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In vivo repopulation of cytoplasmically gene transferred hematopoietic cells by temperature-sensitive mutant of recombinant Sendai viral vector ☆

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Abstract

Recent clinical studies revealed 'proof of concept' of gene therapy targeting hematopoietic stem cells (HSCs) to treat hematopoietic disorders. However, vector integration-related adverse events of retroviral vectors have slowed progress in this field. As an initial step to overcoming this hurdle, we examined the potential of an improved cytoplasmic RNA vector, temperature-sensitive mutant non-transmissible recombinant Sendai virus (ts-rSeV/dF), for gene transfer to murine HSCs and progenitors. Both conventional vector and ts-rSeV/dF-GFP showed efficient gene transfer to T-lymphocyte-depleted syngeneic bone marrow cells (BMCs) (>85%), but only BMCs treated with ts-rSeV/dF-GFP but not with conventional vector efficiently repopulated in the recipient mice, associated with multilineage differentiation *in vitro* and *in vivo*. To our knowledge, this is the first demonstration of the *in vivo* reconstruction of hematopoietic series by cytoplasmically gene transferred BMCs, that warrants further investigation to realize this strategy in clinical settings.

Keywords: Sendai viral vector; Hematopoietic stem cells; Cytoplasmic gene therapy; Severe immunodeficiency syndrome; Bone marrow transplantation

Gene therapy targeting hematopoietic cells is a promising strategy for treating inherited hematopoietic disorders. Hematopoietic stem cells (HSCs) in particular are an ideal target, because they can reconstitute the entire hematopoietic system in a recipient during a lifetime. Recent clinical success in clinical gene therapy for X-linked SCID using a retroviral vector expressing the common γ-chain revealed the 'proof of concept' of the strategy and has given encouragement to physicians, patients, and scientists, but a serious adverse event, T-cell leukemia, which is related to the insertion of a pro-viral genome into the host chromosome

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mediated by retroviral vector, is an obstacle to developing the gene therapy strategies in this field [1–4].

We recently developed a novel viral vector for efficient gene transfer, recombinant Sendai virus (rSeV), and demonstrated highly efficient gene transfer to various organs *in vivo*, including airway epithelial cells, vascular tissue, skeletal muscle, and joint synovium [5–8] by using replication-competent additive-type rSeV; these findings are also consistent with a clinically available fusion gene-deleted non-transmissible rSeV (rSeV/dF) [9]. Since rSeVs uses a cytoplasmic transcription system, it can mediate gene transfer to a cytoplasmic location without the DNA phase [10] and therefore theoretically avoid a vector integration-related adverse event. In addition, there are technical advantages in the use of rSeVs for gene transfer to HSCs. First, vector particles can be easily concentrated to high titers, unlike retroviral vectors. Second, and most importantly, the modalities of target cell processing and viral transduction are technically non-demanding and feasible in clinical situations that require transduction into large numbers of target cells. Using replication-competent additive-type rSeV, we previously demonstrated a highly efficient gene transfer to HSCs derived from human cord blood preserving their functions to differentiate into the entire hematopoiesis series *in vitro* [11]; however, repopulation of HSCs has been unsuccessful when these cells were treated with additive-type rSeV as well as rSeV/dF (unpublished data). Therefore, we have again turned our focus to improving the rSeV system in view of reduced immunogenicity and cytotoxicity.

Recently, we newly developed temperature-sensitive mutant non-transmissible rSeV (rSeV/dF$P^{ts}M^{ts}HN^{ts}L^{ts}$, abbreviated as ts-rSeV/dF) [12,13], as an improved version of rSeV/dF. This new vector loses the expression of envelope-related genes M and HN at 37 °C [12,13], resulting in dramatically reduced cytotoxicity as well as innate immune responses in the murine lung [14].

In this study, therefore, we examined the potential of SeV as a cytoplasmic transcription system for gene transfer to HSCs and hematopoietic progenitors.

Materials and methods

Animals. Female 7-week-old C57BL/6 mice of Charles River grade were obtained from KBT Orientals Co., Ltd. (Tosu, Saga, Japan) and kept under specific pathogen-free and humane conditions. The GFP-TG mice (C57BL/6-TgN(act-EGFP) OsbC14-Y01-FM131) [15] were a gift from Prof. Okabe, Osaka University, Osaka, Japan. These mice were used for all experiments except the CTL assay. All animal experiments were carried out according to the protocols approved by the Institutional Committee for Animal Experiments and by the Institutional Committee for Recombinant DNA and Infectious Pathogen Experiments, Kyushu University. The experiments were carried out in accordance with recommendations for the proper care and use of laboratory animals and according to The Law (No. 105) and Notification (No. 6) of the Japanese Government.

rSeV vectors. rSeV/dF-GFP and ts-rSeV/dF-GFP were constructed as previously described [9,12,13]. A series of F-deficient SeV vectors were prepared by using recombinant LLC-MK_2cells carrying the F gene (LLC-MK_2/F7). An adenovirus vector, AxCANCre, expressing Cre recombinase was used for the induction of F protein in LLC-MK_2/F7 cells (referred to as LLC-MK_2/F7/A). Recombinant vaccinia virus vTF7-3 carrying T7 RNA polymerase was inactivated with psoralen and long-wave UV irradiation, and then used for the ribonucleoprotein complex recovery. The viral vectors were further amplified by several rounds of propagation. The titers of the recovered viral vectors were expressed as cell infectious units [9].

Gene transfer to BM cells and BMT. BMCs from donor mice were flushed under sterile conditions with RPMI 1640 with 10% fetal calf serum from the medullary cavities of tibiae and femurs using a 23-gauge needle. Red blood cells were depleted using Lysing buffer (0.38% NH_4Cl, Tris–HCl, pH 7.65), and mature T-cells were depleted with anti-Thy1.2 monoclonal antibody (Sigma, St. Louis) plus selected rabbit complement (Cedalene, Ontario, Canada). Gene transfer was carried out by simply adding MOI = 10 of vector solution to the media at 37 °C for 1 h, and 2×10^7 (C57BL/6) BMCs were given by tail vein injection. For bone marrow ablation, the recipient mice were treated by whole-body γ-irradiation at lethal dose (10 Gy) 4–5 h before BMT. For blood cell count, PBCs were obtained via the tail vein, diluted, and counted by an automatic cytometer (CelltacαMEK-6158 NIHON KOHDEN, Tokyo, Japan).

Flow cytometry analyses. At appropriate time points, PBCs or tissue samples were obtained and subjected to flow cytometry analysis. For immune cell subset analysis, cells from each organ were stained with CD3-APC/DX5-PE (for pan-T-cells), CD3-APC/CD4-PE (CD4/T-cells), CD3-APC/CD8-PE (CD8/T-cells), CD11b-PE/CD11c-APC (monocyte/macrophages), and B220-APC/IgM-PE (B-cells) (all antibodies were from BD Pharmingen, CA) by FACSCalibur (Becton Dickinson, CA). For *in vitro* analysis of each progenitor population, whole BMCs, 48 h after ts-rSeV/dF-GFP exposure, were stained with biotin-conjugated anti-lineage (lineage panel; BD pharmingen), anti-Sca-1-APC, anti-c-kit-PE antibodies and streptavidin for 30 min. Propidium iodide-positive dead cells were excluded. Data were evaluated using Cellquest™ (Becton Dickinson) or Flowjo (Tree Star Inc., CA) software.

Enrichment of HSCs and colony assay. Enrichment of HSC via FACS Aria (Becton Dickinson) was previously described [16,17]. Briefly, mononuclear cells were obtained from BMCs supplemented with Lympholyte-M (Cedalene), and lineage$^+$ cells were removed with sheep anti-rat IgG conjugated magnetic beads (Dynal, A.S.), and with anti-c-kit-APC antibody and anti-APC microbeads (Dynal, A.S.). The remaining cells were stained with biotin-conjugated anti-lineage-cocktail (BD Pharmingen), streptavidin-perCP-Cy5, anti-c-Kit-APC, and anti-Sca-1-PE, and were sorted in duplicate. For the colony assays, an enriched HSC population was cultured in MethoCult™ Media (M3434; StemCell Technologies, Vancouver, BC, Canada), and exposed to ts-rSeV/dF-GFP for 1 h. After washing, 1000 cells were cultured in 1 ml Methocult media per dish (35 mm) for 10–12 days. CFU-Mix colonies were observed and analysed by BZ-8000 and BZ-Analyzer (KYENECE, Osaka, Japan).

Statistical analysis. All data are expressed as the mean ± SD, and were analyzed by one-way ANOVA with Fisher's adjustment, with the exception of the data on animal survival. Survival was plotted using Kaplan–Meier curves, and statistical relevance was determined using a log–rank comparison. $P < 0.05$ was considered significant.

Results

Efficient engraftment and repopulation of cytoplasmically gene transferred murine bone marrow cells by ts-rSeV/dF in C57BL/6 syngeneic recipient mice

We first evaluated the gene transfer efficiency of recombinant ts-rSeV/dF-GFP compared to that with rSeV/dF-GFP mediated gene transfer to murine (7-week-old female C57BL/6) BMCs depleted with erythrocyte and mature T-cells. We here used whole BMCs instead of an enriched stem cell fraction to avoid the loss of experimental animals

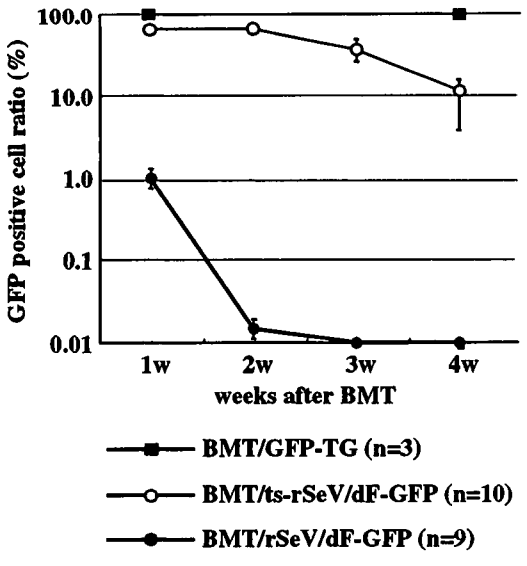
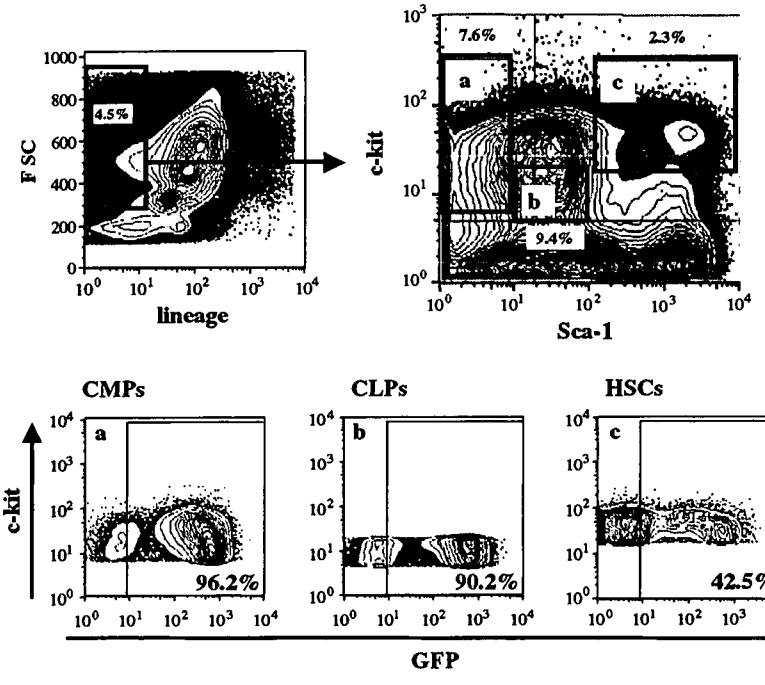


Fig. 1. Time course of GFP gene transferred cell ratio in peripheral blood cells (PBCs) after bone marrow transplantation (BMT). After bone marrow transplantation of 2×10^7 BMCs treated with ts-rSeV/dF-GFP (BMT/ts-rSeV/dF-GFP, $n = 10$) or conventional rSeV/dF-GFP (BMT/rSeV/dF-GFP, $n = 9$), the PBCs were collected via the tail vein of C57BL/6 recipient mice at each time point, and GFP expression was determined by FACS analysis. BMCs from GFP-transgenic mice ($n = 3$) were used as a positive control.

from unsuccessful reconstruction after lethal irradiation. BMCs were isolated from medullary cavities of tibiae and femurs. Erythrocytes were depleted in lysing buffer (0.38% NH_4Cl in Tris–HCl, pH 7.65) and mature T-lymphocytes were removed by anti-Thy1.2 antibody (mouse ascitis IgM monoclonal antibody) with rabbit complement; the removal of more than 99.9% of T-cells was constantly done and checked by FACS analysis. These BMCs were incubated with vectors at a multiplicity of infections 10 (MOI = 10) for only 1 h at 37 °C, and GFP expression was determined 48 h later, as described previously [15]. Both vectors constantly showed high gene transfer efficiency (>85%) to murine BMCs in repeated experiments (data not shown; similar data are shown in Fig. 2A).

Next, we engrafted these BMCs (2×10^7 cells/200 μl/head, 1 h after virus exposure) to lethally irradiated (γ-X ray 10 Gy) female C57BL/6 mice via the tail vein, and then the GFP expression in the cells from peripheral blood (PBCs) was monitored every week. BMCs from β-actin promoter-driven GFP transgenic mice (C57BL/6-TgN(act-EGFP)OsbC14-Y01-FM131) [15] were also used as a positive control of successful bone marrow transplantation (BMT).

As shown in Fig. 1, all recipients receiving BMCs from GFP transgenic mice (GFP-TG) demonstrated nearly



	CMPs	CLPs	HSCs	whole BMCs
GFP-positive ratio (%)	97.5 ± 1.3	77.3 ± 13.0	63.9 ± 21.4	91.7 ± 5.0

(n=3)

Fig. 2. *In vitro* gene transfer efficiencies of ts-rSeV/dF-GFP to populations of hematopoietic stem cells (HSC) and common myeloid and lymphoid progenitors (CMPs and CLPs). A typical result of the FACS analyses is given as panels, and the summary of triplicate experiments is demonstrated in the bottom table. Each enriched population was gated by Lin^-/c-Kit^+/$Sca\text{-}1^-$ for CMPs, Lin^-/c-Kit^{low}/$Sca\text{-}1^{low}$ for CLPs, and Lin^-/c-Kit^{high}/$Sca\text{-}1^{high}$ for HSCs. Data are expressed as mean ± SD.

100% GFP-positive cells in PBCs, indicating successful engraftment of, and almost total replacement by, donor cells under this experimental condition. In contrast, PBCs from mice of BMT/rSeV/dF-GFP showed very low repopulation of GFP-positive cells (~1%) at 1 week, and were rapidly eliminated during the experimental course, a representative result of our previous experiments (unpublished data). Importantly, recipient mice with BMT/ts-rSeV/dF-GFP exhibited a relatively high repopulation of PBCs (50–70%) 1 week after BMT; the ratio gradually declined to ~10% at 4 weeks.

To our knowledge, this is the first demonstration of the efficient repopulation of donor cells that were transferred by cytoplasmic RNA vector.

Efficient ts-rSeV/dF-mediated gene transfer to HSCs and progenitor populations

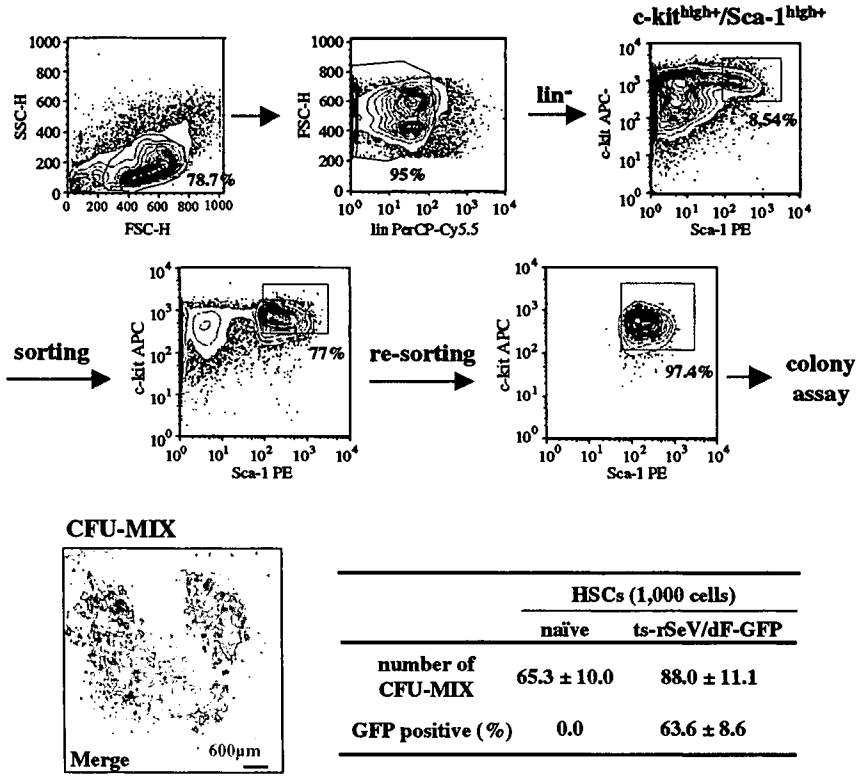
We examined the gene transfer efficiencies of ts-rSeV/dF-GFP to common myeloid progenitors (CMPs: enriched in $Lin^-Sca\text{-}1^-c\text{-}Kit^+$) [16], common lymphoid progenitors (CLPs: enriched in $Lin^-Sca\text{-}1^{low}c\text{-}Kit^{low}$) [17], and HSCs (enriched in $Lin^-Sca\text{-}1^{high}c\text{-}Kit^{high}$) by flow-cytometric analysis (Fig. 2). Constant high gene transfer, over 90%, was seen in T-cell-depleted BMCs by ts-rSeV/dF-GFP, and relatively high gene transfer also occurred in these populations (mean = 97.5% of CMPs, 77.3% of CLPs, and 63.9% in HSCs, $n = 3$).

Multilineage differentiation of cytoplasmically gene transferred HSCs in vitro

Subsequently, we investigated whether gene transferred HSCs by ts-rSeV/dF-GFP might sufficiently differentiate to various types of colonies by a colony-forming assay. As shown in Fig. 3, 1000 cells of highly enriched HSCs were sorted from BMCs twice and transfected with the GFP gene, and then sparsely cultured for 12 days [16]. Colony-forming units (CFU) were then assessed by fluorescence microscope. We counted the number of multilineage mixed colonies (CFU-Mix) as CFU from single cells with pluripotency. The result in Fig. 3 shows, a reduction in the number of CFU-Mix was not seen in cells treated with ts-rSeV/dF-GFP, suggesting no significant effect of gene transduction to cell differentiation. In addition, the ratio of GFP-positive colonies was almost comparable to gene transfer efficiency to HSCs, suggesting no significant effect on cell growth by ts-rSeV/dF-GFP, at least *in vitro*.

In vivo repopulation and multilineage differentiation of cytoplasmically gene transferred BMCs

Using six animals 5 weeks after BMT with BMC treated ts-rSeV/dF-GFP, we next examined the distribution, repopulation and differentiation of GFP-positive cells in typical lymphoid organs (thymus, spleen, bone marrow)



	HSCs (1,000 cells)	
	naïve	ts-rSeV/dF-GFP
number of CFU-MIX	65.3 ± 10.0	88.0 ± 11.1
GFP positive (%)	0.0	63.6 ± 8.6

Fig. 3. *In vitro* colony-formation assay. Cells were sorted twice (FACS panels) in order to enrich HSC fraction, a typical photomicrograph of GFP-positive CFU-Mix (bottom left), suggesting multilineage differentiation, and the table summary of the quantitative data (bottom right) are given. Data are expressed as mean ± SD.

as well as PBCs by flow cytometry. As shown in Fig. 4A, these six mice showed a representatively high GFP-positive ratio (55.9 ± 5.9%, $n = 6$) in PBCs, and the thymus, spleen, and bone marrow of these animals also exhibited a mean = 47.7%, 79.3% and 17.1% of GFP-positive cells, respectively, confirming the repopulation of transplanted cells in each lymphoid organ. A simultaneous experiment using the BMT/SeV/dF-GFP of three animals did not show any GFP-positive cells at all 5 weeks after BMT (data not shown).

Subsequently, we investigated the GFP-positive ratio of each cell type in the spleen and bone marrow by FACS analyses. Cells from each organ were determined by antibodies specific for the following surface-markers: NK cells = $CD3^-/DX5^+$, NKT cells = $CD3^+/DX5^+$, pan-T-cells = $CD3^+/DX5^-$, CD4/T-cells = $CD3^+/CD4^+$, CD8/T-cells = $CD3^+/CD8^+$, dendritic cells (DCs) = $CD11b^+/CD11c^+$, monocytes/macrophages (Mφ) = $CD11b^+/CD11c^-$, B-cells = $B220^+/IgM^+$. As shown in Fig. 4B, even though BMCs were used after complete depletion of T-cells, all these subsets contained GFP-positive cells at various ratios including T-cell lineages, suggesting that these GFP-positive cells were from HSCs and progenitors. Interestingly, DCs and Mφs, both from CMPs [16], included a relatively large number of GFP-positive cells in both the spleen and bone marrow, probably reflecting the high gene transfer to CMPs (Fig. 2).

These findings, therefore, strongly suggested that BMCs transfected with ts-rSeV/dF-GFP were capable of differentiating the whole hematopoietic series *in vivo*.

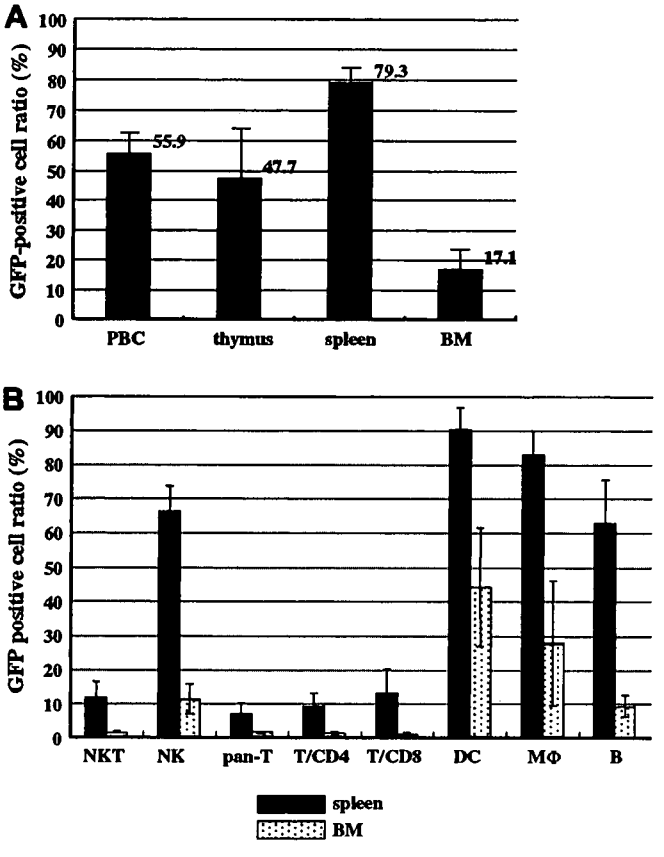


Fig. 4. *In vivo* repopulation and multilineage differentiation of BMCs transfected by ts-rSeV/dF-GFP after BMT. Data are expressed as mean ± SD. (A) Repopulation efficiency of ts-rSeV/dF-GFP treated BMCs in typical lymphoid organs at 4 weeks after BMT *in vivo*. (B) FACS analysis of ration of GFP expression in various cellular subsets in spleen and bone marrow of recipient mice at 5 weeks after BMT. Each cell subset was determined as follows: NK = $CD3^-/DX5^+$, NKT = $CD3^+/DX5^+$, pan-T-lymphocytes = $CD3^+/DX5^-$, CD4/T-cells = $CD3^+/CD4^+$, CD8/T-cells = $CD3^+/CD8^+$, DCs = $CD11b^+/CD11c^+$, Mφ = $CD11b^+/CD11c^-$, and B-lymphocytes = $B220^+/IgM^+$.

Discussion

Physicians and scientists in the gene therapy community have been greatly encouraged by the dramatic outcome of clinical studies to treat X-linked SCID conducted in France [1]. However, this trial has also raised safety concerns about vector integration to the host chromosomes.

Using vectors that express the transgene in cellular cytoplasm is one possible way of addressing safety concerns; in the present study, therefore, we attempted to seek the potential of a cytoplasmic RNA vector, rSeV, as a new modality for gene therapy to treat hematopoietic disorders. To the best of our knowledge, this is the first study to make use of a newly developed version of the cytoplasmic RNA vector, ts-rSeV/dF, to show the high gene transfer efficiency to hematopoietic stem/progenitor populations and their successful repopulation associated with differentiation to multiple lineages of hematopoietic series.

Apparently, an advance seen in this study is that the repopulation of cytoplasmically gene transferred BMCs could be done by using an improved design of rSeV/dF, a temperature-sensitive mutant ts-rSeV/dF, but not of conventional rSeV/dF (and prototype additive rSeV, unpublished data). However, there is no definitive explanation as to why ts-rSeV/dF realized an efficient repopulation. As previously demonstrated, ts-rSeV/dF shows a reduced cytotoxic effect on some types of cells, but this is not a likely explanation for its ability to realize BMC repopulation because our *in vitro* study using murine HSCs, which is shown in Fig. 3, along with a previous study using human HSCs [11], could not demonstrate the apparent cytotoxicity and the disturbance of their differentiation caused by any type of rSeV vector. It is therefore possible that the reduced expression of HN protein due to the insertion of temperature-sensitive mutations enabled BMCs to repopulate in the recipient.

The findings obtained in this study suggested the possible application of 'cytoplasmic gene therapy' for hematopoietic disorders; however, our preliminary study assessing long-term engraftment also revealed the potential limitations of the current construct *in vivo* in view of its clinical application. In other words, more than 40% of recipient mice showing efficient hematopoietic gene transfer were dead in later phase due to severe pancytopenia (Yoshida K and Yonemitsu Y, unpublished data), therefore, more studies and biological information are thus

needed to realize cytoplasmic gene therapy for hematopietic diseases.

In summary, we here demonstrated the successful repopulation and reconstruction of hematopoietic series by cytoplasmically gene transferred BMCs using newly developed ts-rSeV/dF. This study is, to the best of our knowledge, the first report reconstituting whole hematopoietic series using a bone marrow gene transferred by cytoplasmic transcription, but the transient repopulation is an issue that must be resolved. Since 'cytoplasmic gene therapy' is an attractive and challenging strategy for physicians and scientists in this field, further studies are needed to overcome the current problems.

Competing interest statement

Dr. Yonemitsu is a member of the Scientific Advisory Board of DNAVEC Corporation.

Acknowledgments

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Development of immunostimulatory virotherapy using non-transmissible Sendai virus-activated dendritic cells ☆

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Abstract

Dendritic cell (DC)-based immunotherapy has been clinically evaluated, however, still requires modification to improve its outcomes. We previously demonstrated that DCs activated by replication competent recombinant Sendai virus (SeV) showed dramatic efficacy over that seen in use of current DC vaccine for immunotherapy against malignancies; however, application of replication-deficient vector is more relevant in clinical setting. We here show that F-gene-deleted non-transmissible Sendai virus (SeV/dF)-activated DCs (DCs/SeV/dF) has strong antitumor effects against murine SCCVII tumor, that was well-known as a less immunogenic cell line. SeV/dF shows high transfection efficiency to DCs and leads them to upregulate costimulatory molecules. Intratumoral injection of DCs/SeV/dF resulted in a marked and representative inhibition of the tumor, even when the tumors were well-vascularized. This is the first demonstration that non-transmissible SeV vector, SeV/dF, could be a DC-activator; DC/SeV/dF-based cancer immunotherapy may, therefore, warrant further investigation.

Keywords: Squamous cell carcinoma; Non-transmissible Sendai virus; Dendritic cell

Despite the continuous efforts of physicians, the overall survival for patients with squamous cell carcinoma (SCC), including esophageal cancers, have not shown significant improvement over the last decade [1,2]. Among the clinically available therapeutics, surgical treatment has been the most effective when cancer foci could be resected without the involvement of or metastasis to other vital organs [3–5]. However, in the case of esophageal carcinomas, anatomical loss of serosa of the esophagus would be a cause of accelerated invasiveness of SCC to the aorta, trachea, etc., resulting in advanced diseases. Extensive surgical approaches, including extended esophagectomy with 3-field lymph node dissection could improve patient prognosis, but quality of life is occasionally reduced due to impairment of deglutition, phonation, and respiration [3,4]. Since such advanced esophageal SCC has also remained intractable to chemo-radiotherapy, the development of novel and less invasive therapeutic strategies has been much desired to treat patients with advanced SCC.

☆ *Competing interest statement:* The authors declare that they have no competing financial interests.

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Much attention has been paid to cancer immunotherapy using dendritic cells (DCs) that has shown good potential for antitumor immunity [6,7]; DC-based cancer immunotherapy is, therefore, currently being tested in clinical trials all over the world. Although reported clinical trials have shown the safety and feasibility of DC therapy in patients bearing advanced malignancies, objective clinical responses have remained limited [8,9].

The poor clinical response of DC therapy might be due to the insufficient activation, and, in turn, the state of activation of DCs for cancer vaccine is still under debate [10]. Immature DCs (iDCs) display a phenotype reflecting their specialized function as antigen-capturing cells; however, the activity of antigen presentation of this subset of DCs to T cells, however, is not efficient [10]. Thus, to achieve a dramatic improvement in the clinical efficacy of DC-based cancer immunotherapy, knowledge regarding the most appropriate condition of DCs for cancer immunization should be obtained.

Sendai virus (SeV) is a negative-strand RNA virus and a member of the *paramyxoviridae* family, and the genomic replication and transcription are carried out in the cytoplasm by its own RNA polymerase. Using a prototype vector based on SeV, replication competent SeV, we recently demonstrated that DCs activated by SeV showed dramatically enhanced antitumor effects on various murine tumors [11], suggesting the possible utility of SeV vector system for cancer immunotherapy. As a next step, we subsequently developed a new mode of non-transmissible viral vector based on SeV-lacking fusion (F) gene (SeV/dF) that is more relevant for clinical use [12]. Some previous observations have demonstrated that immature DCs are dramatically activated and leads to a maturation state by the infection of SeV, thus acquiring the capacity to promote cell-mediated immunity in a replication-dependent and toll-like receptor (TLR)-independent fashion [13,14].

We, therefore, investigated the therapeutic value and immune consequences of intratumoral administration of activated DCs by SeV/dF.

Methods

Mice and cell line. Female C3H/He mice (6–7 weeks old) were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Shizuoka, Japan) and kept under specific pathogen-free conditions. All animal experiments were performed according to approved protocols and in accordance with recommendations for the proper care and use of laboratory animals by the Committee for Animals, Recombinant DNA, and Experiments Using Infectious Pathogens at Chiba University, and according to The Law (No. 105) and Notification (No. 6) of the Japanese Government. SCCVII (kindly provided by Dr. Shibamoto, Nagoya City University) is a spontaneously arising murine SCC that has been shown to be poorly immunogenic [15].

Rescue of SeV/dF. Preparation and recovery of F-defective and non-transmissible recombinant SeV used in this study (SeV/dF-GFP and SeV/dF-null) were performed as previously described [12,16]. Briefly, LLC-MK2 cells stably expressing F-gene were transfected with a plasmid mixture containing pSeV18+b(+)/F-EGFP, pGEM-NP, pGEM-P, and pGEM-L. The transfected cells were collected, resuspended, and lysed by three cycles of freezing and thawing. Subsequent genomic RNA–nuclear protein complex (RNP) transfection was performed by cationic lipid into F-expressing LLC-MK2/F7 cells. Virus yield is expressed in PFU and cell infectious units (CIU).

Murine bone marrow-derived DCs (mBM-DCs). DCs were obtained from mouse bone marrow precursors as described previously [11,17] with minor modification. Briefly, bone marrow cells were harvested from femurs and tibias. After washing, lineage antigen-positive (CD45R, CD5, CD11b, Gr-1, TER119, 7/4) cells were removed by using the SpinSep mouse hematopoietic progenitor enrichment kit (StemCell technologies, Canada). These lineage-negative cells were cultured under 20 ng/ml murine GM-CSF and 20 ng/ml murine IL-4 in RPMI 1640 medium. On day 4, the cultures were refreshed by adding a half volume of culture medium supplemented with GM-CSF and IL-4 at the same concentrations. On day 7, DCs were collected and seeded at 1×10^6/ml and then incubated with SeV vectors at each MOI or lipopolysaccaride (LPS) (1 μg/ml).

Flow cytometric analysis. DCs were replated in fresh medium and incubated with SeV-GFP at each MOI or 1 μg/ml LPS for 48 h. Biotin-conjugated anti-mouse H-2K, CD40, CD80, CD86, and FITC-conjugated anti-CD11c (Pharmingen, San Diego, CA) monoclonal antibodies (mAbs) were used for each primary antibody, and biotinylated Abs were detected by subsequent staining with streptavidin-PE (Pharmingen). Cells were analyzed using a FACScalibur (Becton–Dickinson Tokyo, Japan).

Cytotoxic assay. DCs were replated and incubated with SeV/dF-GFP at each MOI. At 48 h, cell viability was assessed with 7-amino-actinommycin D (7AAD) to count living cells for FACS analysis, as previously described [18].

Fluorescein isothiocyanate (FITC)–dextran uptake. FITC–dextran uptake was performed to assess the endo-/phagocytotic activity of DCs. Cells were then incubated with 1 mg/ml of FITC–dextran (MW = 40,000, Sigma–Aldrich, Tokyo, Japan) for 30 min at 4 or 37 °C, and washed with ice-cold phosphate-buffered saline (PBS) and labeled on ice with PE-conjugated mAb for CD11c. The uptake was measured by FACS at different times after stimulation, and was calculated as the change in MFI between cell samples incubated at 37 and 4 °C.

DC immunotherapy of the established tumor. SCCVII cells were harvested and processed by three rapid cycles of freezing and thawing. DCs were pulsed with tumor lysate (DC number: number of tumor cells for lysate = 1:3) for 18 h and were then incubated with SeV/dF or LPS for 8 h. Intradermal implantation was performed in the abdomen on day 0, and 1×10^6 DCs were injected intratumorally on days 10, 17, and 24. For all injections, materials were suspended in a 100-μl volume of PBS. The tumor size was assessed using microcalipers three times a week, and the volume was calculated by the following formula: (tumor volume; mm^3) = 0.5236 × (long axis) × (short axis) × (height).

Immunohistochemical staining. Standard immunohistochemical staining was performed using 5-μm-thick paraffin-embedded sections by standard avidin–biotin complex method. Blocking was done using 3% hydrogen peroxide and non-immune serum, and the primary antibodies (anti-vWF, Chemicon international, CA) was applied for 1 h at room temperature. Visualization was done using 3,3-diaminobenzidine substrate solution, and the sections were counter-stained with hematoxylin.

CTL activity. Seven days after the last immunization, spleen cells were pooled, and restimulated with irradiated (100 Gy) SCCVII cells for 6 days. These cells were harvested on day 6 and used as effector cells in a standard 4-h ^{51}Cr release assay. Target tumor cells (1×10^6) were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ for 90 min at 37 °C. The labeled target cells were incubated with the effector cells for 4 h at 37 °C in 96-well plates in 200 μl of T-cell medium at various E:T ratios. The radioactivity of the supernatants was counted using a γ-counter. Cytolytic activity was calculated using the following formula: percentage of specific Cr^{51} release = (experimental release − spontaneous release) × 100/(maximum release − spontaneous release). Assays were performed in triplicated wells.

Statistical analysis. The data were evaluated statistically by repeated measures one-way ANOVA. The statistical significance of difference groups was determined using the Scheff's test, and $P < 0.05$ was considered statistically significant.

Results

Characterization of DCs modified by SeV/dF

DCs infected by SeV/dF-GFP at each MOI were analyzed by flow cytometry for GFP expression. The ratio of GFP-expressing DCs increased in a MOI-dependent manner, with the transfection efficiency at MOI = 20 being estimated at approximately 80% based on similar findings in repeated experiments (Fig. 1A). Interestingly, LPS-activation of DCs reduced the transfection efficiency of SeV/dF-GFP (data not shown), indicating that the susceptibility of mBM-DCs to SeV/dF may depend on their maturation state. Higher viral doses of SeV/dF-GFP did not result in a significant cytopathic effect (Fig. 1A).

Phenotype of mBM-DCs treated by SeV/dF

We next evaluated the effects of SeV/dF transfection on the maturation and activation of DCs. Here, we used SeV/dF without a foreign gene (SeV/dF-null).

Two-day stimulation of iDCs with SeV/dF-null or LPS, a well-known strong DC-activator that is not clinically available, increased the expression of these costimulatory molecules. The expression levels of surface markers on the C3H strain-derived DCs after SeV/dF-null infection were milder than for those treated with LPS (Fig. 1B); a different finding indicating that C57BL6-derived DC/SeV/dF-null show comparable levels of expression to those seen with LPS [11].

We subsequently assessed the effects of SeV/dF on endo-/phagocytotic activity of mBM-DCs as compared with that of LPS (Fig. 1C). Endo-/phagocytosis of DC/LPS was mildly and transiently enhanced, peaked at 1 h after LPS stimulation, and then markedly downregulated in its time course. In contrast, endo-/phagocytosis of DC/SeV/dF-null was transiently decreased, and then caught up to levels comparable to those of immature DCs. Forty-eight hours after stimulation, the endo-/phagocytotic activity of DCs-SeV/dF-null was significantly higher than that of DC/LPS ($P < 0.05$, $n = 4$, each), which was equal to the levels of unstimulated DCs.

These results suggest that SeV/dF-null may activate mBM-DCs without any significant reduction in their endo-/phagocytotic activity.

Antitumor effects of SeV-activated DC in vivo

We next examined the antitumor effects of intratumoral administration of DCs activated with SeV/dF *in vivo*. We here used established (7–10 mm in diameter on day 10) and well-vascularized SCCVII tumors (Fig. 2A); because 'there are no cancer vaccine models that reproducibly demonstrate that vascularized tumors can be rejected' at present, as noted by Rosenburg et al. [9].

Tumor-bearing mice were given tumor lysate-pulsed mBM-DCs which were activated with LPS or SeV/dF-GFP three times (Fig. 2B and C). Administration of DCs without stimulation showed a modest antitumor response, probably due to their mild spontaneous activation during *ex vivo* cultivation. Antitumor activity was significantly enhanced with the use of DCs/LPS, and one animal showed a complete rejection of the tumor. Intratumoral injection of DCs/SeV/dF resulted in a marked inhibition of tumor growth in all animals, and with this regimen, 2 of 7 tumors were completely eliminated, a finding determined by histopathology.

Next, the cytolytic activity of spleen cells was investigated. Cytolytic activity against SCCVII cells was augmented in unstimulated DCs, DC/LPS, and the DCs/SeV/dF treatment group compared with that in the no-treatment groups (Fig. 3). The highest cytolytic activity was observed in the mice that received DCs/SeV/dF treatment.

These results confirmed that intratumor injection of DCs/SeV/dF enhanced the tumor-specific CTL response.

Antitumor effects of SeV-activated DC without pulsing tumor lysate

As shown in Fig. 1, endo-/phagocytosis of DCs was not impaired by SeV/dF treatment *in vitro*; we, therefore, evaluated the antitumor effects of SeV-activated DCs without pretreatment with tumor lysate. As shown in Fig. 4, the antitumor effects of DCs activated by SeV/dF were not impaired without pulsing tumor lysate, whereas no tumor showed complete elimination. These results suggest that intratumor injection of DC/SeV/dF may not always require the uptake of tumor antigen during preparation.

Discussion

The key observations of the current study were as follows: (1) SeV/dF efficiently transfected immature mBM-DCs without a significant reduction of cell viability; (2) SeV/dF leads mBM-DCs to a mature and activated state without a significant reduction in endo-/phagocytotic activity; (3) DC/SeV/dF-GFP demonstrates superior antitumor effects on less immunogenic murine SCCs, which were treated as well-established and well-vascularized, in comparison with the use of DC/LPS. This is the first report suggesting the potential utility of SeV/dF as an alternative activator for DCs in clinical cancer immunotherapy.

We demonstrate that SeV/dF efficiently transfects DCs at relatively lower MOI without significant cytopathic effects. Although other investigators have focused on adenovirus vectors for gene transfer to DCs, much higher titer (over MOI = 300) has been required [19,20] to achieve the comparable efficiency of SeV at lower MOI. It has been reported that adenoviral infection induces cytotoxicity and impairs antigen uptake [20], although there are some conflicting reports regarding this point. iDCs actively internalize antigen, and in contrast, mature DCs are poorly endocytotic and function to present antigens to T-cells [21,22]. Our findings, however, have demonstrated that DCs activated by SeV/dF can efficiently uptake antigens

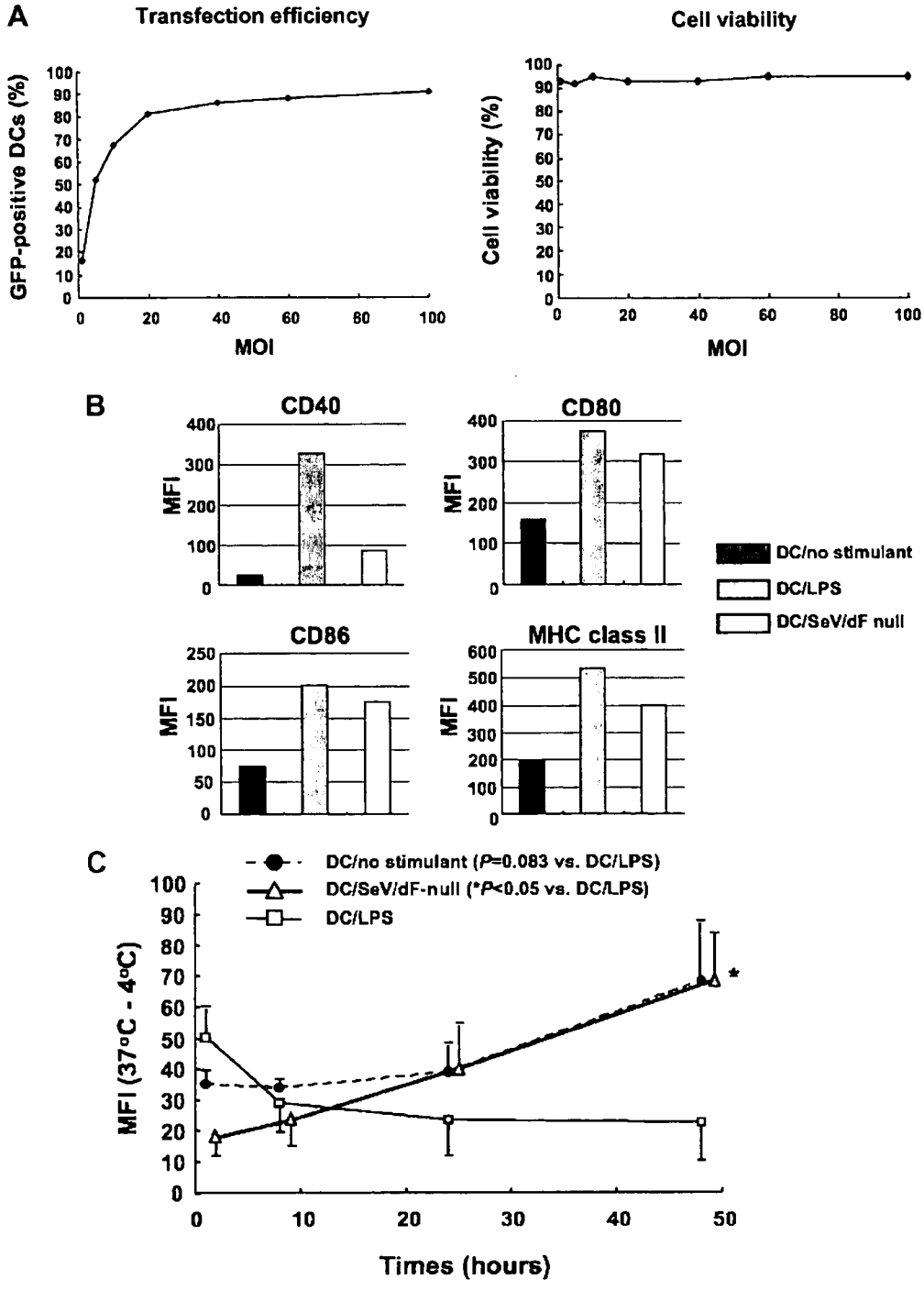

Fig. 1. Characterization of mBM-DCs modified by SeV/dF. (A) Gene transfer efficiency and cytopathic effects. Forty-eight hours after SeV/dF-GFP exposure, DCs were subjected to FACS analysis assessing the gene transfer efficiency (left), or to an assessment of cell viability by an early apoptotic cell-detecting technique using 7AAD (right). These experiments were performed at least in duplicate, and all produced similar results. (B) Effects of SeV/dF on surface markers of mBM-DCs. DCs were collected and transfected by SeV/dF-null at MOI = 40 or exposed to LPS (1 μg/ml), and 48 h later, DCs were subjected to FACS analysis assessing the expression of surface markers. Bar graph indicating the corresponding mean fluorescent intensity (MFI) is shown. The experiment was carried out in triplicate, produing similar results. (C) Effects of SeV/dF on endo-/phagocytic activities of mBM-DCs. DCs were transfected by SeV/dF-null at MOI = 40 or stimulated by LPS (1 μg/ml), and then exposed to 1 mg/ml of FITC–dextran for 30 min at 4 or 37 °C. The uptake was measured at each time and expressed MFI between cell samples incubated at 37 and 4 °C. The graph contains all data from four independent experiments using the same procedure.

and that the antitumor effects of DC/SeV/dF via intratumor injection were not impaired without pulsing tumor lysate, suggesting that the DC/SeV/dF may still have antigen uptake and processing activity *in vivo*.

Despite the higher expression of costimulatory molecules, CD40, CD80, and CD86 in DC/LPS than in DC/SeV/dF, the intratumor administration of DCs/SeV to established SCCVII tumors *in vivo* showed stronger antitu-

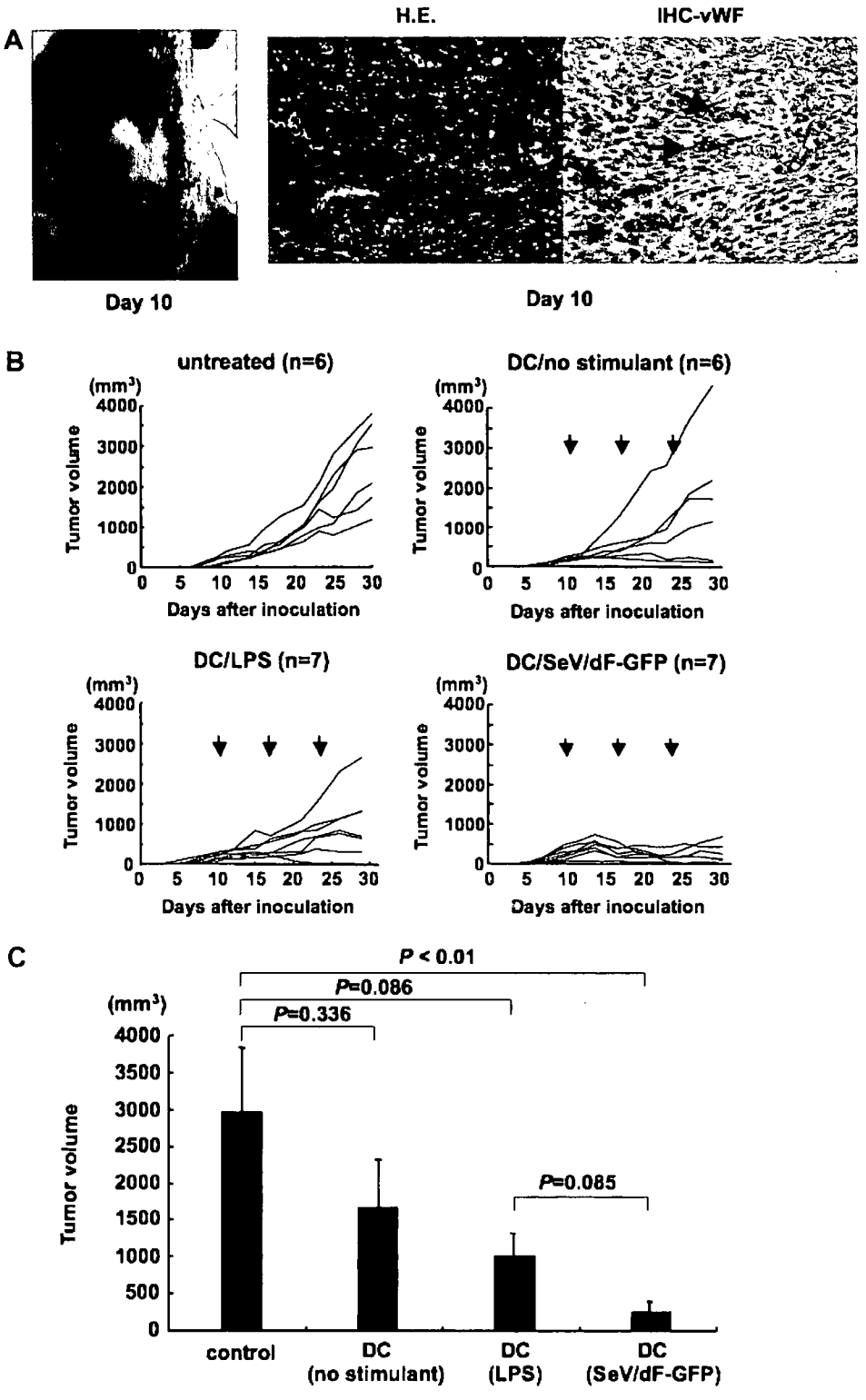

Fig. 2. Antitumor effects of intratumor administration of lysate-pulsed DC/SeV/dF-GFP against well-vascularized SCCVII tumors. (A) Characterization of the established SCCVII tumors at the time of the start of DC-therapy. Left: representative gross observation of intradermally inoculated SCCVII tumor of C3H mice. The size of the tumor is 7–10 mm in diameter. Middle and right (original magnification: 200×): histopathological findings in hematoxylin-eosin (H.E.: middle) and in immunohistochemistry for von Willebrand Factor (IHC-vWF) identifying intratumor neovascularization (arrows). (B) The time course of tumor volume of each animal. Ten days after tumor inoculation, DC-therapy (left upper: no treatment; right upper: DCs without stimulant; left bottom: DCs activated with LPS; and right bottom: DCs activated with SeV/dF-GFP) was performed three times at weekly intervals (indicated as arrows). One animal of the DC/LPS group and 2 of the DC/SeV/dF-GFP were completely eliminated. (C) Bar graph providing a direct comparison of tumor volume on day 30.

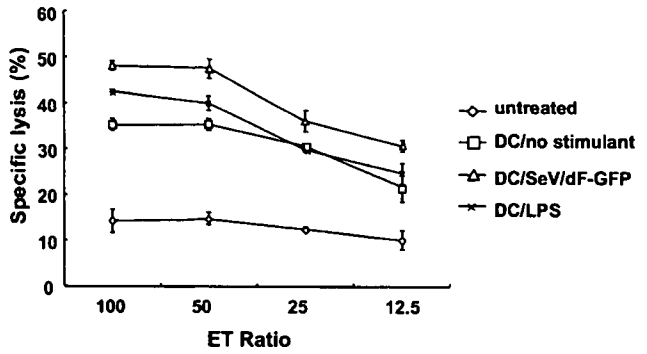

Fig. 3. Cr release assay to determine the CTL activity. Seven days after the third immunization, spleen cells isolated from immunized mice and ^{51}Cr release assay was performed.

mor effects and CTL activity than that of DC/LPS. The exact reason for these stronger effects remain unknown, but a possible mechanism related to the continuity of activity of DCs can be explained as follows. Recent studies related to the activity of DCs have shown that DCs exposed to stimuli for long periods undergo functional paralysis or exhaustion [23,24]. Considering the maintained endocytotic activity and modest upregulation of costimulatory molecules on DC/SeV, it may be possible that the paralysis or exhaustion of DC/LPS expressing higher surface markers resulted in more modest antitumor effects than those seen in the use of DC/SeV/dF.

Related to the above discussion, several important studies regarding the mechanisms of negative-strand RNA virus-induced activation of DCs have been reported in recent years. These are categorized as having two independent systems, namely toll-like receptors (TLRs) and TLR-independent systems; the former recognize dsRNA and ssRNA presuming pathogen-associated molecular patterns for viruses [25], and the latter is a TLR-independent pathway using RNA helicases, including RIG-I [26]. The latter pathway well explains that activated DCs by SeV are more powerful than DCs that are exposed to LPS *in vivo*; therefore, further characterization should provide a better understanding of the mechanism to improve the efficacy of cancer vaccines.

Immunotherapy for SCCs has not been well-studied, and there is at present sparse information regarding the response of SCCs to CTLs. Although previous experimental studies demonstrated that the pre-administration of lymphokine-activated killer cells prevented pulmonary metastases of SCCVII cells, which are a less immunogenic cell line used in this study, they have not demonstrated any significant reduction in established pulmonary metastases [15,27]. Since 'there are no cancer vaccine models that reproducibly demonstrate that vascularized tumors can be rejected' at present, as noted by Rosenburg et al. [9,28], the current results, indicating that the well-vascularized tumors were eliminated in more than 25 % of mice treated with DC/SeV/dF-GFP, suggest the potential utility of DC/SeV/dF.

In summary, we here demonstrated that mBM-DCs treated with non-transmissible SeV/dF without therapeutic

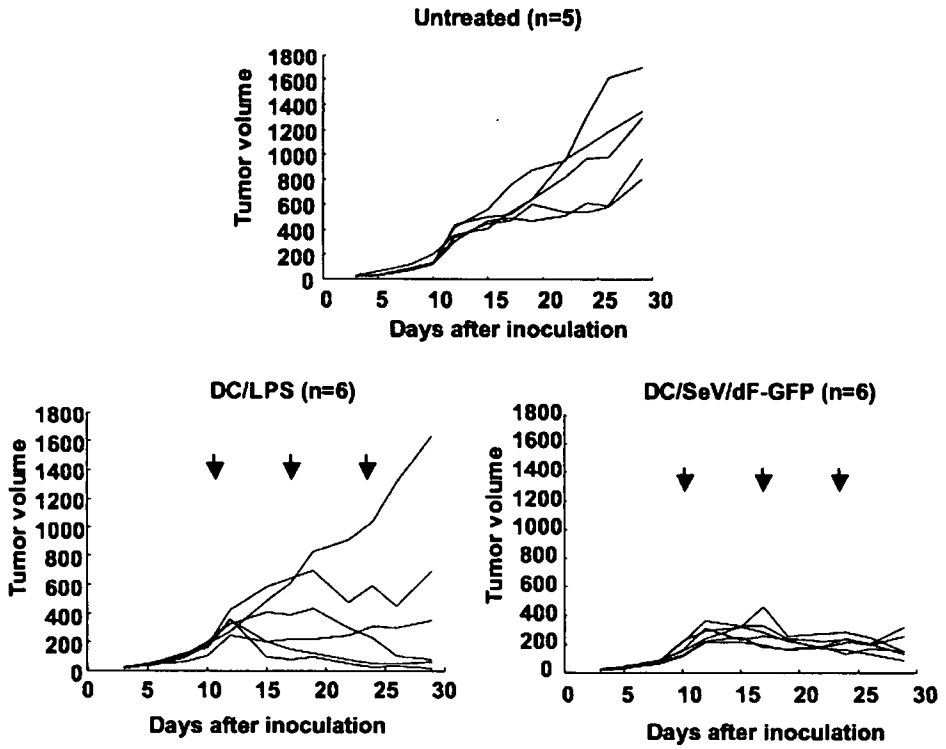

Fig. 4. The time course of tumor volume of each animal in response to DC-therapy without *ex vivo* tumor lysate pulsing. DC-therapy without pulse (upper: no treatment; left bottom: DC/LPS; and right bottom: DC/SeV/dF-GFP) was performed three times at weekly intervals from 10 days after tumor inoculation.

gene induced strong antitumor effects against less immunogenic murine SCC tumors. These results thus indicate for the first time that SeV/dF could be a promising candidate for DC-activator to treat intractable malignancies; therefore, DC/SeV/dF warrants further investigation for enhancing antitumor effects in a clinical setting.

Acknowledgments

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Neurospheres From Human Adipose Tissue Transplanted Into Cultured Mouse Embryos can Contribute to Craniofacial Morphogenesis: A Preliminary Report

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Adipose-derived stromal cells (ASCs) are one of the most promising stem cell populations that differentiate into the mesodermal as well as neural lineages in vitro. In this study, we examined the neural differentiating potential of human ASCs by a neurosphere culture method. Neurospheres derived from human ASCs expressed *Nestin* and *Musashi-1* genes, which are marker genes for neural stem cells. When these cells were labeled with *green fluorescent protein* gene transfection by Sendai virus vector and transplanted into the head region of mouse embryos using a whole embryo culture system, these cells were incorporated into the craniofacial development. Some transplanted cells appeared to migrate along the second branchial arches, implicating some similarity to the cranial neural crest cells. Although preliminary, our results support an idea that ASC-derived neurospheres have properties of neural progenitors in vitro and in vivo.

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Adipose-derived stromal cells (ASCs) were originally reported as a subtype of the mesenchymal stem cells (MSCs) isolated from liposuction aspirates differentiating into the mesodermal tissues such as bone, cartilage, and adipose tissue.[1] Characterization of ASCs has recently been studied world wide by many groups, including ours.[2–4] ASCs are now regarded as one of the most promising adult stem cells for regenerative medicine because they can be harvested safely by liposuction, and a good yield can be anticipated.

Advances in stem cell research have resulted in a novel concept of cellular plasticity of differentiation beyond the boundary of germ layers. MSCs and ASCs can differentiate into neuronal (and thus ectodermal) derivatives, although these cells are primarily mesodermal.[5,6] Recent reports further indicate that stem cells with neural characteristics can be isolated from the mesodermal tissues such as the dermis and the heart.[7–9] In these cases, the cells were harvested by a neurosphere method, which was originally developed as a culture method of isolating spheres of neural stem cells from the embryonic and adult brain.[10–12] However, it is to be elucidated whether this method is also applicable for obtaining neural stem cells from the adipose tissue or ASCs.

In this study, the neurospheres expressing neural stem cell marker genes were obtained from human ASCs. We also transplanted these cells into mouse embryos cultured in vitro to examine whether these cells behave similar to neuronal cells in vivo.

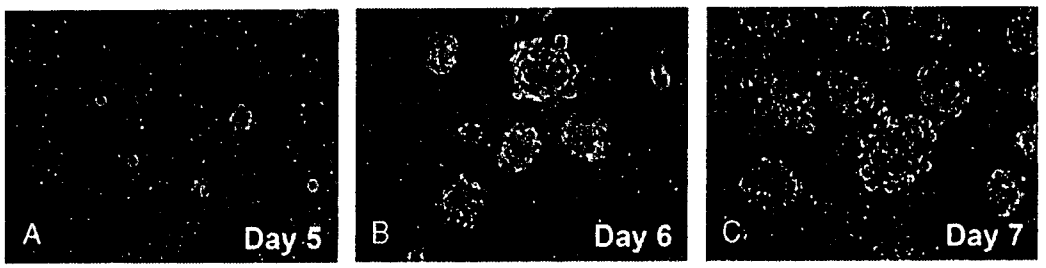

Fig 1 Neurosphere formation of adipose-derived stromal cells cultured in neurosphere medium for 5 days (A), 6 days (B), and 7 days (C) (magnification ×200).

MATERIALS AND METHODS

Isolation of Human ASCs and Neurosphere Cell Culture

ASCs were isolated from the human liposuction aspirates as reported previously.[3] The suctioned fat was digested with 0.075% collagenase in phosphate-buffered saline (PBS) for 30 minutes on a shaker at 37°C. Mature adipocytes and connective tissues were eliminated by centrifugation. Blood cells were also eliminated by treating with erythrocyte lysis buffer, and resultant ASC pellets were obtained. Alternatively, ASCs could be isolated from the fluid portions of liposuction aspirates by treating with erythrocyte lysis buffer and density gradient centrifugation with Ficoll (GE Healthcare Bio-sciences, Piscataway, NJ).

Neurosphere culture was performed as described previously with slight modification.[12] Freshly isolated ASCs were plated at a density of 2×10^7 cells in 10 cm uncoated dishes and cultured in the neurosphere culture medium at 37°C in an atmosphere of 5% CO_2 in humid air. The neurosphere medium was a Dulbecco's Modified Eagle's Medium/F12 (1:1)-based medium supplemented with human recombinant epidermal growth factor (EGF, 20 ng/mL, PeproTech, Rocky Hill, NJ), human recombinant basic fibroblast growth factor (FGF, 20 ng/mL, Kaken Pharmaceutical, Tokyo, Japan), 2% B27 supplement (Gibco, Carlsbad, CA), 100 U/mL penicillin, and 100 μg/mL streptomycin. Half of the medium was replaced with a fresh medium on the fourth to fifth day, and the passaging was performed on the eighth day.

Quantitative Real-Time Reverse-Transcriptase Polymerase Chain Reaction

Total mRNA was extracted using RNeasy-mini kit (Qiagen, Hilden, Germany) from the neurosphere cells derived from passage one ASCs, which were precultured in the normal medium containing M199 medium and 10% fetal bovine serum (FBS). The preculturing was necessary for reducing the contamination of blood cells. Control mRNA was also extracted from the passage one undifferentiated ASCs cultured in M199 plus 10% FBS.

Expressions of undifferentiated neural stem cell marker genes *Nestin* and *Musashi-1*[13] and adipogenic differentiation marker *Leptin* were analyzed by real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR) using an ABI PRISM 7700

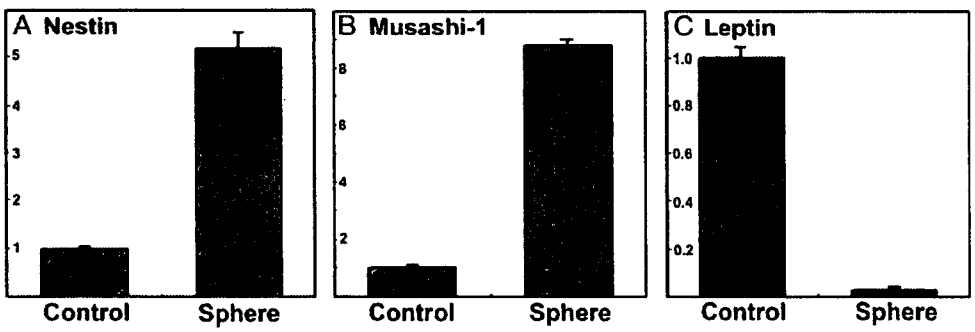

Fig 2 Quantitative real-time reverse-transcription polymerase chain reaction analysis of gene expressions of neural stem cell marker *Nestin* (A), *Musashi-1* (B), and adipogenic differentiation marker *Leptin* (C). Control = undifferentiated adipose-derived stromal cells; Sphere = neurospheres. Assays were performed in triplicate, and standard errors are indicated by error bars.

(Applied Biosystems, Foster City, CA), as reported previously. Gene expression of the target sequence was normalized to that of the housekeeping gene *β-actin*. Transcript level in the control (undifferentiated ASC) group was arbitrarily expressed as 1. TaqMan chemistry and assay by design primers and probe sets were used for human *Nestin*, *Musashi-1*, *Leptin*, and *β-actin*. All the primers and probe sets were purchased from Applied Biosystems.

Mouse Whole Embryo Culture and Transplantation of Neurosphere-Like Cells

Neurospheres derived from human ASCs were transfected with *green fluorescent protein* (*GFP*) gene using the Sendai virus vector (DNAVEC Corp. Tsukuba, Japan), as reported previously.[14,15] The original vector SeV/ΔF lacks the F gene encoding fusion protein necessary for penetration of ribonucleoprotein complex into infected cells, and is thus nontransmissible and nonpathogenic.[14] The modified SeV/ΔF vector has additional mutations to reduce its cytotoxicity,[15] and we used the modified vector in the present study. Neurospheres were incubated for 1 hour in the medium with the modified SeV/ΔF carrying the *GFP* gene at a multiplicity of infection of 250 and rinsed with PBS.

Mouse whole embryo culture was performed as reported previously.[16–19] Nine mouse embryos at embryonic day (E) 8 were dissected out without damaging yolk sacs, and the GFP-transfected neurosphere cells were transplanted using micropipettes into the head region of the embryos. The embryos were cultured for approximately 40 hours, and presence or absence of the GFP-positive transplanted cells was investigated under a fluorescent dissecting microscope. All experimental procedures were performed at the University of Tokyo under approval of the ethical committee.

RESULTS

We first cultured human ASCs in the neurosphere culture medium containing EGF and basic FGF without serum. On the third day of culture of freshly prepared ASCs, the floating ASCs started to form small masses (data not shown). The neurosphere-like cellular aggregates were clearly observed on the fifth day (Fig 1A). The number and the size of the spheres became increasingly larger within the next 2 days (Fig 1, B and C). The passaging was performed on the eighth day when the spheres were dissociated and resuspended in the new medium. The spheroids were newly formed after culturing again for several days (data not shown), suggesting self renewal of the neurosphere cells.

To characterize the neurosphere cells, we next examined expressions of neural stem cell marker *Nestin* and *Musashi-1* genes and adipocyte marker *Leptin* by quantitative real-time RT-PCR. Expressions of *Nestin* and *Musashi-1* genes were remarkably upregulated in the neurosphere cells compared with the control ASCs without culturing in the neurosphere medium (Fig 2, A and B), suggesting characteristics of neural progenitor. Conversely, *Leptin* expression

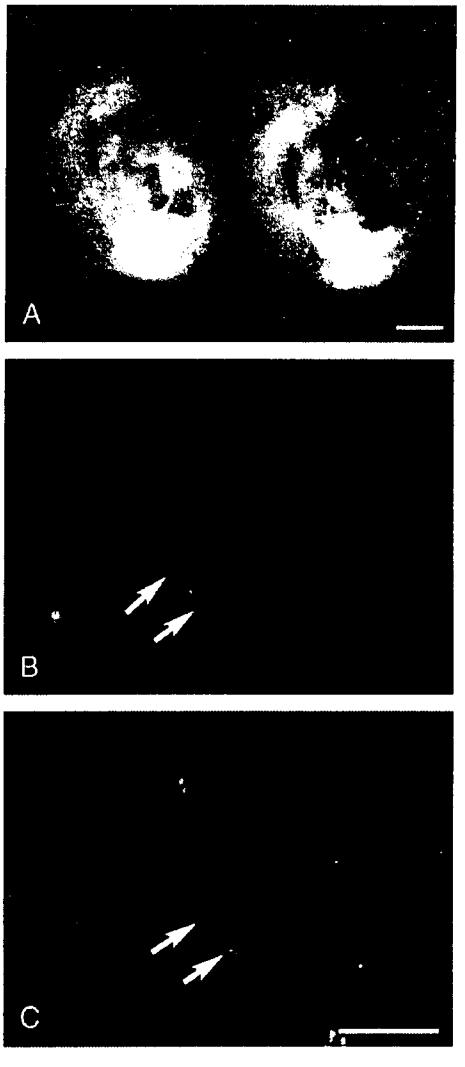

Fig 3 Neural crest-like migrations of green fluorescent protein (GFP)-transfected, adipose-derived stromal cell-derived neurospheres grafted into mouse embryo cultured in vitro. (A) Appearances of mouse embryos cultured for 40 hours from embryonic day 8. (B and C) Fluorescent views of embryos. GFP-positive neurosphere cells were arranged in a row (arrows), suggesting their migration along second branchial arch. Bars = 500 μm.

was dramatically reduced in the neurospheres (Fig 2C), indicating loss of adipogenic potential.

To investigate functions of the neurosphere cells in vivo, we labeled these cells by the modified Sendai virus vector carrying the *GFP* gene and transplanted them into the head region of the E8 mouse embryos. After the embryos were cultured for approximately 40 hours in vitro, the transplanted GFP-positive cells were clearly observed and appeared viable in only two embryos of the nine cultured embryos. The GFP-positive cells were incorporated into the craniofacial region as well as the heart and the trunk in these two embryos (Fig 3). Notably, the transplanted cells were arranged in a row along the second branchial arch (arrows in Fig 3, B and C) in a quite similar pattern to the neural crest cells migrating within the second branchial arch. Although not confirmatory, this result suggests a intriguing possibility that neurosphere cells derived from ASCs have neural crest-like properties.

Discussion

ASCs are probably one of the most well-known stem cells among plastic surgeons. ASCs were originally reported by Zuk et al[1] from the clinical samples of liposuction aspirates. According to their broad spectrum of differentiation potential, ASCs have been used in a number of preclinical animal studies of in vivo regeneration of a various tissues such as bone,[20,21] cartilage,[22] vessels,[4,23,24] soft tissue,[4] bone marrow,[25] and so on. Even a clinical case was reported, in which a calvarial defect was repaired by ASCs combined with scaffold.[26] Several groups reported neural differentiation of ASCs in vitro,[5,6,27] and Kang et al[28] reported functional recovery of the rat model with cerebral infarction after ASC transplantation in vivo.

The neurosphere method was originally reported by Reynolds et al[10,11] and is one of the most frequently used methods for isolating neural stem cells from the embryo or from the adult central nervous systems. However, this method has not yet been applied for obtaining neural stem cells from adipose tissue or the ASC population. In this preliminary study, we obtained neurospheres from the ASCs in human liposuction aspirates. Proliferation of these cells was quite rapid, possibly faster than other neurospheres from various tissue origins such as the dermis and the heart,[7–9] suggesting advantages of ASCs as a origin of neuronal progenitors for regenerative medicine. These neurosphere cells expressed *Nestin* and *Musashi-1*, marker genes for neural stem cells, probably reflecting their tendency of differentiating into neuronal progenitors. This view is further supported by inhibition of their expression of *Leptin*, a marker for adipogenic differentiation and maturation.

Do the ASC-derived neurosphere cells behave as neuronal progenitors in vivo? Our attempt of grafting these cells into the cultured mouse embryo revealed that some of the cells migrate along the second branchial arch and contribute to craniofacial morphogenesis. Their migratory pattern is quite similar to that of cranial neural crest cells, as we reported previously.[16] The neural crest cells are an embryonic cellular population characterized by extensive migration and a unique repertoire of differentiation.[29] The neural crest cells are often regarded as stem or progenitor cells for peripheral neurons and Schwann cells, and the craniofacial skeletal mesenchyme is also neural-crest derived.[17,19,29,30] Recent studies indicate that the neural crest stem cells can be harvested from the seemingly "mesodermal" tissues of adult animals, such as the dermis,[7] the hair follicular dermal papilla,[8] or the heart,[9] by means of the neurosphere method, implicating that it is also the case in the adipose tissue. Because our data are preliminary and we have a small sample size, further studies such as those with detailed expression analysis of neural/neural crest marker genes and large-scale in vivo grafting are necessary to confirm this interesting idea.

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Sendai Virus-Mediated Gene Delivery into Hepatocytes *via* Isolated Hepatic Perfusion

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The recombinant Sendai virus vector is a promising tool for human gene therapy, capable of inducing high-level expression of therapeutic genes in tissue cells *in situ*. The target tissues include airway epithelium, blood vessels, skeletal muscle, retina and the central nervous system, but application to hepatic tissues has not yet been achieved, because direct intraportal injection of the vector is not feasible. We report an efficient and harmless procedure of gene delivery by recombinant Sendai virus into rat parenchymal hepatocytes, based on isolated hepatic perfusion with controlled inflow. Critical parameters for successful hepatic gene delivery are a brief preperfusion period (25 °C, 5 min); appropriate vector concentration in the perfusate (10^7 pfu/ml); moderate portal vein pressure (12 mmHg) and a brief hyperthermic postperfusion period (42 °C, 5 min). Under these optimized conditions, marker genes were expressed in most parenchymal hepatocytes without significant damage to hepatic tissues. Furthermore, expression of the marker genes was undetectable in nonhepatic tissues, including the gonads, indicating that this approach strictly targets hepatic tissues and thus offers good clinical potential for human gene therapy.

Key words gene delivery; hepatic perfusion; Sendai virus vector; vector targeting

The Sendai virus is a nonsegmented negative strand RNA virus belonging to the *Paramyxoviridae*.[1] It has a unilamellar lipoid envelope encapsulating the nucleocapsid (RNA–protein complex), and infects the host cell by delivering the nucleocapsid *via* fusion between the envelope and the cell membrane.[1] Recombinant Sendai virus has received attention as a unique viral vector for human gene therapy, capable of producing a large amount of therapeutic proteins,[2,3] with exceptionally broad cell and tissue specificities.[4] For example, recombinant Sendai virus has been used successfully to deliver therapeutic genes *in situ* into airway epithelium,[5] blood vessels,[6] skeletal muscle,[7] retina[8] and the central nervous system[9] of various animal species. This broad host specificity partly depends on the early stage of infection, using sialic acid, a ubiquitous component of the animal cell membrane, as a primary receptor.[10] Nevertheless, the application of this vector to the parenchymal hepatocyte, an important target cell in gene therapy for treating metabolic diseases, has not yet been established.

As the native Sendai virus can infect nondividing parenchymal hepatocytes *in vitro*,[4,9] derivative vectors are potentially applicable to the hepatic tissue *in vivo*. However, direct *intravenous* or *intraportal* injection of Sendai virus vectors is not feasible because of their hemagglutinating and hemolytic activities: the injected vectors immediately aggregate and destroy red blood cells, and may form a harmful blood coagulation. Furthermore, Sendai virus particles (<230 nm in diameter) are larger than the mesh of the hepatic sinusoids (*ca.* 100 nm), a physiological sieve that restricts the access of large serum components to parenchymal hepatocytes;[11] this also hinders their application to the hepatic tissue.

In this article, we describe a procedure of isolated hepatic perfusion that enables reproducible and tissue-specific gene delivery by Sendai virus vectors. Isolated hepatic perfusion is a surgical procedure originally developed for treating nonresectable liver tumors by regional high-dose administration of anticancer drugs,[12,13] and has been used for delivering gene transfer vectors into hepatic tissues.[14—17] This procedure is useful for administrating Sendai virus vectors because a brief preperfusion can flush interfering blood cells from the hepatic tissue. Furthermore, perfusion under controlled portal pressure alters the mean mesh size of the hepatic sinusoids so that the Sendai virus particles can path through this physiological sieve. As isolated hepatic perfusion has been successfully applied to chemotherapy for nonresectable hepatic tumors in clinical trials, this approach should be feasible for human gene therapy.

MATERIALS AND METHODS

Purification and Physical Characterization of Sendai Virus Vectors Recombinant Sendai viruses carrying the firefly luciferase gene (SeV-luc)[18] and the gene for enhanced green fluorescent protein (SeV-EGFP)[19] were propagated by inoculating 100 μl of the diluted working virus seed contain-

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ing *ca.* 10 plaque-forming units (pfu) of the virus into the chorio-allantoic cavity of 10-d-old fertilized domestic chicken eggs. After 72 h incubation, the viral particles were recovered from the chorio-allantoic fluid by high-speed centrifugation and suspended in BSS buffer (150 mM NaCl, 10 mM Tris–HCl, pH 7.6) as previously described.[20]

Viral particles were purified using sucrose two-step centrifugation (20% and 50% sucrose in BSS; 60000 ***g*** for 60 min at 4 °C in a Beckman SW28Ti rotor), and recovered from the interface between the sucrose layers.[21] The purified viral particles were then subjected to gel filtration for sizing,[22] using Sephacryl S1000 (2.0×45 cm diameter columns; Amersham Biotechnology, Piscataway, New Jersey, U.S.A.) as a medium, with HBS (150 mM NaCl, 10 mM HEPES–NaOH, pH 7.6) as running buffer. Gel filtration using Sephacryl S1000 has been successfully used for sizing large unilamellar liposomes with diameters of 200—400 nm.[22] The flow rate was kept at 0.1 ml/min, and aliquots of 2 ml were collected. All purification procedures were performed under a sterile environment.

The diameter of viral particles in each fraction was determined by the cumulant method using a dynamic light scattering spectrophotometer (model DLS-700, Otsuka Electronics Co., Ltd., Osaka, Japan).[23] The infectious titer of the purified virus was determined by plaque assays using $LLCMK_2$ cells.[24] Purified viral particles were frozen at −80 °C in the presence of 10% dimethyl sulfoxide.

For examining the purified Sendai virus particles under scanning electron microscopy, the samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) (2 h at 25 °C, then 14 h at 4 °C), and trapped on Nuclepore polycarbonate membrane filters (pore size 0.05 μm, Whatman Inc., Clifton, NJ, U.S.A.). The samples were then dehydrated, dried, coated with an osmium plasma coater (thickness *ca.* 2 μm) and examined as described.[25]

Surgical Procedure for Isolated Hepatic Perfusion Specific pathogen free, 8- to 10-week-old male Wistar rats (Japan SLC, Shizuoka, Japan) were used throughout the study. All the animal experiments were performed according to our institutional guidelines for the care and use of laboratory animals. Anesthesia was induced and maintained using ether inhalation.

Abdominal exposure was carried out through a midline incision. The liver and the suprahepatic caval vein were freed from ligamentous attachments, then the main phrenic vein and right adrenal vein were ligated with 6-0 silk sutures and opened using an electric knife. A 24-gauge cannula was inserted into the pyloric branch of the portal vein, with the tip in the portal lumen. The right renal vein was ligated using 6-0 silk sutures and the same cannula was inserted *via* the vein into the inferior *vena cava* until the tip lay near the caudal side of the liver. The distal part of the portal vein, suprahepatic and distal part of the inferior *vena cava* were clamped to isolate the hepatic circulation. Portal pressure was determined by inserting a micro pressure probe together with the cannula into the portal vein.

The perfusion system consisted of a peristaltic pump (Gilson, Inc., Middleton, WI, U.S.A.), and a perfusion circuit with the syringes filled with perfusate. For the basic perfusate, we used lactate-Ringer solution (LRS) containing heparin (3000 IU/l) as an anticoagulant. The perfusion procedure consisted of three sequential steps: preperfusion (10 ml) for flushing out the blood components; perfusion (10 ml) for delivering the vectors, and postperfusion (10 ml) for washing out the excess vectors. Flow rate was calibrated before each perfusion experiment.

After the perfusion, all the clamped vessels were released. The pyloric branch and the right renal vein were decannulated and ligated. The intestines were warmed to 37 °C, and the abdomen was closed using standard procedures. After surgery, each rat was allowed to recover in a warmed cage with free access to water.

Detection of EGFP Activity Four rats were killed 12 h after the administration of SeV-EGFP (10^8 pfu) by hepatic perfusion. The liver was fixed *in situ* by perfusing with 4% paraformaldehyde in phosphate buffered saline (PBS) (pH 7.4) and then divided into 5 mm-thick cubes. These were incubated in 5% to 30% sucrose in PBS, flash frozen in O.C.T. compound (Miles Inc., Elkhart, Indiana, U.S.A.) and sectioned (5 μm) using a cryostat. EGFP was detected under fluorescence microscopy using a GFPA optical filter cube (Olympus, Tokyo, Japan). For histological examinations, tissue sections were stained with hematoxylin and eosin.

Determination of Firefly Luciferase Activity Rats were killed 4 h after the administration of SeV-luc (10^8 pfu) by hepatic perfusion, and the liver, lungs, heart, left kidney, spleen, thymus and testes were removed. Luciferase activities in each tissue were determined as described,[26] except that the samples were first homogenized using a microhomogenizer (Nichion, Chiba, Japan) in lysis solution containing protease inhibitors (10 μg/ml each of E-64, aprotinin, leupeptin and pepstatin A, and 0.5 mM phenylmethylsulfonylfluoride) and the homogenates were clarified by brief centrifugation (1000 ***g*** for 10 min). Protein was determined using a BCA protein assay kit (Pierce, Rockford, U.S.A.).

RESULTS

The physical size of the gene transfer vectors is one of the critical parameters determining their accessibility to the parenchymal hepatocytes *in vivo*. Viral vectors successfully used for delivering genes into the hepatocyte (adenovirus, lentivirus and adeno-associated virus vectors), have the sizes (30—90 nm in diameter) well below the inner diameter of the mesh of the hepatic sinusoids (*ca.* 100 nm). However, the Sendai virus particle is much larger, with a diameter of over 200 nm. Furthermore, Sendai viruses show extensive heterogeneity in size, with diameters ranging from 160 to 600 nm, depending on the number of RNA genomes encapsulated in a single particle.[27] Therefore, selecting and using homogenous Sendai virus particles is a key factor for evaluating delivery through the hepatic sinusoid.

To obtain a homogenous virus preparation, we purified viral particles on gel filtration using Sephacryl S1000,[22] and the sizes of the purified particles in each fraction were determined by dynamic light scattering. As shown in Figs. 1a and b, the viral particles recovered in major peak fractions on gel filtration were acceptably homogenous in size (235±5 nm in diameter, with a polydispersity index below 0.1). The purified virus suspensions were frozen in small aliquots in the presence of dimethyl sulfoxide, and were thawed just before use. This freezing and thawing did not alter the infectivity or