4 mg/kg) administered for three hours with a stepwise increase (0.01 mg/kg for first 60 min, 0.03 mg/kg for the next 60 min, and 0.36 mg/kg for the last 60 min) gradually decreased BP and HR. (C) Infusion of 100 mL of saline prior to rh-HGF administration prevented a decrease in BP during exposure to rh-HGF.

of capacitance vessels is a cause of HGF-induced BP reduction.

Evaluation of renal toxicity induced by repeated dose of rh-HGF in rats

Repeated dose toxicity tests using rats or cynomolgus monkeys identified an increase in urinary excretion of albumin and protein as a potential adverse event in a clinical trial. Therefore, we further examined whether renal toxicity induced by repeated rh-HGF dosing for 14 days was reversible. We intravenously administered 0.4, 1.0, and 4.0 mg/kg/day of rh-HGF to rats for 14 days, followed by a 14-day observation. Urinary excretion of albumin increased in rats treated with rh-HGF from day 4 in a dose dependent manner (Figure 2). In animals treated with 0.4 or 1.0 mg/kg/day of rh-HGF, excretion of urinary albumin preceded an increase in proteinuria (Figure 2A and 2B). Conversely, neither serum creatinine nor BUN were affected throughout the experimental period, and increased urinary excretion of albumin gradually decreased after the completion of rh-HGF dosing during the 14-day observation period. In histological analysis, mesangial expansion, hyaline droplet deposition in glomeruli and tubules, and renal hypertrophy were observed after repeated doses of rh-HGF for 14 days; however, these histological findings were in the slight-to-mild range, and still identified as reversible changes (data not shown). In a clinical trial, the clinical dose of rh-HGF, 0.6 mg/m^2, corresponds to 0.1 mg/kg in rodents. Therefore, renal toxicity, induced by repeated rh-HGF dosing for 14 days, would be predicted to be reversible; furthermore, excretion of urinary albumin is a useful way to monitor renal toxicity.

Patient characteristics

Between September 2005 and June 2008, 20 patients with FHSA or LOHF were evaluated for participation in the clinical trial of rh-HGF. Sixteen patients were excluded because they met one or more of the exclusion criteria. Consequently, four patients were enrolled; despite a dose-escalation study, only the initial dose of rh-HGF (0.6 mg/m^2) was administered. Among the

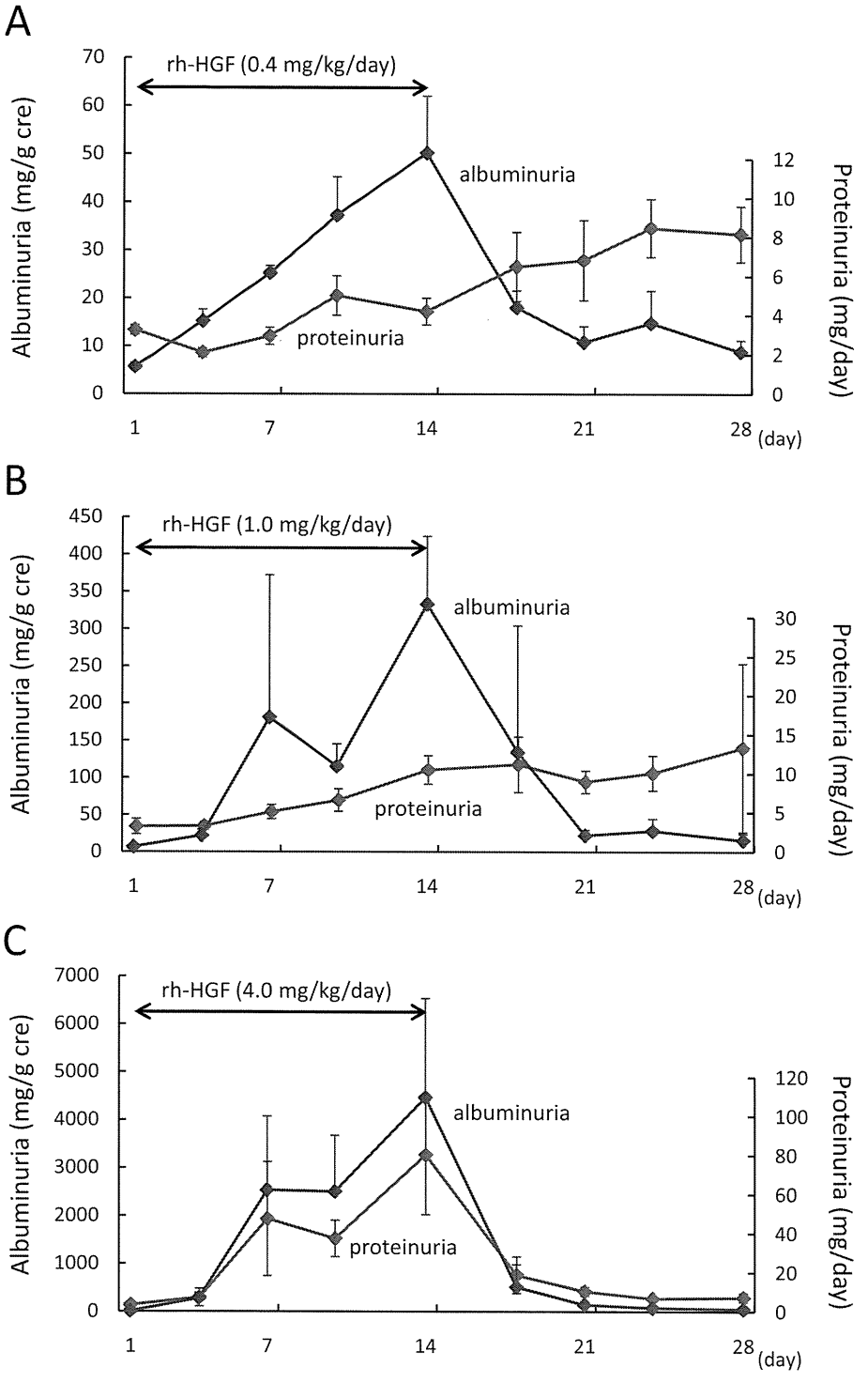

Figure 2 Repeated dose of rh-HGF induced an increase in urinary excretion of albumin and protein in rats. Rats were administered rh-HGF, 0.4 (A), 1.0 (B), and 4.0 mg/kg/day (C) (n = 4 for each), intravenously for 14 days, and urinary excretion of albumin and protein was measured before (day 1), during (days 7 and 14), and 7 and 14 days after HGF administration. Repeated doses of rh-HGF induced an increase in urinary albumin excretion in dose dependent manner. Urinary excretion of albumin was reversible even when dosing 4.0 mg/kg/day of rh-HGF (C), and preceded an increase in proteinuira in rats treated with 0.4 and 1.0 mg/kg of rh-HGF (A and B, respectively).

participating subjects, the age was between 40 and 71, and two were male (Table 1). Patients 1, 2 and 4 were diagnosed as having FHSA, and patient 3 as having LOHF. These four patients were not able to receive liver transplantation, because patients 1, 3, and 4 lacked appropriate donors, and patient 2 was over 70 years old. FHSA in patients 1 and 4 was caused by HEV and a supplement containing coenzyme Q-10, respectively, whereas the cause of hepatic failure in patients 2 and 3 was undetermined. Two patients with FHSA (patients 1 and 2) and one with LOHF (patient 3) exhibited hepatic encephalopathy at grade II and V, respectively, whereas the consciousness level of patient 4 with FHSA was not impaired at the time of enrollment. In all patients, markedly prolonged PT and an increase in T-Bil and serum HGF were observed. Patient 2, with FHSA, and patient 3, with LOHF, exhibited reduced liver volume as determined by CT volumetry at enrollment. Treatment with rh-HGF was started between five and seven days after appearance of hepatic encephalopathy. rh-HGF (0.6 mg/m^2/day) was intravenously administered for 14 days in patients 2 and 4. Patients 1 and 3 required cessation of rh-HGF on days 14 and 13, respectively, because of increased serum creatinine (2.1 mg/dL) and oliguria, respectively. Both of these symptoms were determined to accompany hepatic failure, but not rh-HGF dosing. Thus, these patients were subject to a total of 13- and 12-day HGF administration regimens, respectively. Plasma exchange was performed in all patients. Three patients, except for patient 1 with FHSA caused by HEV, were treated with corticosteroid (Additional file 1, Additional file 2, Additional file 3, Additional file 4). Finally, two of the patients with FHSA (2 and 4) survived, whereas the other two patients died. Patient 1, who had FHSA, died after the study period; patient 3, who had LOHF, died during the study period (Table 1).

Pharmacokinetics of stepwise infusion of rh-HGF for three hours

In patients 1, 2, and 3, rh-HGF was administered after plasma exchange. Serum levels of HGF increased in parallel with a stepwise increase of rh-HGF dosing, and reached maximum drug concentration (Cmax) at the end of a three-hour rh-HGF injection (Figure 3). Cmax gradually increased from 18.8 ± 6.0 ng/mL on day 1 to 22.3 ± 9.6 ng/mL on day 11 during the HGF dosing period (Table 2). The mean value of half-life ($T_{1/2}$) was approximately 630 to 840 min. The area under the blood concentration-time curve (AUC) gradually increased, and the clearance (CL) and steady-state volume of distribution (Vdss) appeared to gradually decrease, during the HGF dosing period.

Intravenous rh-HGF was well tolerated in all patients with FH or LOHF

Preclinical safety studies revealed that a decrease in BP during rh-HGF infusion and renal toxicity induced by repeated rh-HGF dosing, including an increase in urinary excretion of albumin, were potential adverse events in a human study. In the phase I/II study of patients

Table 1 Patient characteristics

Patient No.	1	2	3	4
Age/Gender	67/M	71/F	64/F	40/M
Diagnosis/Etiology	FHSA/HEV	FHSA/unknown	LOHF/unknown	FHSA/drug
Reason for not receiving LT	donor[1]	age[2]	donor[1]	donor[1]
Before rh-HGF administration				
Grade of HE	II	II	V	0
Prothrombin time INR (%)	2.07 (33)	1.55 (49)	1.78 (37)	1.62 (43)
Albumin (g/dL)	2.9	3.2	2.9	2.9
T-Bil (mg/dL)	11.2	6.9	11.7	27.6
Direct/total bilirubin ratio	0.58	0.41	0.44	0.71
ALT (IU/L)	32	131	260	253
Serum HGF (ng/mL)	0.77	1.94	1.07	1.88
AFP (ng/ml)	7.0	22.9	3.9	39.7
Liver volume (mL)	1055	595	640	1110
Days between HE and rh-HGF administration (days)	7	5	5	5
Duration of rh-HGF dosing (days)	13	14	12	14
Outcome				
during the study period	alive	alive	dead	alive
during the follow-up period	dead	alive	-	alive

FHSA, fulminant hepatitis subacute type; LOHF, late onset hepatc failure; HEV, hepatitis E virus; LT, liver transplantation; HE, hepatic encephalopathy. [1]lack of an appropriate donor; [2]age 70 or over.

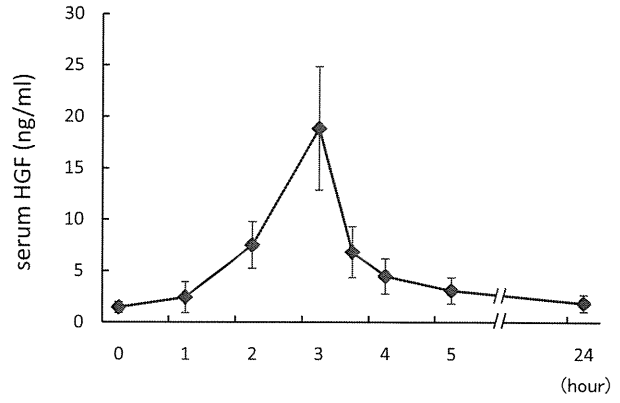

Figure 3 Sequential changes in serum HGF concentration during and after rh-HGF administration. rh-HGF (0.6 mg/m^2) was administered intravenously with a stepwise increase for three hours (0.06 mg/m^2 for 60 min, 0.18 mg/m^2 for next 60 min, and 0.36 mg/m^2 for last 60 min). Serum levels of HGF were measured by ELISA. Sequential changes in (A) serum HGF levels on day 1 of rh-HGF dosing period.

with FH or LOHF, respiratory status was not affected by rh-HGF administration in any patient, but BP was decreased mildly to moderately from approximately one hour after the beginning of HGF injection in patients 1, 2 and 3 (Figure 4). As HGF reduces BP through dilatation of capacitance vessels, the HR increased up to 30%. However, this decrease in BP did not require cessation of rh-HGF or any vasopressor therapy, and BP returned to resting levels after the completion of HGF administration. Patient 2, who awoke from hepatic encephalopathy on day 3 of the HGF dosing period, did not suffer from any symptoms during HGF administration, even though the HR increased up to ~30% (Figure 4).

Table 2 Pharmacokinetic parameters of rh-HGF

parameters	Estimate values	95% confidence interval	
Day 1			
C_{max} (ng/mL)	18.8	13.0	24.7
$AUC_{0\text{-}300}$ (ng/mL*min)	1485.6	991.3	1979.8
$AUC_{0\text{-}\infty}$ (ng/mL*min)	1994.0	1214.6	2773.3
$T_{1/2}$ (min)	756.2	526.8	985.7
CL (mL/m^2/min)	0.000361	0.000160	0.000561
V_{dss} (mL/m^2)	0.125	0.063	0.186
Day 5			
C_{max} (ng/mL)	21.3	12.8	29.9
$AUC_{0\text{-}300}$ (ng/mL*min)	1727.2	1099.7	2354.7
$AUC_{0\text{-}\infty}$ (ng/mL*min)	2493.8	1647.0	3340.5
$T_{1/2}$ (min)	843.6	540.5	1146.6
CL (mL/m^2/min)	0.000277	0.000138	0.000416
V_{dss} (mL/m^2)	0.106	0.059	0.153
Day 11			
C_{max} (ng/mL)	22.3	11.4	33.1
$AUC_{0\text{-}300}$ (ng/mL*min)	1965.5	801.6	3129.5
$AUC_{0\text{-}\infty}$ (ng/mL*min)	3126.4	1355.2	4897.5
$T_{1/2}$ (min)	633.3	318.0	948.6
CL (mL/m^2/min)	0.000230	0.000095	0.000365
V_{dss} (mL/m^2)	0.088	0.031	0.146

All patients showed slight to mild increase in urinary excretion of albumin at enrollment and a decrease in urine volume during the rh-HGF study period. However, repeated doses of rh-HGF did not increase urinary excretion of albumin, and urine volume was affected by several factors other than rh-HGF administration, including volume of infusion, amount of circulating plasma, and diuretic dosing. Although hypokalemia, anemia, a decrease in platelet count, prolonged PT, a decrease in anti-thrombin III, and hematuria were also observed in three of four patients, there was no apparent evidence for a causal relationship between these adverse events and rh-HGF administration. Patient 3, who died of advanced hepatic failure during the observation period, exhibited respiratory failure. However, this severe adverse event was associated with progression of hepatic failure, not rh-HGF; no other severe adverse events directly caused by single or repeated doses of rh-HGF were observed during the study period.

HGF administration did not show a beneficial effect on hepatic encephalopathy, laboratory data results, or patient survival

Three out of four patients exhibited hepatic encephalopathy at enrollment (Table 1). Patient 1 presented with grade II hepatic encephalopathy at the beginning of protocol therapy. This patient did not recover from hepatic encephalopathy either during or after the study period. The patient ultimately died 68 days after the onset of hepatic encephalopathy (Additional file 1). In patient 2, who had FHSA and ultimately survived, plasma exchange was performed on days 2, 4, and 8 during the HGF dosing period (Additional file 2), and hepatic encephalopathy had improved by day 3. Patient 3 showed advanced hepatic encephalopathy at enrollment. Although the consciousness level was transiently alleviated during the rh-HGF dosing period, hepatic encephalopathy continued to progress during the observation period; the patient died 28 days after the onset of hepatic encephalopathy (Additional file 3). Patient 4 had already recovered from hepatic encephalopathy at enrollment, and did not show any impairment of consciousness level during the study period (Additional file 4). Consequently, we did not observe a definite effect of rh-HGF administration on hepatic encephalopathy.

Laboratory data results, including PT-INR, T-Bil, serum albumin, and ALT, were not affected during the rh-HGF dosing and observation period (Figure 5). In

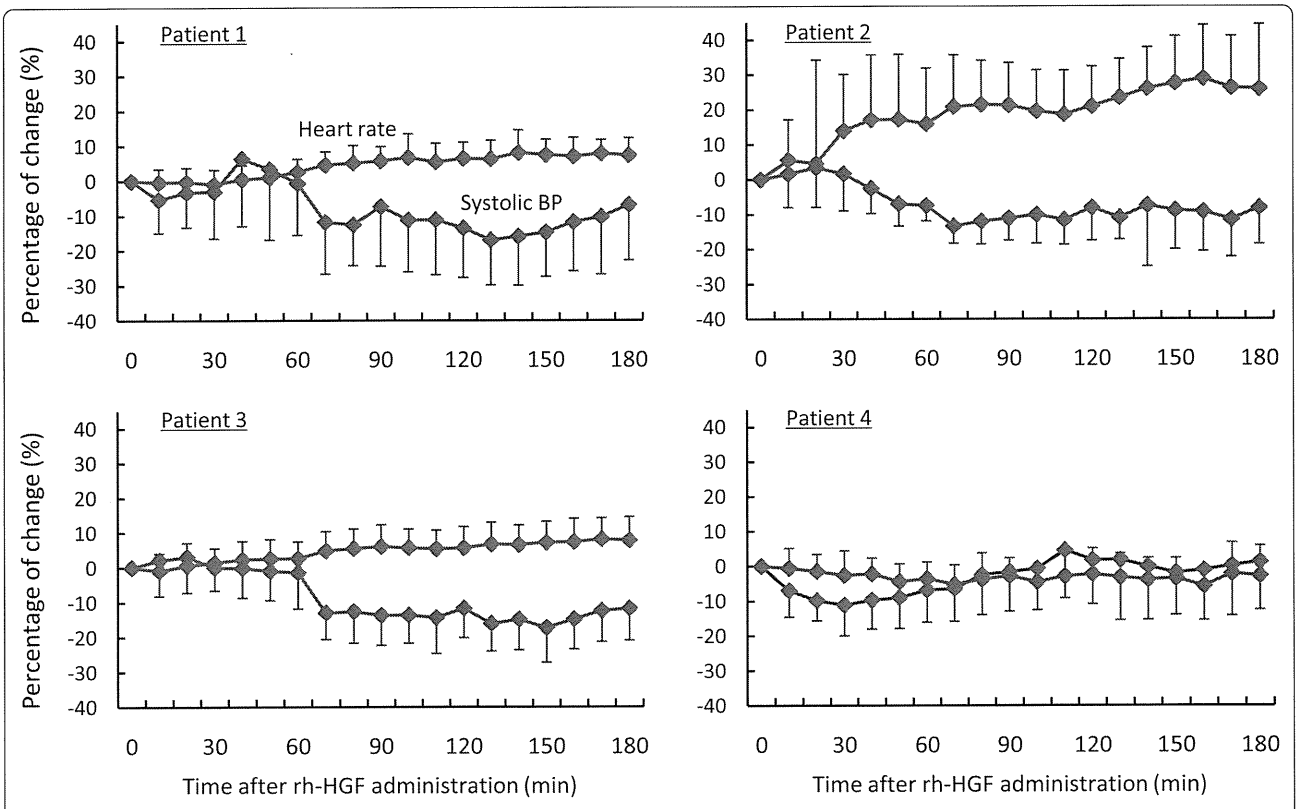

Figure 4 Blood pressure decreased during infusion of rh-HGF in patients with FH or LOHF. BP and HR were monitored during rh-HGF infusion for three hours. Intravenous rh-HGF (0.6 mg/m^2) reduced systolic BP, and increased HR in patients 1, 2 and 3. BP reduction during rh-HGF infusion did not affected patients' general condition. BP immediately recovered following the completion of rh-HGF administration.

patient 1, serum AFP, which is known to increase not only during development of hepatocellular carcinoma but also liver regeneration, modestly increased during the rh-HGF dosing period, followed by a gradual decrease during the observation period. Conversely, patients 2 and 4, who ultimately survived, exhibited an increase in serum AFP at enrollment, whereas AFP levels gradually decreased throughout the study period. However, no definite effect of rh-HGF dosing on serum AFP levels was observed.

To assess the effect of administration of rh-HGF on patient survival, we selected subjects as a control, who matched each patient in diagnosis (FHSA or LOHF), age (≥45 or <45), gender, PT (<10% or ≥10%), T-Bil (≤18.0 or >18.0 mg/dL) and direct/total bilirubin ratio (≤0.67 or >0.67), from the data of national survey of FH and LOHF in Japan between 1998 and 2006. Consequently, we set 57 control subjects for patients 1 and 2, 13 for patient 3, and 17 for patient 4, and estimated hazard ratios using the stratified proportional hazards model. The survival time from the onset of hepatic encephalopathy or disease in patients treated with rh-HGF was slightly longer than that in control subjects, but the difference was not statistically significant (Table 3).

Discussion

This clinical trial covered patients with FH, an extremely severe and fatal liver disease: subjects enrolled in this trial are predicted to die without liver transplantation. Indeed, a nationwide survey of the patients with FH or LOHF (1998-2002) in Japan revealed that the survival rate of the patients (n = 192) who met this study's inclusion criteria was 17.7% (n = 34). Additionally, FH is a relatively rare syndrome in Japan (698 patients between 1998 and 2003) [4]; patients with severe complications, especially renal dysfunction and heart failure, were excluded in order to more precisely evaluate the safety and efficacy of the proposed therapy. Therefore, we had difficulty with recruitment of trial subjects. Ultimately, we recruited only four patients to our institute, Kyoto University Hospital, for treatment with the initial dose of rh-HGF.

Predicted adverse events included a decrease in BP, by dilatation of capacitance vessels, and proteinuria. Therefore, we established a stepwise infusion method to avoid a rapid reduction of BP, and confirmed reversibility of renal toxicity through additional preclinical studies. In this clinical trial, rh-HGF was administered intravenously for 12 to 14 days, and severe side effects and