Efficient Ex vivo Expansion of Vα24+ NKT Cells Derived From G-CSF-mobilized Blood Cells

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Summary: Natural killer T (NKT) cells are involved in the function of innate immune systems and also play an important role in regulating acquired immune responses. In previous reports, we showed that Vα24+ NKT cells proliferated more efficiently from granulocyte-colony stimulating factor (G-CSF)-mobilized peripheral blood mononuclear cells (PBMC) than from non-mobilized PBMC. However, the mechanism of this enhanced NKT cell expansion is not yet clear. The goal of this research was to develop culture conditions for the more efficient ex vivo expansion of NKT cells. G-CSF-mobilized PBMC was cultured in AIM-V medium supplemented with 10% autoplasma, 100 ng/mL α-galactosylceramide (α-GalCer) and 100 IU/mL recombinant human (rh) interleukin (IL)-2. The efficiency of the expansion of Vα24+ NKT cells was evaluated on day 12. The expansion-fold of Vα24+ NKT cells was augmented depending on the proportion of CD14+ cells at the beginning of culture. The depletion of Vα24+ NKT cells abrogated the expansion of Vα24+ NKT cells. Depletion of CD56+ NK cells from mobilized PBMC enhanced, and add-back of purified CD56+ NK cells suppressed the expansion of Vα24+ NKT cells. Experiments with different timings for the addition of cells, IL-2 and α-GalCer suggested that follow-up supplementation with IL-2 or CD14+ cells should be avoided for the efficient expansion of Vα24+ NKT cells. These results should be useful for the development of an efficient and practical expansion protocol for adoptive immunotherapy with Vα24+ NKT cells.

Key Words: Vα24+ NKT cells, α-galactosylceramide, CD14+ cells, CD56+ NK cells

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INTRODUCTION

NKT cells are lymphocyte lineage and show characteristics of both T cells and NK cells.[1] NKT cells coexpress T cell receptors (TCRs) and NK cell markers, and display an extremely restricted TCR repertoire, consisting of Vα24 chain preferentially paired with Vβ11 chain. Upon activation by a specific ligand, NKT cells produce high levels of interferon-gγ (IFN-γ) and interleukin-4 (IL-4), and yield a strong immune response against several types of tumor cells.[2] Therefore, these invariant NKT cells are considered key effector cells, and play critical roles in immunity against microbial infection, tumor and autoantigens.

The marine sponge-derived glycosphingolipid α-galactosylceramide (α-GalCer) specifically activates human and mouse invariant NKT cells.[3,4] In vivo activation of NKT cells by α-GalCer induced strong cytotoxicity and the production of several cytokines in mice,[5] and it is well known that NKT cells differentiate efficiently with the in vitro administration of α-GalCer to acquire cytotoxic activities.[6] Therefore, this glycolipid agent may be able to effectively expand and activate NKT cells, and thus may be a useful tool for clinical immunotherapy.

For the clinical application of NKT cells in cancer immunotherapy, efficient expansion of the cells is very important. We previously reported that granulocyte colony-stimulating factor (G-CSF)-mobilized PBMC showed a higher efficacy of expansion of NKT cells,[7] and a fetal bovine serum (FBS)-free culture system has been developed.[8] In this study, we further attempted to improve the culture system by evaluating the effects of other cell components and interleukin (IL)-2.

MATERIALS AND METHODS

Cells and Plasma Preparation

Peripheral blood (PB) or apheresis products were obtained from normal healthy donors for allogeneic peripheral blood stem cell transplantation (PBSCT) after written informed consent was obtained. Healthy donors were administered G-CSF (filgrastim) 10 μg/kg subcutaneously for 4 continuous days, and leukapheresis was performed on the 4th day. PB was collected in a heparin-containing collection tube before and after G-CSF mobilization. The plasma was separated from cell components by centrifugation at 3,000 rpm for 15

minutes. The cells were loaded on lymphocyte separation medium (Ficoll-Conray, Immuno-Biologic Laboratories, Gunma, Japan), and centrifuged at 2,000 rpm for 20 minutes. PBMC were collected from the intermediate layer of Ficoll-Conray density gradient centrifugation and washed twice with PBS. The plasma was subjected to heat-inactivation and stored at -20°C until use. A cell separator (COBE-Spectra, GANBRO, Stockholm, Sweden) was used for leukapheresis. Any residual mononuclear cells were collected from apheresis tubes and bags by washing with PBS after cells were collected for clinical transplantation, and separated by Ficoll-Conray density gradient centrifugation. The apheresis plasma was also collected from the collection bags.

Expansion of Vα24+ NKT Cells

In this manuscript, we use the term Vα24+ NKT cells to refer to Vα24+ CD3+ double-positive NKT cells and confirmed the co-expression of Vβ11 chain. Isolated PBMC were cultured in 6-well culture plates (Costar, Corning, NY) at 2.0×10^5 cells/mL (each well filled with 4 mL media) in AIM-V media (Life Technologies, Rockville, MD) containing 10% autologous plasma, supplemented with 100 ng/mL α-galactosylceramide (α-GalCer, supplied by Kirin Brewery Co., Tokyo, Japan) and 100 IU/mL recombinant human (rh) IL-2 (R&D Systems, Minneapolis, MN) for 12 days. IL-2 was freshly added every 3 days to maintain its biologic activity. In the first experiment to define the efficacy for Vα24+ NKT cells expansion between before and after G-CSF mobilization, we used steady-state autologous plasma before G-CSF administration (pre-G-CSF), autologous plasma derived from PB after G-CSF administration (post-G-CSF PB) and autologous plasma obtained from apheresis product after G-CSF administration (post-G-CSF apheresis). In other experiments, we uniformly used autologous plasma obtained from apheresis product.

Monoclonal Antibodies

For flow cytometry analysis, anti-CD3-APC, anti-CD14-FITC, anti-CD16-PE, anti-CD56-FITC, anti-CD161-PE, anti-CD20-FITC and anti-CD19-PE monoclonal antibodies (mAbs) were purchased from BD Biosciences (Mountain View, CA). IgG1-FITC and IgG1-PE (cocktail), anti-Vα 24-FITC, anti-Vα24-PE, anti-Vβ11-PE and anti-CD4−FITC and anti-CD8−PE (cocktail) mAbs were from Immunotech (Marseilles, France). Anti-CD3-FITC mAb was from BD Pharmingen (San Diego, CA). For cell separation, anti-CD34-FITC, anti-CD56-FITC and anti-CD14-FITC mAbs were purchased from BD Biosciences (Mountain View, CA). Anti-Vα24-FITC mAb was from Immunotech (Marseilles, France). Anti-CD3-FITC mAb was from BD Pharmingen (San Diego, CA).

Cell Surface Antigen Analysis

For cell surface antigen staining, cells were incubated with FITC-, PE- or APC- conjugated mouse anti-human mAbs for 30 minutes on ice. After staining, cells

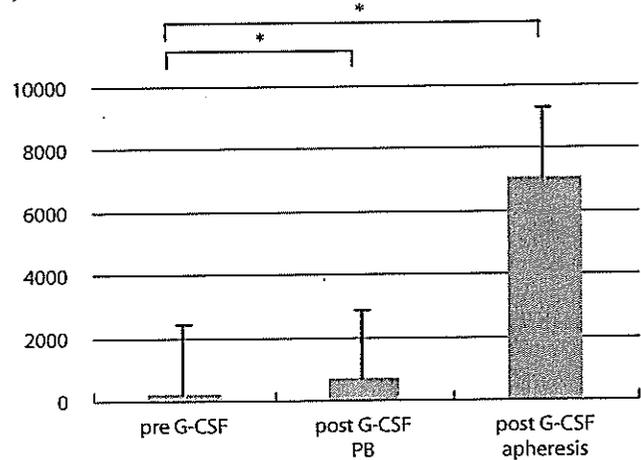**(A) proportion**
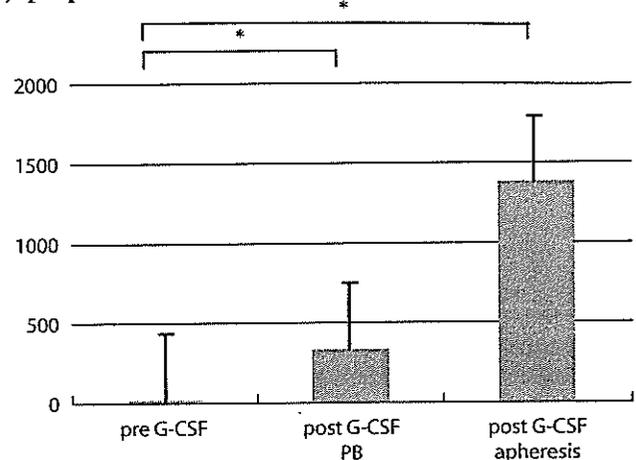
(B) absolute number

FIGURE 1. Proportion and absolute number of Vα24+ NKT cells on day 12 The proportion (A) and absolute number (B) of Vα24+ NKT cells increased 18(SD±23)- and 182(±158)-fold at the end of 12 days of culture for cells harvested before G-CSF administration, whereas these values were 333(±347)- and 669(±925)-fold in cells harvested after treatment with G-CSF. The highest increase was observed with apheresis product, which showed values of 1384(±1434)- to 7091(±2160)-fold respectively. The results were based on data obtained from 20 healthy donors. The bar means standard deviation. (*; P<0.05)

were washed twice and re-suspended in PBS. Staining with propidium iodide (PI; Sigma-Aldrich, St. Louis, MO) preceded all experiments to remove dead cells. Data were acquired by flow cytometry (FACSCalibur; BD Biosciences) and analyzed using CellQuest software (BD Biosciences). In this manuscript, we considered "CD56+ cells" as NK cells and use the phrase "CD56+ NK" cells.

Cell Separation and Coculture

PBSC Obtained from apheresis products were stained with FITC-conjugated mAbs against CD34,

Vα24, CD14, and CD56 for 20 minutes at 4°C and washed once with 5 mM EDTA-PBS. Anti-FITC-microbeads (Miltenyl Biotec, Gladbach, Germany) were then added to PBSC. After target cells were reacted with anti-FITC-microbeads, they were sorted by a magnetic cell separation system (Super MACS; Miltenyl Biotec), according to the manufacturer's protocol. The purity of isolated cells in the positive fraction was monitored and assured to be higher than 90% by flow cytometry, except for Vα24$^+$ NKT cells, which are difficult to obtain in high purity because of their rarity in PB. Although Vα24$^+$ NKT cells had a low purity (20% at most) after isolation by MACS, they were still considered enriched Vα24$^+$ NKT cells. On the other hand, contamination by CD14$^+$, CD 56$^+$, CD34$^+$, or Vα24$^+$ cells in their respective negative fractions was less than 10%.

To evaluate the influence of each cell population on Vα24$^+$ NKT cell expansion, we depleted and/or added back CD34$^+$ cells, Vα24$^+$ NKT cells, CD14$^+$ cells or CD56$^+$ NK cells, and evaluated the results on days 3, 6, 9 and 12. To evaluate the direct cell-cell interaction between CD56$^+$ NK cells and others, we used a Cell Culture Insert System with a 3 μm-pore membrane (Transwell, Corning, NY), and placed the CD56$^+$ NK fraction in the upper chamber and the CD56$^-$ fraction in the lower chamber. On day 12, the cells in the lower chamber were analyzed.

Contribution of CD14$^+$ Cells to Vα24$^+$ NKT Cell Expansion

To evaluate the contribution of CD14$^+$ cells to Vα24$^+$ NKT cell expansion and to optimize the CD14$^+$ cell conditions in our culture system, we depleted and added back CD14$^+$ cells to CD14$^-$ cells on day 0, on day 3, on day 6 or on day 9. CD14$^+$ cell was depleted by MACS (described above) and each added-back cells were 4.0×10^5 cells with optimized medium to maintain final concentration of IL-2 and autologous plasma. We also evaluated changes of concentration of CD14$^+$ cells before and after G-CSF administration and also evaluated the effects of them between different CD14$^+$ cell/CD14$^-$ cell ratio on Vα24$^+$ NKT cell expansion using the following culture conditions. The whole cell number was adjusted to 2.0×10^5 cells/ml in all wells, and the ratio of CD14$^+$
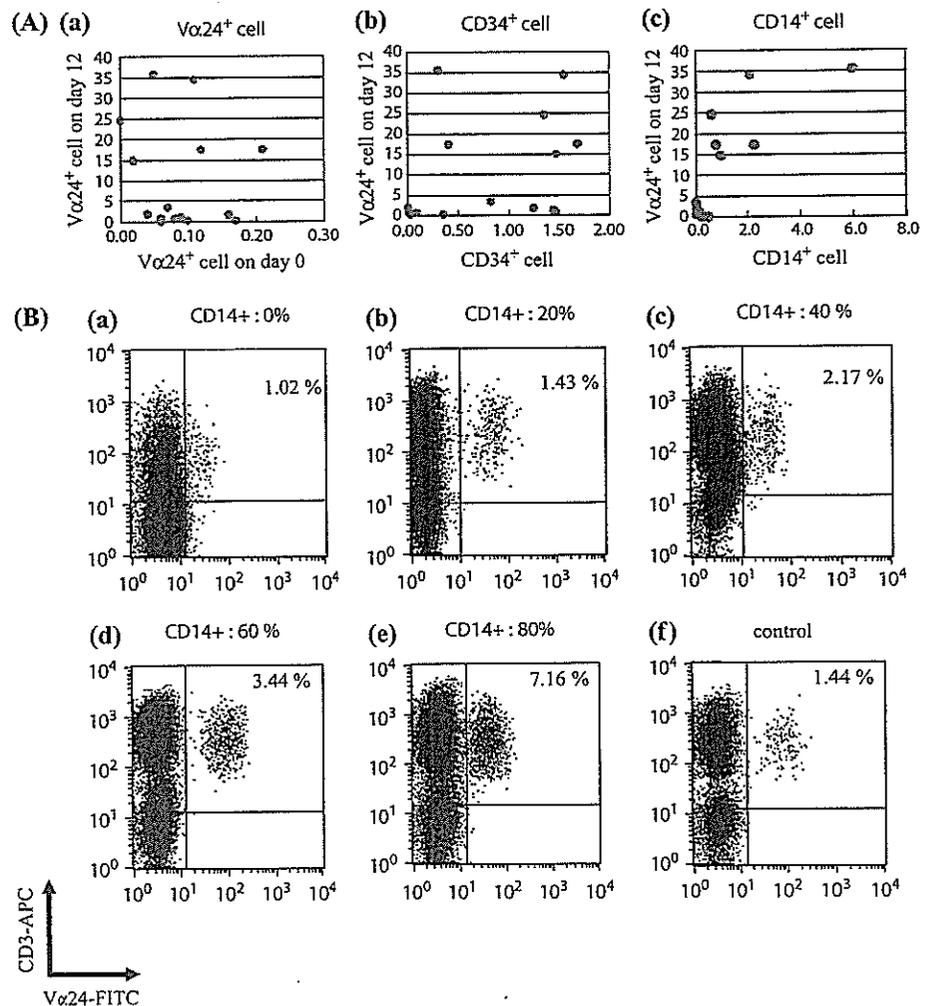
FIGURE 2. Effect of CD34$^+$, Vα24$^+$, and CD14$^+$ cells on expansion of Vα24$^+$ NKT cells (A) The proportion of (a) CD34$^+$, (b) Vα24$^+$ on day 0 were not associated with the expansion efficacy of Vα24$^+$ NKT cells ($r^2 = 0.171$, 0.016, respectively). Only CD14$^+$ cells (c) in the initial cell mixture had a relatively strong correlation ($r^2 = 0.545$) with the proliferation of cultured Vα24$^+$ NKT cells. These results were analyzed in 16 healthy donors. (B) The efficacy of Vα24$^+$ NKT expansion depended on the proportion of CD14$^+$ cells in apheresis products. The proportion of CD14$^+$ cells was as follows: (a) 0, (b) 20, (c) 40, (d) 60 and (e) 80% with a fixed total cell number of 2.0×10^5 cells/ml. The control means the result by using apheresis product without manupiration. These results are representative data from four experiments.

cells: $CD14^-$ cells was 0:5, 1:4, 2:3, 3:2, 4:1 or 5:0. The purpose of these manipulation was to detect the contribution of $CD14^+$ cells in the different timing of culture process and by the different proportion.

Modification of IL-2 Supplementation Schedule

In our original protocol established by Mikami and Harada, we added IL-2 to the cell culture medium every 3 days to maintain its biologic activity. However, in this study, we modified the schedule of IL-2 administration to determine the suitable culture conditions for $V\alpha24^+$ NKT expansion as follows: addition of IL-2 i) only on day 0, ii) days 0 and 3, iii) days 0, 3 & 6, and iv) days 0, 3, 6 & 9. Each supplementation of IL-2 was oriented to 100 IU/mL as a final concentration. The cell numbers and their phenotypes were analyzed on day 12. α-GalCer was also supplemented at final concentration 100 ng/mL.

Statistical Analysis

Student's t test was used to compare 2 groups and P values of < 0.05 were considered statistically significant. Correlation was estimated by the ordinary least squares method. Correlation coefficients are shown as squared values (r^2).

RESULTS

Efficient Expansion of Vα24⁺ NKT Cells Derived from G-CSF-Mobilized PBSCT of Normal Healthy Donors

We compared the expansion-fold of $V\alpha24^+$ NKT cells in PBSCT before and after G-CSF mobilization in 20 healthy donors. The expansion fold of percentage and absolute number of $V\alpha24^+$ NKT cells increased, respectively, 18(SD ± 23)- and 182(± 158)-fold in PBMC before G-CSF mobilization, whereas these were 333(± 347)- and 669(± 925)-fold in G-CSF-mobilized PBMC. Apheresis products from collection bags showed more efficient expansion capacities, from 1384(± 1434)- to 7091(± 2160)-fold (Figs. 1A, B). Thus, G-CSF mobilization significantly increased the capacity for $V\alpha24^+$ NKT cell expansion.

Relationship Between the Concentration of CD34⁺, Vα24⁺ and CD14⁺ Cells on V α 24⁺ NKT Expansion

To analyze the contribution of $CD34^+$, $V\alpha24^+$ and $CD14^+$ cells on the proliferation of $V\alpha24^+$ NKT cells in apheresis product, we compared the percentage of $CD34^+$, $V\alpha24^+$ and $CD14^+$ cells on day 0 and $V\alpha24^+$ NKT expansion efficacy on day 12. The results suggested only $CD14^+$ cells showed the correlation with the expansion of $V\alpha24^+$ NKT cells. (Fig. 2A).

Contribution of CD14⁺ Cells to the Ex Vivo Expansion of Vα24⁺ NKT Cells

It has been reported that $CD14^+$ cells, dendritic cells and monocytes play a critical role in the initiation of proliferation of $V\alpha24^+$ NKT cells.[9] In PB after G-CSF
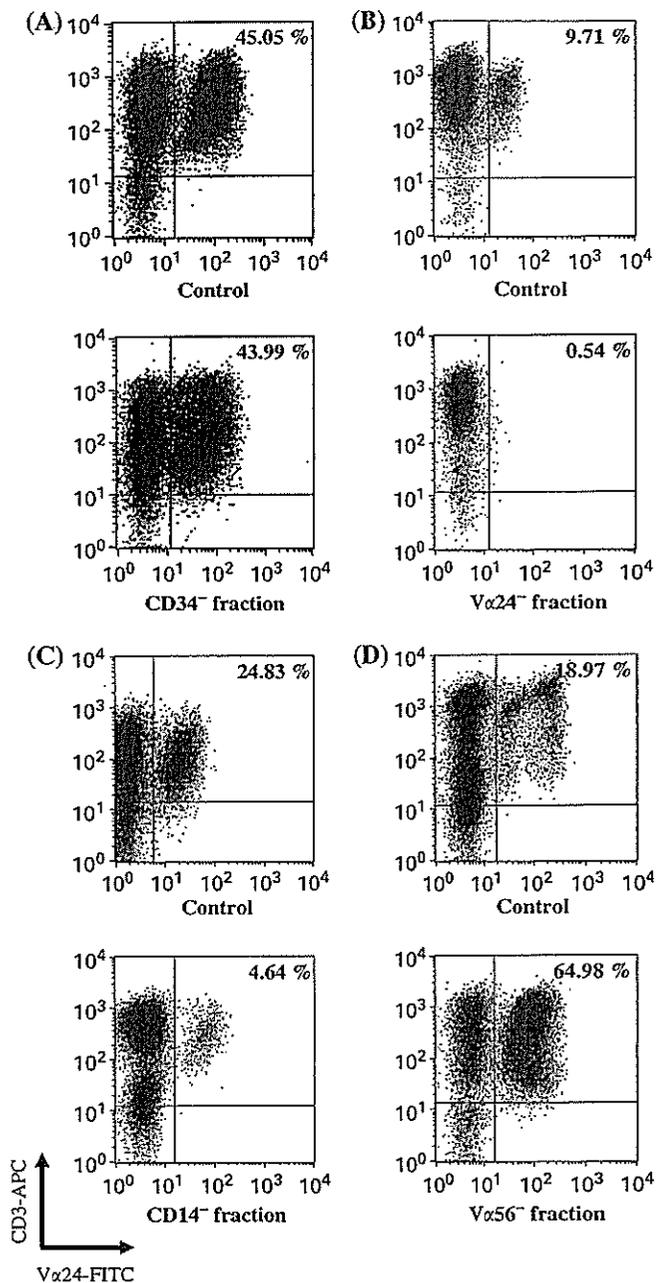

FIGURE 3. Effects of $CD34^+$, $V\alpha24^+$ NKT, $CD14^+$ and $CD56^+$ NK cell depletion on the expansion of $V\alpha24^+$ NKT cells $CD34^+$, $V\alpha24^+$ NKT, $CD14^+$, and $CD56^+$ NK cells were depleted using a MACS sorting system. (A) When $CD34^+$ cells were depleted, $V\alpha24^+$ NKT cells proliferated the same as in culture without $CD34^+$ cell-depletion. When (B) $V\alpha24^+$ NKT cells or (C) $CD14^+$ cells were depleted, $V\alpha24^+$ NKT cells did not expand. (D) When $CD56^+$ NK cells were depleted, the expansion efficiency of $V\alpha24^+$ NKT cells improved. These are each representative results from four experiments. The control in this experiment means the result by using apheresis product without target cell depletion.

treatment, the absolute number of $CD14^+$ cells significantly increased (from 350 ± 81 to $2353 \pm 1220 /\mu L$), although their percentage in PB did not change (from 7.24 ± 5.07 to $5.53 \pm 2.10\%$) due to an overwhelming increase in granulocytes. In apheresis products, the proportion of $CD14^+$ cells in nuclear cells also increased 5.7- to 38-fold compared with before G-CSF mobilization, because the apheresis products included low granulocyte contaminations, less than 20%. We obtained $CD14^+$ cells using the MACS system with a purity of >95%, and made a $CD14^+$ cell gradation (0%, 20%, 40%, 60%, 80% and 100%) under a fixed total cell count of 2.0×10^5 cells/mL/well. The efficacy of $V\alpha24^+$ NKT expansion was related to the initial proportion of $CD14^+$ cells, and the percentage of $V\alpha24^+$ NKT after expansion was increased in $CD14^+$ cell dose dependent manner (Fig. 2B).

Effect of Depletion of Cells, Including CD34⁺, Vα24⁺ NKT, CD14⁺ and CD56⁺ Cells, on Vα24⁺ NKT Cell Expansion

To determine the origin of $V\alpha24^+$ NKT cells and the contribution of each cell population on $V\alpha24^+$ NKT cell expansion, we tested the following cell culture conditions with apheresis products: 1) $CD34^+$ cell-depleted, 2) $V\alpha24^+$ NKT cell-depleted, 3) $CD14^+$ cell-depleted, and 4) $CD56^+$ cell-depleted culture. When $CD34^+$ cells were depleted, $V\alpha24^+$ NKT cells proliferated the same as in non-depleted culture (Fig. 3A).

However, the depletion of $V\alpha24^+$ NKT cells completely abrogated the expansion of $V\alpha24^+$ NKT cells (Fig. 3B). Depletion of $CD14^+$ cells also abrogated $V\alpha24^+$ NKT cell expansion to result in the complete disappearance of $V\alpha24^+$ NKT cells on day 12 (Fig. 3C). Interestingly, when $CD56^+$ NK cells were depleted, a remarkable improvement in $V\alpha24^+$ NKT cell proliferation was observed (Fig. 3D). In experiments with $CD56^+$ NK cells separated from $CD56^-$ fraction using a 3.0 μm-pore membrane, the proliferation of $V\alpha24^+$ NKT cells was maintained in $CD56^-$ fractions. The mixed culture of $CD56^+$ NK cells with $CD56^-$ fraction in the same wells resulted in the suppressed proliferation of $V\alpha24^+$ NKT cells, even though there were 1.0×10^5 $CD14^+$ cells (data not shown).

Add-Back of Cells, Including CD14⁺ Cells, to Vα24⁺ NKT Cell Cultures

The analysis of cell kinetics during culture suggested that $CD14^+$ cells gradually decreased in the early phase (days 0–3), whereas $V\alpha24^+$ NKT cells gradually increased in the latter phase of culture (days 9–12). With regard to $CD56^+$ NK cell kinetics, cell numbers continued to increase during culture in good responders (Fig. 4A), whereas they peaked on day 9 in poor responders (Fig. 4B). To evaluate the effects of $CD14^+$ NK cells in the early phase and late phase of $V\alpha24^+$ NKT cell expansion, we depleted and added back $CD14^+$ cells to the $CD14^-$ cell population, which included $V\alpha24^+$ NKT cells, on

FIGURE 4. Cell kinetics of Vα24⁺ NKT cells and CD56⁺ NK cells in good and poor expanders (A) In a good-expanding donor, both CD56⁺ NK cells and Vα24⁺ NKT cells continued to proliferate without decline (representative results from four experiments). The right hand y-axis is used for the cell number of Vα24⁺ NKT cells. (B) In a poor-expanding donor, CD56⁺ NK cells proliferated more efficiently than Vα24⁺ NKT cells, with a peak on day 9, concomitant with a suppression of Vα24⁺ NKT cell proliferation on day 12 (representative results from four experiments). The right hand y-axis is used for the cell number of Vα24⁺ NKT cells. (C) We added-back CD14⁺ cells to CD14⁻ cells on days 0, 3, 6, and 9. (D) Add-back of CD14⁺ cells before day 3 enhanced the proliferation of Vα24⁺ NKT cells. These are representative results from four independent experiments. All of four experiments were comparable and had a same tendency.
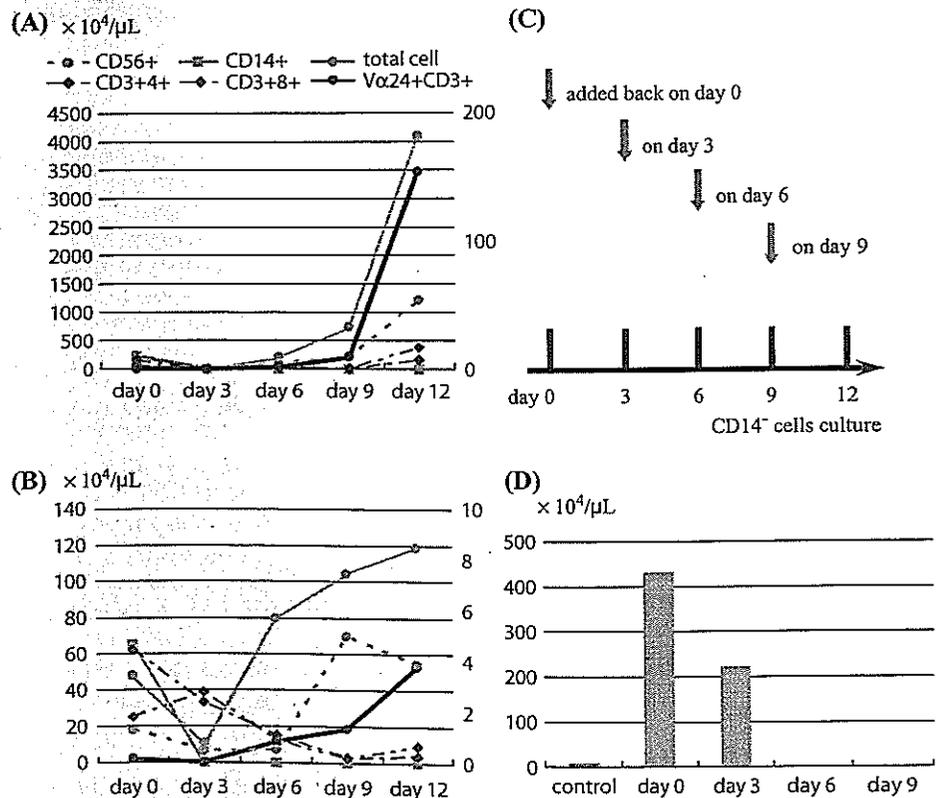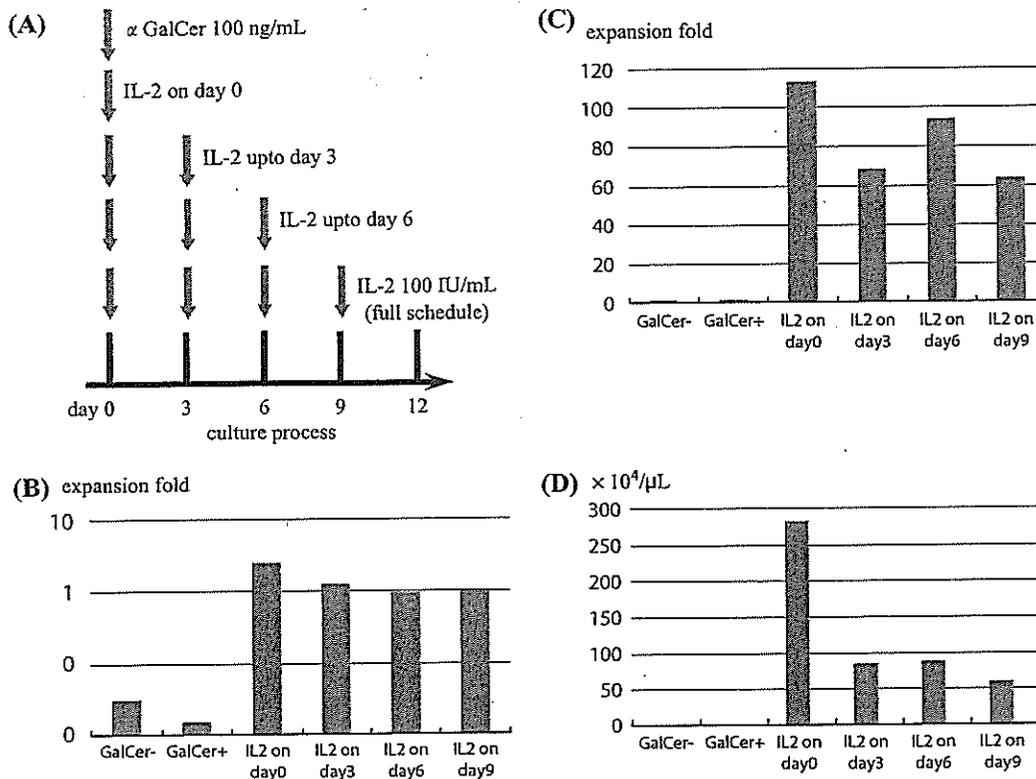
FIGURE 5. Effects of treatment with IL-2 on the expansion of $V\alpha24^+$ NKT cells (A) We tested different schedules for the administration of IL-2, as follows: on day 0 only, on days 0 and 3, on days 0, 3 and 6, and on days 0, 3, 6 and 9. We found that (B) the expansion-fold of whole cells, and the expansion-fold of the proportion (C) and absolute number (D) of $V\alpha24^+$ NKT cells were higher when IL-2 was supplemented on day 0 only (representative results from four experiments). All of four experiments were comparable and had a same tendency. In this experiments, α-GalCer was also supplemented at the concentration of 100 ng/mL without non-α-GalCer supplemented control.

days 0, 3, 6 and 9, respectively (Fig. 4C). Figure 4D shows that add-back of $CD14^+$ cells on day 0 induced the highest expansion of $V\alpha24^+$ NKT cells, whereas the addition of $CD14^+$ cells in the late phase did not show any remarkable benefit.

Effect of IL-2 Supplementation on the Expansion of $CD56^+$ NK Cells and $V\alpha24^+$ NKT Cells

We hypothesized that repeated IL-2 supplementation could result in the enhancement of $CD56^+$ NK activity to suppress the proliferation of $V\alpha24^+$ NKT cells.[10] In Figure 5, we tested four different schedules of IL-2 administration: on day 0 only, on days 0 and 3, on days 0, 3 and 6, and on days 0, 3, 6 and 9 (Fig. 5A). We found that whole cells and $V\alpha24^+$ NKT cells expanded most effectively when IL-2 was added on day 0 only (Figs. 5B–D).

DISCUSSION

The methods that have been used for the ex vivo expansion of human NKT cells can be divided into two

categories: simple culture of PBMC with α-GalCer,[11,12] and a two-step culture method that uses α-GalCer-pulsed monocytes as feeder cells.[13-15] A single culture system has the benefit of simplicity and a low risk of contamination, and a major obstacle in a two-step culture system is the availability of a large number of feeder cells. Hence, in this study of the former type, we intended to improve and establish culture conditions for realistic clinical application. Previously, we used a single stimulation of α-GalCer on the initial day, and then administered IL-2 every 3 days to obtain satisfactory expansion of human $V\alpha24^+$ NKT cells.[7] We have also reported that the addition of 5% autologous plasma was also effective.[8] G-CSF mobilization increased the efficacy of $V\alpha24^+$ NKT cell expansion, and our data suggested that this was due to a change in cellular component including $CD14^+$ cells[16] and serous factors in the blood. In our present study, we found that $CD14^+$ cells, which are effectively mobilized together with $CD34^+$ cells by G-CSF,[16] are one of the candidates hat contribute to the effective ex vivo expansion of $V\alpha24^+$ NKT cells. Only the number of pre-cultured $CD14^+$ cells affected the magnitude of the expansion of $V\alpha24^+$ NKT cells, and this agreed with a

previous report by van der Vliet et al that dendritic cells (DC) derived from monocytes including CD14^{+} cells could efficiently mediate the expansion of Vα24^{+} NKT cells.[17,18] Additionally, we showed that 1) depletion of CD14^{+} cells resulted in the loss of Vα24^{+} NKT cell expansion, and 2) the expansion efficacy of Vα24^{+} NKT cells depended on the ratio of CD14^{+} cells at the initiation of culture. Based on these observations, we speculated that the initial presence of CD14^{+} cells plays an important role in the subsequent effective expansion of Vα24^{+} NKT cells. We observed that the intensity of CD1d molecules on CD14^{+} cells, which is critical for interaction with α-GalCer for the expansion of Vα24^{+} NKT cells,[19] increased after G-CSF mobilization (data not shown). Hence, it is reasonable to speculate that more CD14^{+} cells with a high intensity of CD1d molecules plays a key role in NKT cell expansion. The higher expansion efficiency in apheresis products compared with G-CSF-mobilized PB may be secondary to a higher concentration of CD14^{+} cells.

The removal of Vα24^{+} NKT cells before culture resulted in the loss of Vα24^{+} NKT cell proliferation, and this supported previous reports that ex vivo-expanded Vα24^{+} NKT cells were neither committed nor supported by CD34^{+} cells, but were derived from peripheral circulating V α 24^{+} NKT cells.[17] Whereas CD34^{+} cells do not appear to be directly involved in the expansion of Vα24^{+} NKT cells, they might make the circumstances suitable for Vα24^{+} NKT cell expansion, through the secretion of unidentified soluble factors from bone marrow-derived stromal cells, as suggested by Johnston et al.[20] Although the presence of Vα24^{+} NKT cells on day 0 is critical for the expansion of Vα24^{+} NKT cells, no correlation was found between the proportion of Vα24^{+} cells before culture and the proportion of Vα24^{+} NKT cells at the end of culture. This suggests that some other factor(s) might regulate the expansion kinetics of Vα24^{+} NKT cells. The inhibition of cell expansion by CD56^{+} NK cells was restored when direct cell-to-cell contact was interrupted, which suggests that direct interaction between Vα24^{+} NKT cell and CD56^{+} NK cells plays a role. This hypothesis was indirectly supported by the phenomina that IL-2 supplementation in every 3 days suppressed expansion of Vα24^{+} NKT cells. Indeed, NK cell-mediated interference of NKT cells is well known to be a primary immune regulatory mechanism.[21] Another possibility is indirect inhibition through the modulation of DC functions. It has been reported that NK cells could yield cytolytic activity against DC during their expansion.[22–24] NKT cells were also activated by DC, resulting in the suppression and killing of DC[25,26] in the same manner as NK cells.

In conclusion, for the efficient ex vivo expansion of Vα24^{+} NKT cells, the presence of Vα24^{+} cells and CD14^{+} cells at the initiation of culture is critical. NK cells may interact with antigen presenting cells (APC) and interfere with the expansion of NKT cells by hindering the function of antigen presentation or providing direct

cytotoxicity against APC. We believe that these findings may be useful for the development of an efficient system for the expansion of NKT cells for future adaptive immunotherapy.

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Allogeneic MHC Gene Transfer Enhances Antitumor Activity of Allogeneic Hematopoietic Stem Cell Transplantation without Exacerbating Graft-versus-Host Disease

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Abstract   Enhancement of the specific antitumor activity of allogeneic hematopoietic stem cell transplantation (alloHSCT) against solid cancers is a major issue in the clinical oncology. In this study, we examined whether intratumoral allogeneic MHC (alloMHC) gene transfer can enhance the recognition of tumor-associated antigens by donor T cells and augment the antitumor activity of alloHSCT. In minor histocompatibility antigen – mismatched alloHSCT (DBA/2→BALB/c: H-2^d) recipients, alloMHC gene (*H-2K^b*) was transduced directly into a s.c. tumor of CT26 colon cancer cells. Because CT26 cells have an aggressive tumorigenicity in syngeneic BALB/c mice, an *H-2K^b* gene transfer provides only a limited antitumor effect after syngeneic (BALB/ c→BALB/c) HSCT. By contrast, the *H-2K^b* gene transfer caused significant tumor suppression in the alloHSCT recipients, and this suppression was evident not only in the gene-transduced tumors but also in simultaneously inoculated distant tumors without gene transduction. *In vitro* cytotoxicity assay showed specific tumor cell lysis by donor T cells responding to the *H-2K^b* gene transfer. Graft-versus-host disease was not exacerbated serologically or clinically in the treated mice, demonstrating that alloMHC gene transfer enhances the antitumor effects of alloHSCT without exacerbating graft-versus-host disease. This combination strategy has important implications for the development of therapies for human solid cancers.

Allogeneic hematopoietic stem cell transplantation (alloHSCT) often leads to a significant graft-versus-tumor (GVT) effect, and has proved to be an effective therapeutic approach for several types of leukemia, particularly acute and chronic myelogenous leukemia. Recently, alloHSCT has been applied not only for hematologic malignancies but also for solid cancers, such as renal and breast cancers (1–8). However, the benefit of the GVT effect is often offset by the occurrence of graft-versus-host disease (GVHD), a potentially fatal adverse effect primarily mediated by donor T cells. It is commonly believed that in MHC-matched alloHSCTs, the target antigens for a GVT effect

include tumor-associated antigens (TAA) and ubiquitously or tissue-specifically expressed minor histocompatibility antigens (mHA), whereas the targets for GVHD are mHAs. Therefore, efforts to selectively enhance a donor T-cell response to TAAs may provide a means to augment antitumor activity without a concomitant increase in toxicity.

Intratumoral transfer of an allogeneic MHC (alloMHC) gene modifies tumor cells to express the alloMHC molecule, a highly immunogenic antigen that causes an allogeneic rejection response. In the process of this response, cytolytic T lymphocytes are generated not only against the modified tumor cells but also against unmodified tumor cells (9). A putative mechanism for the induction of specific tumor immunity is that the allogeneic response increases local production of cytokines, facilitates antigen presentation and T-cell accumulation, and consequently causes sensitization to previously unrecognized TAAs. Clinical trials with direct intratumoral injection of the human leukocyte antigen-*B7* gene – expressing plasmid DNA complexed with liposome revealed a higher safety profile and systemic response in substantial populations (10-15%) in patients with melanoma and head and neck cancer (10 – 16). The treatment seemed to be promising but showed a limited clinical efficacy, for which the immune tolerance established between tumor and host may be one of the plausible explanations (17).

We expect that an alloMHC gene transfer could enhance the GVT effect by promoting recognition of TAAs by the donor immune system in alloHSCT recipients, and also that alloHSCT, on the other hand, could augment the therapeutic efficacy of an alloMHC gene transfer by providing a "fresh"

immune system in which tolerance to tumor cells is not yet induced. In this study, using an MHC (H-2^d)–matched mouse alloHSCT model, we found that an intratumoral alloMHC (H-$2K^b$) gene transfer significantly enhanced the antitumor effects of alloHSCT against a murine colon cancer. Importantly, GVHD was not exacerbated in any of the treated mice, suggesting the augmentation of tumor-specific immunity of donor T cells by the H-$2K^b$ gene transfer.

Materials and Methods

Animals and transplantation. Seven- to 9-week-old female BALB/c (H-2^d, Ly-1.2) and DBA/2 (H-2^d, Ly-1.1) mice were purchased from Charles River Japan, Inc. (Kanagawa, Japan) and were housed under sterilized conditions. Nine- to 10-week-old BALB/c mice received a lethal dose (9 Gy) of total body irradiation on the day of transplantation. The irradiated BALB/c mice were injected i.v. with 5 × 10^6 of T cell–depleted bone marrow cells and 2 × 10^6 splenic T cells from donor DBA/2 or BALB/c mice in a total volume of 0.2 mL Dulbecco's PBS solution, and the transplanted mice were designated as alloBMT or synBMT, respectively. Bone marrow cells were isolated from donors by flushing each femur and tibia with RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum (ICN Biomedicals, Inc., Irvine, CA), and splenic cells were prepared by macerating the spleens with a pair of tweezers. After lysis of the erythrocytes, the bone marrow and splenic cells were incubated with anti-Thy-1.2 immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) at 4°C for 15 minutes, followed by depletion and selection of T cells by AutoMACS (Miltenyi Biotec), respectively. More than 90% of T cells were depleted from the bone marrow cells.

Tumor cell lines. CT26 and Renca are weakly immunogenic BALB/c-derived colon and renal cancer cell lines, respectively, and were obtained from the American Type Culture Collection (Rockville, MD). Both cell lines were confirmed to express MHC class I molecules (H-$2K^d$ and H-$2D^d$) abundantly by flow cytometry (data not shown). Cells were maintained in RPMI containing 10% fetal bovine serum, 2 mmol/L L-glutamine, and 0.15% sodium bicarbonate (complete RPMI). A CT26 cell line that stably expresses the H-$2K^b$ gene was generated by retrovirus vector-mediated transduction and designated as CT26/H-2K^b.

Fluorescence-activated cell sorting analysis. FITC-conjugated monoclonal antibodies (mAb) to identify mouse H-2K^b, CD4, CD8, and phycoerythrin-conjugated mAb to CD3 were purchased from BD PharMingen (San Diego, CA), and FITC-conjugated mAbs to Ly-1.1 and Ly-1.2 were purchased from Meiji Dairies Co. (Tokyo, Japan). Cells were incubated with the relevant mAbs in a total volume of 100 µL PBS with 5% fetal bovine serum for 30 minutes at 4°C. Cells were then washed twice with PBS containing 5% fetal bovine serum, suspended in PBS, and analyzed by FACSCalibur (BD Biosciences, San Jose, CA). Irrelevant IgG mAbs were used as a negative control. Ten thousand live events were acquired for analysis. Allogeneic donor (DBA/2) T-cell engraftment was determined by the percentages of Ly-1.1$^+$ cells among CD3$^+$ cells.

Evaluation of GVHD. The degree of clinical GVHD in transplant recipients was assessed weekly by a scoring system that sums changes in five variables: weight loss, posture, activity, fur texture, and skin integrity (maximum index, 10) as previously described (18). In some recipients, selected serum chemistry was also examined for evaluation of GVHD.

In vitro cell proliferation assay. CT26/H-2K^b and CT26 cells were seeded at 2 × 10^3 per well in 96-well flat plates (Nunc A/S, Roskilde, Denmark). Cell numbers were assessed by a colorimetric cell viability assay using a water-soluble tetrazolium salt (Tetracolor One; Seikagaku Co., Tokyo, Japan) for 4 days after the seeding. The absorbance was determined by spectrophotometry using a wavelength of 450 nm with 595 nm as a reference using ELISA Analyzer (Toyo Sokki, Tokyo, Japan).

The assays (carried out in eight wells) were repeated a minimum of twice and the means ± SD were plotted.

In vivo tumor inoculation and alloMHC gene transfer. Tumor cells were prepared in a total volume of 50 µL PBS and injected s.c. on the right or left leg. A plasmid DNA expressing the H-$2K^b$ gene under the control of the Rous sarcoma virus long terminal repeat promotor was used for intratumoral gene transfer. The same vector plasmid DNA without a transgene was used as a negative control. Plasmid DNA-liposome complex per mouse was prepared by addition of 10 µg plasmid DNA into a total of 25 µL PBS, followed by the addition of 25 µL of 0.15 mmol/L DMRIE/DOPE [(+/−)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide/dioleoylphosphatidylethanolamine], which was provided from Vical, Inc. (San Diego, CA). The mixture solution was incubated at room temperature for 15 minutes, and then injected directly into the s.c. tumor. In previous report, presensitization of mice with alloMHC gene–expressing CT26 cells could maximize the therapeutic efficacy of alloMHC gene transfer (9). In this study also, preimmunization with CT26/H-2K^b followed by the treatment with H-$2K^b$ DNA-liposome complex was done in some of the transplanted mice as an optimized therapeutic control. As a preimmunization treatment, 1 × 10^6 of 30 Gy–irradiated CT26/H-2K^b cells were i.p. injected twice (at 14 and 7 days) before tumor inoculation. The shortest (r) and longest (l) tumor diameters were measured at indicated days and the tumor volume was determined as $r^2 l$ / 2. Data are presented as means ± SD.

Cytotoxicity assays. Using 24-well plates (Nunc), responder splenocytes (5 × 10^6/mL) were cultured with 30 Gy–irradiated CT26/H-2K^b stimulators (3-4 × 10^5/mL) in a complete RPMI containing 50 µmol/L 2-mercaptoethanol for 5 days. The responder cells were then collected and used as effector cells in a 4-hour chromium release assay against indicated target cells. Concanavalin A lymphoblasts were prepared by stimulating the splenocytes of naïve BALB/c mice for 3 days with 5 µg/mL concanavalin A at 2 × 10^6/mL in the complete RPMI containing 2-mercaptoethanol. Indicated target cells were labeled by combining 5 × 10^6 cells with 50 µCi ^{51}Cr (Perkin-Elmer Japan Co., Kanagawa, Japan) in a total volume of 0.2 mL complete RPMI for 1 hour at 37°C, followed by washing thrice with plain RPMI. For the chromium release assay, 5 × 10^5 effector cells were mixed with 2 × 10^4 target cells (effector-to-target ratio, 25) in a total volume of 0.2 mL complete RPMI in 96-well round-bottom plates (BD Biosciences). To evaluate the relative contributions of CD4$^+$ and CD8$^+$ T cells to tumor cell lysis, effector cells were incubated with mAb to mouse CD4 (L3L4; PharMingen), CD8 (Ly-2; PharMingen), or both for 30 minutes at 37°C before mixture with target cells. Supernatants were harvested with the Skatron harvesting system (Skatron, Sterling, VA) and counted in a gamma counter (Packard Bioscience Company, Meriden, CT). Percentage of cytotoxicity was calculated as [(experimental cpm − spontaneous cpm) / (maximum cpm − spontaneous cpm)] × 100. Spontaneous cpm was obtained from targets cultured in medium alone, and maximum cpm was obtained from targets incubated in 1% NP40. Each assay was done in triplicate.

In vivo depletion of T-cell function. To deplete specific immune effector cell subsets before and during treatment with H-$2K^b$ gene transfer, the transplanted mice received i.p. injections of 0.3 mg mAbs from the anti-CD4$^+$ hybridoma (clone GK1.5, rat IgG$_{2b}$) and/or anti-CD8$^+$ hybridoma (clone Lyt-2.1, mouse IgG$_{2b}$; ref. 19). Injections started 5 days before the inoculation with CT26 cells and the treatment repeated every 5 to 6 days throughout the entire experimental period to ensure depletion of the targeted cell type. CD4$^+$ and CD8$^+$ T-cell depletion was confirmed by flow cytometry of splenic suspensions at the time of tumor injection and weekly afterward.

Statistical analysis. Comparative analyses of the data were done by the Student's t test using SPSS statistical software (SPSS Japan, Inc., Tokyo, Japan). $P < 0.05$ was considered as a significant difference.

Results

AlloHSCT causes GVHD and GVT effects. We first assessed the posttransplant immune reconstitution of T cells and donor chimerism of splenic CD3$^+$ T cells in alloBMT recipients (DBA/2→BALB/c). To exclude biases related to transplant procedures, including lethal irradiation, we conducted synBMT (BALB/c→BALB/c) as a control. The reconstitution of both CD4$^+$ and CD8$^+$ T cells was delayed in alloBMT recipients compared with that in synBMT recipients at 8 weeks posttransplantation (Fig. 1A), which was consistent with other reports (20–22). The early (≤2 weeks) posttransplant mortality, most likely due to acute GVHD or graft failure, was usually <15% in transplant recipients. Analysis of donor engraftment showed 95.7 ± 1.5% donor type in alloBMT recipients (*n* = 3) at 8 weeks posttransplantation.

We then examined whether our alloHSCT models generate any GVHD and GVT effects. The clinical score of GVHD severity at 8 weeks was ~4 in alloBMT recipients, whereas it was <1 in
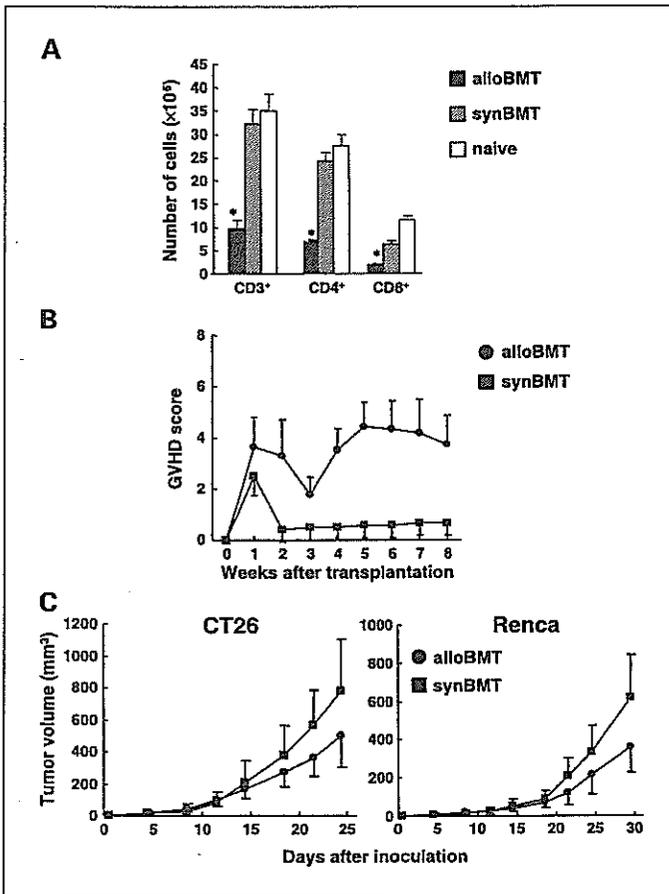

Fig. 1. Clinical GVHD score and s.c. tumor growth in all BMT or synBMT recipients. *A,* T-cell immune reconstitution in alloBMT or synBMT mice. The absolute number of splenic T cells was measured 8 weeks after the transplantation (*n* = 3 per group). *, *P* < 0.05 compared with synBMT. *B,* clinical course of GVHD in the recipient mice. Clinical GVHD scores were assessed weekly after the transplantation by a scoring system that sums changes in five clinical variables as described in the text (maximum index, 10; synBMT, *n* = 13; alloBMT, *n* = 11). *C,* growth curves of CT26 and Renca s.c. tumors in the recipient mice. Eight weeks after the transplantation, CT26 and Renca cells were inoculated s.c. and tumor sizes were measured on the days indicated (*n* = 4-6 per group). Representative of at least three independent experiments.
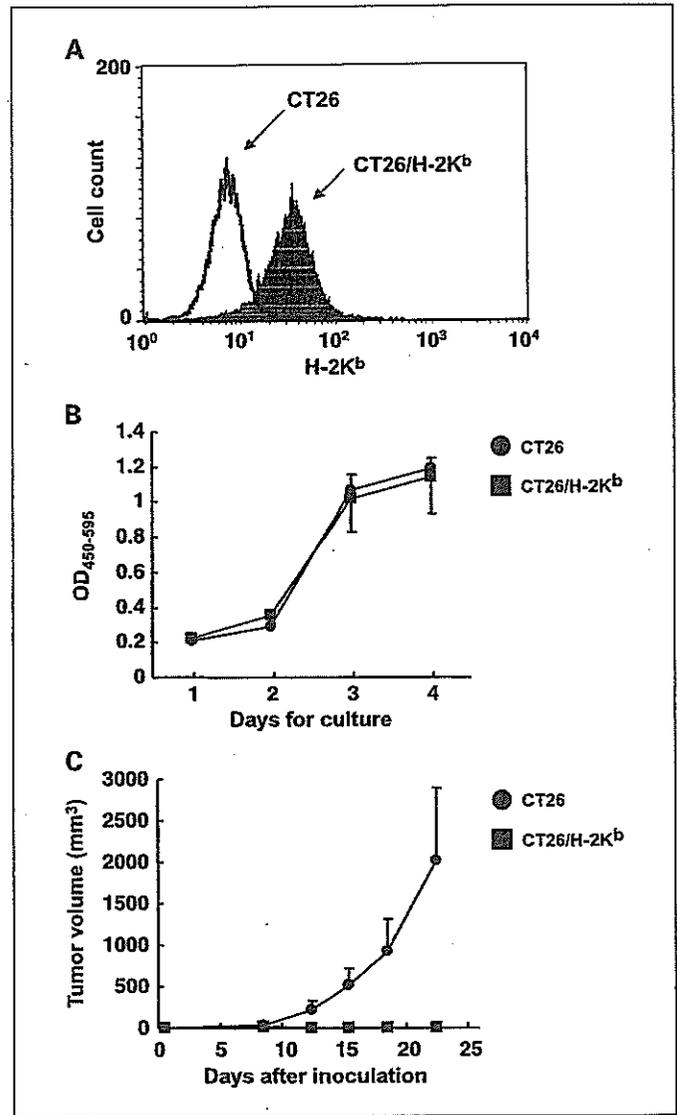

Fig. 2. Immune rejection of CT26/H-2K^b cell line in BALB/c mice. *A,* flow cytometric analysis of CT26/H-2K^b and unmodified CT26 cells. Cells were incubated with FITC-conjugated antibody to mouse H-2K^b and flow cytometry was carried out by FACSCalibur. *B, in vitro* cell proliferation assay. Number of CT26 and CT26/H-2K^b cells was assessed by a colorimetric cell viability assay. Each assay was done in eight wells. OD$_{450-595}$, absorbance at 450 to 595 nm. *C, in vivo* rejection of CT26/H-2K^b tumors. CT26 and CT26/H-2K^b cells were injected s.c. into the legs of naive BALB/c mice and tumor sizes were measured on the days indicated (*n* = 5 per group).

synBMT recipients (Fig. 1B). Death from GVHD was rarely observed during the first 3 months after the transplantation. For evaluation of GVT effects, 1 × 10^6 CT26 or 2 × 10^6 Renca cells were s.c. inoculated into the mice 8 to 9 weeks posttransplantation. The growth of the tumors was substantially, although not statistically significantly, suppressed in the alloBMT recipients compared with that in the synBMT recipients (Fig. 1C). Our alloHSCT model was shown to constantly cause GVHD and a limited but detectable level of the GVT effect, which is highly similar to a clinical setting after allogeneic HSCT.

AlloMHC gene–transduced tumor cells cause an immune response in vivo. To determine whether alloMHC gene–transduced CT26 cells could induce an immune response in

mice, the tumorigenicity of CT26/H-2K^b cells was compared with that of unmodified CT26 cells. Flow cytometric analysis confirmed the expression of the H-2K^b molecule in CT26/H-2K^b cells (Fig. 2A), and the *in vitro* proliferation of CT26/H-2K^b cells was compatible with that of unmodified CT26 cells (Fig. 2B). However, when injected s.c. into naïve BALB/c mice, CT26/H-2K^b cells did not form a tumor mass over 3 weeks ($n = 5$), whereas unmodified CT26 cells developed a rapidly growing tumor during the same period ($n = 5$; Fig. 2C), suggesting that the alloMHC gene – transduced tumor cells are highly antigenic and cause an immune response *in vivo*. No apparent toxicity was observed in the CT26/H-2K^b cell-inoculated mice.

AlloMHC gene transfer induces synergistic antitumor effect with alloHSCT. To examine whether alloMHC gene transfer could enhance the antitumor effects of alloHSCT, the mice were inoculated s.c. with 1×10^6 CT26 cells at 8 weeks posttransplantation, and H-2K^b gene – expressing plasmid DNA complexed with liposome was injected into the tumor thrice (at 5, 7, and 9 days) after the inoculation. The tumor

volumes at day 5 were ~60 to 100 mm^3. Preimmunization with irradiated CT26/H-2K^b cells was carried out in a group of transplanted mice as a positive control to maximize the effect of the H-2K^b immune gene therapy (9). A single injection of plasmid DNA-liposome complexes showed ~1% gene transduction efficiency *in vivo* (data not shown). The control empty vector was not immunogenic *in vivo* because the tumor volume of the negative control group without any intratumoral injections was similar to that of the empty plasmid – injected group (Fig. 3A). In the CT26/H-2K^b-preimmunized mice, H-2K^b gene transfer caused significant tumor suppression after either type of transplantation, synBMT or alloBMT (Fig. 3A). In non-preimmunized mice, H-2K^b gene transfer showed only a limited suppressive effect in synBMT recipients, which was probably due to the aggressive tumorigenicity of CT26 cells *in vivo*, whereas significant suppression of tumor growths was recognized in alloBMT recipients (Fig. 3A). The results showed that alloMHC gene transfer can augment the antitumor effects of donor immune cells in the context of alloHSCT.
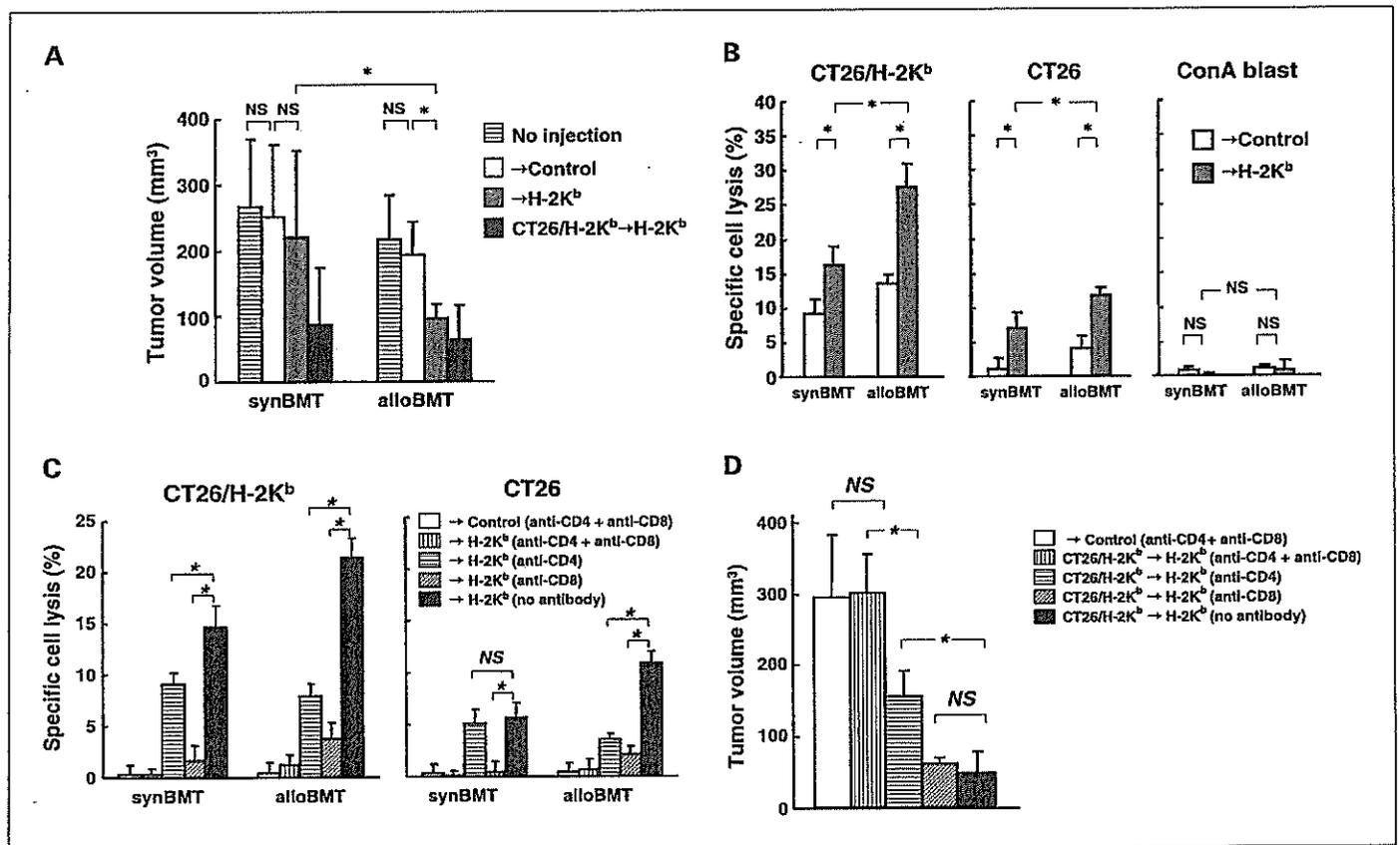

Fig. 3. Synergistic antitumor effect of alloMHC gene transfer in alloBMT recipients. *A,* growth of CT26 s.c. tumors injected with *H-2K^b* vector in alloBMT or synBMT recipients. Eight weeks after the transplantation, CT26 cells were s.c. inoculated and then *H-2K^b* vector or control vector was injected into the tumors thrice. Tumor volume was compared between the groups at 11 days after the completion of vector injection ($n = 5$-7 per group). As a preimmunization treatment, irradiated CT26/H-2K^b cells were i.p. injected twice before tumor inoculation to maximize the therapeutic efficacy of *H-2K^b* gene transfer. →Control, injection of an empty vector without a transgene. CT26/H-2K^b→H-2K^b, *H-2K^b* gene transfer with preimmunization; →H-2K^b, *H-2K^b* gene transfer without preimmunization. *, *P* < 0.05. NS, not significant. Representative of at least three independent experiments. *B, in vitro* cytotoxicity assay of *H-2K^b*-vector injected mice. Splenocytes were collected from alloBMT or synBMT mice ($n = 3$-4 per group) 15 days after the completion of vector injection, and their cytotoxicity was evaluated in a standard 4-hour ^{51}Cr release assay (effector/target ratio, 25) against CT26/H-2K^b, CT26 cells, and BALB/c-derived concanavalin A (*ConA*) lymphoblasts after stimulation with irradiated CT26/H-2K^b cells. *, *P* < 0.05 compared with synBMT. *C,* blocking assay of *in vitro* cytotoxicity. Splenocytes from *H-2K^b* gene – transduced alloBMT or synBMT recipients were analyzed for their cytotoxicity in a standard 4-hour ^{51}Cr release assay (effector/target ratio, 25) against CT26/H-2K^b or CT26 cells with anti-CD4 and/or anti-CD8 antibodies. *, *P* < 0.05. *D,* antitumor effect of *H-2K^b* gene transfer in alloBMT recipients after the *in vivo* depletion of CD4$^+$ and CD8$^+$ T cells. Groups of alloBMT mice were treated with anti-CD4 and/or anti-CD8 antibodies to deplete these cell populations, and the CT26 s.c. tumor was injected with *H-2K^b* vector thrice. Tumor volume was compared between the treatment groups at 11 days after the completion of vector injection ($n = 6$-8). *, *P* < 0.05.
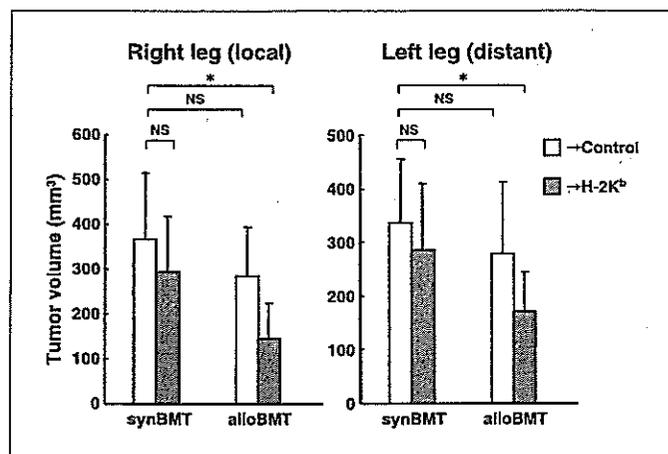
Fig. 4. Suppression of tumors at distant sites by the intratumoral injection of *H-2K^b* vector in alloBMT mice. CT26 cells were s.c. inoculated on both legs of alloBMT or synBMT recipients, and H-2K^b vector were injected into only the right leg tumor thrice without preimmunization. Tumor volume at each leg was compared between the treatment groups at 12 days after the completion of vector injection (*n* = 7-11). *, *P* < 0.05. NS, not significant. Representative of at least three independent experiments.

In vitro cytotoxicity assay was done to assess antitumor cytolytic T-lymphocyte responses induced by alloMHC gene transfer in the non-preimmunized mice. The splenocytes derived from mice bearing *H-2K^b*-transduced tumors recognized and lysed unmodified CT26 cells as well as CT26/H-2K^b cells but not concanavalin A blasts, and the cytotoxic activity was higher in the alloBMT recipients than in the synBMT recipients (Fig. 3B). Splenocytes showed higher cytolytic response to CT26/H-2K^b than to CT26 cells, indicating the donor T cells recognize the H-2K^b molecule as an allogeneic antigen.

Both CD4$^+$ and CD8$^+$ T cells contribute to the antitumor immunity. Then, in the in vitro blocking assays of lymphocyte cytotoxicity with antimurine CD4 and CD8 antibodies, CD8$^+$ T cells were shown to be the dominant effector in synBMT recipients, whereas both CD4$^+$ and CD8$^+$ T cells were apparently contributive to tumor cell lysis in alloBMT recipients (Fig. 3C).

To further explore the role of CD4$^+$ and CD8$^+$ T cells in antitumor immunity, the preimmunized alloBMT mice were treated with anti-CD4 or anti-CD8 antibodies to deplete these cell populations in vivo. The antitumor effect of *H-2K^b* gene transfer was completely cancelled in the transplanted mice with depletion of both CD4$^+$ and CD8$^+$ T cells, whereas the animals depleted of CD4$^+$ or CD8$^+$ T cells showed significant tumor growth inhibition (Fig. 3D). This in vivo depletion study indicated that the CD4$^+$ and CD8$^+$ cytotoxic T cells play central roles in the generation of antitumor immunity. Whereas CD8$^+$ T cells were more contributive to in vitro tumor cell lysis than CD4$^+$ T cells in the in vitro cytotoxicity assay (Fig. 3C), CD4$^+$ T cells might be more contributive to in vivo tumor inhibition than CD8$^+$ T cells (Fig. 3D). One possible explanation is that CD4$^+$ T cells play a variety of roles in vivo, which include enhancement of cellular immunity by interacting with antigen-presenting cells and maintenance of immune memory, as well as direct cytotoxicity. The reason that tumor inhibition in mice depleted of only CD8$^+$ T cells was compatible with that in mice without any T-cell depletion might be that the antitumor effect induced by the cooperative effect of CD4$^+$ and CD8$^+$ cytotoxic T cells was already saturated in the CT26 xenograft model.

AlloMHC gene transfer causes growth suppression of both local and distant tumors in alloHSCT recipients. Next, to evaluate the therapeutic efficacy of alloMHC gene transfer for tumors at distant sites, transplant recipients were s.c. inoculated with 1×10^6 CT26 cells on the right leg and, 5 days later, inoculated with 5×10^5 CT26 cells on the left leg. On the right leg, tumor was then transduced with *H-2K^b* gene thrice. In alloHSCT recipients, significant tumor suppression of the treated tumor on the right leg and the untreated tumor on the opposite leg was observed (Fig. 4), which showed that alloMHC gene transfer causes a systemic antitumor immunity in alloHSCT recipients.

Presence of tumor cells at the time of transplantation does not reduce therapeutic efficacy of alloMHC gene transfer. In these experiments, the recipient immune system was reconstituted in the absence of any tumor. To simulate a clinical situation in which patients have residual tumors at the time of alloHSCT, we injected 1×10^6 of 200 Gy–irradiated CT26 cells i.p. into alloBMT recipients at the time of transplantation (day 0) and also at 7 and 14 days posttransplantation. The mice were inoculated with 1×10^6 wild-type CT26 cells at 8 weeks after the transplantation and then injected with *H-2K^b* plasmid complexed with liposome. It was conceivable that, in this condition, the donor-derived immune system might acquire tolerance to TAAs during its reconstitution. Nonetheless, *H-2K^b* gene transfer, either with or without exposure to wild-type CT26 cells, still led to significant tumor suppression (Fig. 5), indicating that, at least in this HSCT model, the presence of tumor cells at the time of transplantation does not induce immune tolerance to tumor cells and thus does not reduce the efficacy of subsequent alloMHC gene transfer.

AlloMHC gene transfer does not exacerbate GVHD. Although the in vitro cytotoxicity assay showed induction of a tumor-specific cytolytic T-lymphocyte response by *H-2K^b* gene transfer, alloMHC expression in tumor cells could theoretically promote a donor T-cell response not only against TAAs but also against mHAs shared by tumor and normal cells, which might result in GVHD exacerbation. We thus examined serum chemistry and the clinical GVHD score in the transplanted mice with the *H-2K^b* gene transfer. Albumin, total bilirubin, aspartate .
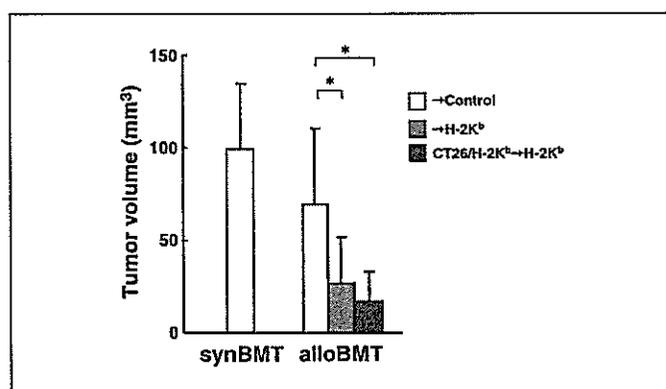
Fig. 5. Preservation of antitumor activity of *H-2K^b* gene transfer in alloBMT recipients with tumor present during the immune reconstitution. Irradiated CT26 cells were i.p. injected at the time of transplantation, and 8 weeks after the transplantation the CT26 s.c. tumor was injected with *H-2K^b* vector thrice in alloBMT recipients. Tumor volume was compared between the groups at 11 days after the completion of vector injection (*n* = 6-8). *, *P* < 0.05.

Table 1. Selected serum chemistry and clinical GVHD score

	CT26/H-2K^b→H-2K^b	→H-2K^b	→Control	P	Normal
AlloBMT					
Serum					
ALB (g/dL)	3.04 ± 0.22	3.2 ± 0.14	3.06 ± 0.28	NS	2.95 ± 0.23
TB (mg/dL)	0.052 ± 0.03	0.036 ± 0.009	0.05 ± 0.014	NS	0.06 ± 0.01
AST (IU/L)	239 ± 47	269 ± 56	314 ± 83	NS	81 ± 11
ALT (IU/L)	153 ± 33	177 ± 43	171 ± 52	NS	41 ± 14
ALP (IU/L)	428 ± 140	448 ± 110	397 ± 93	NS	365 ± 46
GVHD score	5.6 ± 0.5	5.4 ± 0.5	5.4 ± 0.9	NS	
SynBMT					
Serum					
ALB (g/dL)	3 ± 0.32	3.02 ± 0.25	2.92 ± 0.11	NS	
TB (mg/dL)	0.068 ± 0.023	0.046 ± 0.019	0.04 ± 0.024	NS	
AST (IU/L)	210 ± 34	163 ± 57	213 ± 43	NS	
ALT (IU/L)	88 ± 32	94 ± 44	110 ± 38	NS	
ALP (IU/L)	267 ± 95	286 ± 60	295 ± 86	NS	
GVHD score	2.2 ± 0.8	2 ± 0.7	2.2 ± 0.8	NS	

NOTE: *H-2K^b*-expressing or control vector was intratumorally injected thrice in alloBMT or synBMT recipients with or without preimmunization, and 15 days later the serum chemistry and clinical GVHD score were evaluated (*n* = 5 per group). "CT26/H-2K^b→H-2K^b" and "→H-2K^b" indicate *H-2K^b* gene transfer with and without preimmunization, respectively.

Abbreviations: ALB, albumin; TB, total bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; NS, not significant.

aminotransferase, alanine aminotransferase, and alkaline phosphatase are the potential indicators of GVHD-related injury of the hepatobiliary system. GVHD was not serologically nor clinically exacerbated in the *H-2K^b* gene–transduced mice compared with the control vector-injected mice (Table 1).

Intratumoral alloMHC gene transfer might, to some extent, cause the gene transduction into normal cells surrounding the tumor cells. To assess its potential toxicity, 10 μg *H-2K^b*-expressing plasmid DNA complexed with liposome was injected thrice into the s.c. space of non–tumor-bearing alloBMT recipients. Mice were then examined for serum chemistry, clinical GVHD score, and histopathology of the skin, liver, and gut 7 days after the vector injection. All of the mice s.c. injected with *H-2K^b* plasmid did not show any significant exacerbation of GVHD (data not shown).

Tumor-specific immunity induced by alloMHC gene transfer is long-lasting in vivo. The CT26 s.c. tumors disappeared in ~10% to 20% of alloBMT mice by alloMHC gene transfer. To examine *in vivo* longevity of the tumor-specific immunity, a total of 11 alloBMT recipients who survived the initial CT26 challenge with complete tumor remission by *H-2K^b* gene transfer were again inoculated with CT26 cells on the right leg and Renca cells on the left leg. Of the 11 mice, eight had been i.p. injected with irradiated CT26 cells at the time of transplantation and three had not. Renca cells formed a tumor mass in all the mice, whereas CT26 cells were rejected in 10 of 11 mice (91%; Table 2), suggesting that TAAs and/or tissue-restricted mHAs are different between CT26 and Renca cells and that the *H-2K^b* gene transfer to CT26 cells increases the recognition of CT26-specific antigens by donor T cells. *In vitro* cytotoxicity assay also showed lysis of CT26 but not Renca cells or concanavalin A lymphoblasts (data not shown). The results showed that the tumor-specific immunity induced by alloMHC gene transfer is potentially long lasting in HSCT recipients.

Discussion

Several experimental investigations have been made thus far to separate a desirable GVT effect for hematologic or solid malignancies from an undesirable GVHD in alloHSCT recipients. Delayed donor leukocyte infusions into the recipients with mixed chimerism (23, 24), expansion and reinfusion of GVT-specific donor T-cell clones (25, 26), posttransplant immunization of the recipients with tumor cell vaccines (27–29), and pretransplant tumor antigen–specific immunization of the donors (30) were reported to have the potential to selectively enhance the GVT effect. On the other hand, administration of interleukin-11 (31), infusion of CD4$^+$CD25$^+$ regulatory T cells (32, 33), blockade of the function of donor antigen-presenting cells (34), and blockade of the GVHD-specific cytotoxic pathway (Fas-Fas ligand or perforin-granzyme; ref. 35) were shown to be capable of preventing GVHD

Table 2. Tumorigenicity of CT26 and Renca cells in alloBMT recipients cured of initial CT26 challenge

Exposure of CT26 cells	Tumor formation	
	CT26 (right leg)	Renca (left leg)
+	1/8	8/8
−	0/3	3/3
Total	1/11 (9%)	11/11 (100%)

NOTE: Eleven alloBMT recipients who survived the initial CT26 challenge with complete tumor remission by *H-2K^b* gene transfer were again inoculated with CT26 cells and Renca cells. Of the 11 mice, eight had been i.p. injected with irradiated CT26 cells at the time of transplantation and three had not.

while maintaining a GVT effect. In the present study, we showed that intratumoral alloMHC gene transfer could enhance the GVT effect without exacerbating GVHD by inducing systemic tumor-specific immunity in MHC-matched alloHSCT recipients.

Although there have been several animal studies showing the potential efficacy of a combination of alloHSCT and gene-based immunotherapy, such as a tumor vaccine using *granulocyte macrophage colony-stimulating factor* and *interleukin 2/herpes simplex thymidine kinase* genes (27–29), the reports only showed that the antitumor immune activity of tumor vaccines could be reproduced in the context of allogeneic transplantation. To our knowledge, ours is the first report that showed a synergistic antitumor effect of immune gene therapy combined with alloHSCT.

The mechanism for the synergism is yet unclear, but a mixed allogeneic and rejection reactions induced by the combination therapy may be of major importance in creating an environment strongly supporting the activation of an antitumor response. Although the recognition of tumor antigens by donor T cells was not strong enough to induce a significant antitumor immune response in the mice treated with alloHSCT alone, the combination with the alloMHC gene transfer may enhance (*a*) trafficking of immune cells into the tumor, (*b*) local production of various cytokines at the tumor site, and (*c*) presentation of tumor antigenic peptides on antigen-presenting cells through uptake of apoptotic tumor cell bodies induced by the alloMHC gene expression. These effects may facilitate the increased recognition of previously unrecognized or weakly recognized tumor antigens by donor T cells, which leads to a significant tumor specific immunity (36, 37). Furthermore, several murine bone marrow transplantation models have shown that $CD4^+$ and $CD8^+$ T cells both contributed to GVHD through their cytolytic activity (31, 38, 39), and in this study also, although the $CD8^+$ T cells seemed to be major effectors of antitumor immunity by the $H-2K^b$ gene transfer in naïve and synBMT mice (Fig. 3C; ref. 9), the combination of alloHSCT and allo-MHC gene transfer was able to induce effective cooperation of $CD4^+$ and $CD8^+$ cytotoxic T cells in tumor cell killing (Fig. 3C and D). The $CD4^+$ T cells may have an important role in the antitumor immunity as well as GVHD in allogeneic HSCT.

In this study, the tumor cells were inoculated into the mice 8 weeks after the transplantation because the CT26 cell–injected BALB/c mice could not survive >2 months due to the aggressive tumorigenicity of the cell. On the other hand, we need at least a 6-week interval between alloHSCT and the immune therapy to allow a sufficient immune reconstitution necessary for the evaluation of the immune therapy. This experiment model does not exactly replicate the clinical situations in which the recipients usually harbor the relapse or residual cancer cells at the time of HSCT, and one could argue that the immune reconstitution in the presence of tumor cells might induce the acquisition of tolerance to tumor antigens. Therefore, we injected the irradiated CT26 cells at the initial phase after transplantation, and confirmed that the $H-2K^b$ gene transfer elicited effective tumor suppression even in the HSCT recipients exposed to tumor cells during immune reconstitution, suggesting that the alloMHC gene transfer with alloHSCT is a promising therapeutic strategy in clinical setting. As a next step, a combination with other approaches, such as donor lymphocyte infusion and preimmunization with an alloMHC gene–expressing plasmid, might be examined for whether they can further enhance the antitumor effects of alloMHC gene transfer with alloHSCT.

The expression of alloMHC in tumor cells could theoretically promote a donor T-cell response not only for TAAs but also for mHAs shared by tumor and normal host cells, which may cause GVHD. However, in our study, the alloMHC gene transfer did not exacerbate serum enzymes and clinical GVHD scores in alloHSCT recipients. It is possible that much of the immune response was directed against nonimmunodominant mHAs with restricted tissue distribution or possibly even against TAAs, not against immunodominant mHAs. Other reports have also shown that immunization of alloHSCT recipients with a tumor cell vaccine substantially increased GVT activity of donor lymphocytes without exacerbating GVHD (27–30). Luznik et al. hypothesized that the immunogeneic antigen-presenting cells at the vaccine site capture both TAAs and mHAs from tumor cells and promote tumor-specific immunity and GVHD, whereas away from the vaccine site, resting host antigen-presenting cells presenting mHAs induce tolerance in or exhaust alloreactive donor T cells (29, 40). Alloreactive T cells that have been activated at the vaccine site may subsequently become unresponsive to the immunodominant mHAs following an encounter with resting host antigen-presenting cells, whereas tumor-specific T cells would be expected to persist in activated or long-lived memory states. Another possible explanation for the GVT preference over GVHD might be that a major part of potential immunogenic antigens on CT26 tumor cells is TAAs, and indeed it was reported that CT26 cells express high levels of an $H-2L^d$-restricted peptide (AH1) from an endogenous retrovirus, and induce a robust AH-1-specific T-cell response (29, 41). Although we have shown an advantage and safety of the combination therapy in the preclinical study, CT26 cells may not be representative of all clinical human cancers and the occurrence or exacerbation of GVHD should be evaluated carefully in the future stage of the therapeutic development in a clinical study.

Because myeloablative conditioning is associated with a considerable risk of morbidity and mortality and because solid tumors, such as renal cancer, are typically refractory to chemotherapy, the nonmyeloablative alloHSCT is clinically applied against solid tumors (1, 2, 4). Nonmyeloablative alloHSCT often results in a mixed T-cell chimerism, and a donor leukocyte infusion is done for patients with mixed chimerism to achieve exclusively donor-derived hematopoiesis because the establishment of a complete donor chimerism is considered to be crucial for drawing the optimal GVT effect (42, 43). In this study, we used a myeloablative conditioning model to constantly achieve the full donor chimerism, because our primary purpose was to determine the effect of the immune gene therapy on alloHSCT and the full-blown effect after alloHSCT (i.e., complete chimerism). In the clinical setting, the nonmyeloablative conditioning may be optimal to test the potential of the combination therapy to reduce the regimen-related toxicity.

Acknowledgments

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非神経温存前立腺広汎切除術における排尿機能

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前立腺全摘除術における排尿機能については，とかく術後の尿失禁のみが強調される傾向がある。もちろんこれはこの手術における術後後遺症として重要であるが，単に尿禁制のみではなく，排尿困難のない適切な排尿も重要な因子であると考えている。つまり失禁はないが逆に排尿困難があるような症例が見受けられるが，めざすものは，このような点についてもより自然に近い形での機能回復が望ましいと考えている。

かねてから局所進行癌に対しても根治を可能とする広汎前立腺切除術を開発，施行してきた。この方法は確実な切除を可能にすることを目的として開発されたが，結果的に前立腺尖部の把握を，より正確にすることが可能で，確実な切除のみにとどまらず術後の排尿機能についてむしろ良好な方向に作用しているように思われる。

実際には確実な切除断端を確保しながら機能を温存するための尖部の位置関係の把握が重要である。さらにどのような再建を行うかが次のポイントと考えている。具体的に「私はこうして

いる，ここにこだわっている」という点を概説する。

直腸固有筋膜の処理と尖部の確認

広汎前立腺全摘除術についてはすでにいくつかの成書に記載してきた[1,2]。概要を解説する。

まず内骨盤筋膜を切開して直腸固有筋層が認められるまで肛門挙筋を剥離する。前立腺尖部付近では肛門挙筋が前立腺外測に付着していることが多々ある。このような場合にはこの段階では無理に剥離は行わない。直腸固有筋膜をよく観察する。このとき多数の血管が認められる場合，これは前立腺の神経血管束であり，このような場合にはさらに外側に剥離を進める。このような処理を行うことで最終的には固有筋膜に包まれた直腸が濃い黄色調の脂肪を伴って確認される。

次に固有筋膜の縦切開を行う。展開された直腸のちょうど中心付近で血管の疎な場所を選んで切開を開始する。脂肪織の切開を進めると，尾側より脂

肪織が持ち上がってくるような場面に出くわす。これは直腸からの流出静脈であり，この周囲を剥離して静脈を把持する。このことにより直腸筋層への「道しるべ」を得た状態となる。なぜならこの静脈は最終的には直腸筋層から分枝しているからである。この静脈を絹糸で保持することにより後の展開は容易となる。

静脈に沿って，より中枢に剥離を進めると直腸筋層が確認される。直腸筋層付近になると構造が急に粗なfasciaとなる。慣れてくると，このことにより直腸筋層が近いことが認識できる。筋層が確認されたら，末梢に向かい剥離を進める。大切なことは直腸と平行に剥離を進めることである。ともすれば前立腺側に剥離をしがちである。神経血管束から無用な出血をみる原因となる。一般的に生検などの影響が軽微な場合，容易に末梢に向かい剥離が可能である。

剥離を進めると，あるポイントから容易に剥離ができなくなる。これは腱中心に到達したことを意味する。末梢に向かう剥離はここまでとし，直腸を外側に牽引しながら前立腺後面との間の剥離を進めるが，無理に左右を交通することはしない。これはそもそも直腸尿道筋の前立腺との付着のため剥離が難しいことと，盲目的な処理は直腸損傷の危険があるためである。癒着を認めない場合には前立腺尖部後面で容易に左右を貫通することが可能であるが，直視下での処理が困難なこともあり，あまり好まない。

直腸尿道筋は精嚢付近で前立腺に付着しており，この部分をのぞいては筋層を維持することで剥離が可能である。付着部では電気メスで切開することで初めて剥離が可能となる。

最後に最も中枢で直腸筋層の露出を横方向に進める。これは前立腺を逆行性に処理する際，直腸尿道筋，直腸筋層，前立腺を剥離する際の重要なメルクマールとなる。直腸尿道筋と前立腺との付着について模式的に図1に示した。

前立腺尖部の把握と DVCの処理

前立腺尖部の形態はさまざまである。前立腺尖部前面と後面のレベルがずれているような場合には前立腺尖部の把握には相当な技術が必要である。特に後面の前立腺が張り出したような場合，前面のみの情報により処理を行うことは，後面での切除断端陽性の危険があ

る。またPSA eraの現在，前立腺尖部前面が癌の好発部位であり，尖部をいかに的確に把握するかが，確実な切除断端の確保のみならず尿道機能の確保，勃起神経温存において何よりも重要である。

以前はまず陰茎背静脈（dorsal vein complex；DVC）をバンチング処理しながら前立腺尖部の把握を行っていたが，腱中心を利用し，前立腺後面の把握を行う広汎前立腺全摘除術においては側方から前立腺尖部を展開することで，より正確に尖部の把握が可能と考えている。実際には先に剥離した直腸筋層と前立腺の間にクーパーを挿入し，前立腺尖部を左右に振ってみると可動性の乏しい尿道との把握が容易となる。

この段階で前立腺尖部をよく観察すると尖部に肛門挙筋の一部，恥骨尿道筋が前立腺尖部に付着している。さらによく観察すると尿道には尿道を包み込むように輪状に肛門挙筋の成分が存

図1 直腸尿道筋
直腸筋層前面を走行し，末梢は腱中心，中枢側は精嚢基部付近に付着している。したがって図中の矢印の部位で処理することにより，はじめて完全に切除できる。
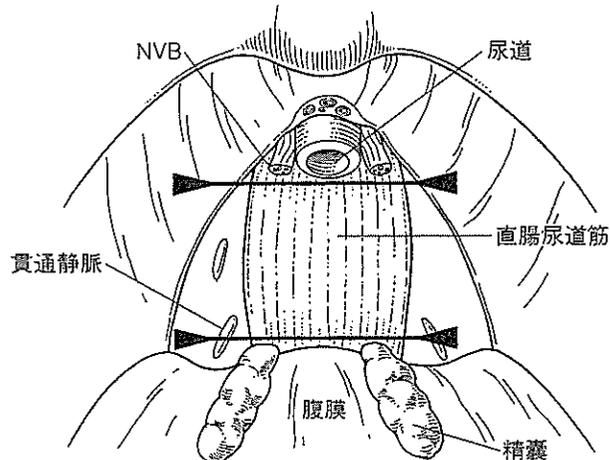
在することが確認される。前立腺尖部に付着する恥骨尿道筋をていねいに前立腺から剥離するが，癒着を認める場合には恥骨尿道筋を切開し前立腺に付着させる。これはこの筋肉を切開することは容易であり，この部位で被膜外浸潤がある危険性を考慮して，このような処理を行っている。バンチング鉗子を用いてDVCを仮の状態で把持し，位置関係の把握をさらに確実にする（図2）。

前立腺尿道移行部を認識し，尿道外側に付着する肛門挙筋を最小限剥離し，尿道後面とおぼしき部位で，尿道外周に沿うようにメッツェンバウムを滑り込ませ，lateral pelvic fasciaを一枚貫通させることで尿道後面に到達できる。左右同様に処理を行い尿道後面に鉗子を通し，血管テープで尿道後面を把持する。

この処理により尿道後面の切断で前立腺後面を不用意に取り残してしまう危険を回避することが可能であり，さらにはDVCの切断部位，あるいはそもそもDVCのバンチング部位のメルクマールが確立したことになり，より的確な処理を可能にすると考えている（図3）。以前の方法では順次DVCをバンチングし，DVCを切断しながら尿道前立腺移行部の把握を行っていたのであるが，どの部位を把持するかメルクマールがないこともあり，技術が必要であり，またいくぶん不正確であった。

DVCの処理ではバンチングは2針程度運針するのみとなっている。これは前述の尿道移行部の把握がすでに終了しているため，必要以上に運針する必要がなくなったからである。前立腺尿道移行部の中枢と末梢それぞれ5mm程度の部位に運針するのみである。その

後，前立腺を牽引してDVCの最も末梢側にstay sutureをおき，出血に際して順次，収束結紮を繰り返し，止血を得るようにしている。最近では他の方法においてもこのような処理が一般的ではと思われる。

前立腺尖部の位置関係を触診・視診あるいは前立腺を可動させて最終確認を行った後，DVCを切開する。DVCはまさに前立腺尿道移行部直上から切開を開始するようにする。以前はいわゆるavascular spaceに鉗子を貫通させていたが，尖部での不要な切除断端陽性となる危険性がありこの処理は行わず，DVCを可能な限り末梢で切断するようにしている。またバンチングされたDVCを手前から切開し尖部に至る処理は行っていない。これも尖部前面における不必要な切除断端陽性につながるためである。
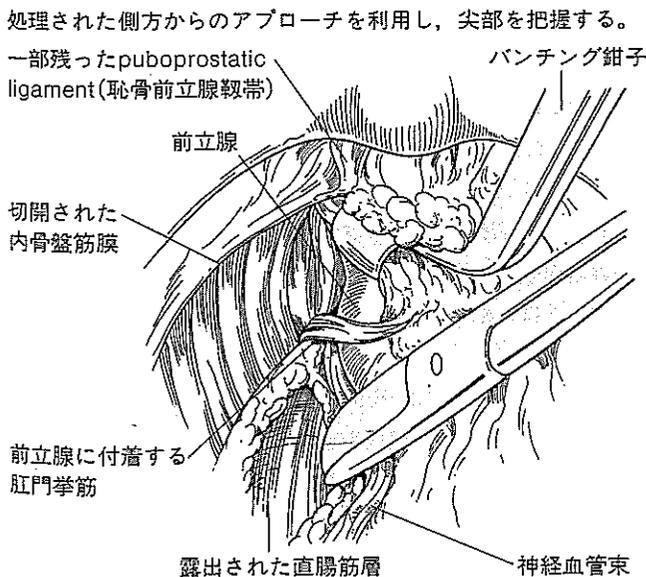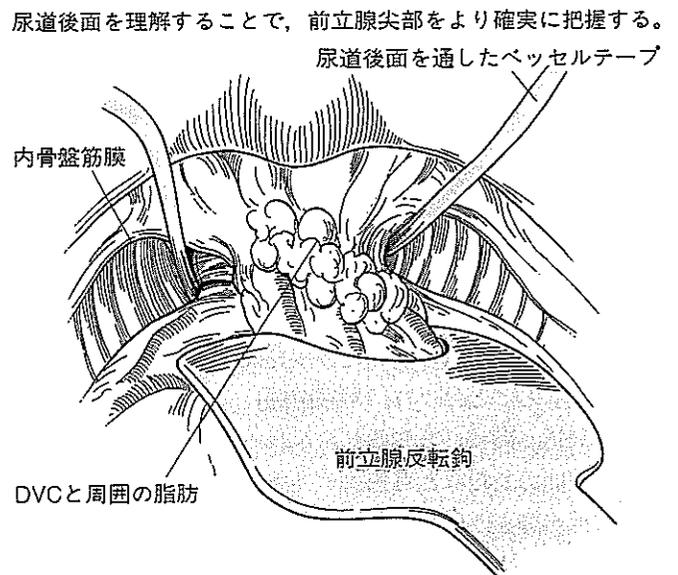
図2　側方アプローチにおける尖部の把握
処理された側方からのアプローチを利用し，尖部を把握する。

一部残ったpuboprostatic ligament（恥骨前立腺靱帯）
バンチング鉗子
前立腺
切開された内骨盤筋膜
前立腺に付着する肛門挙筋
露出された直腸筋層
神経血管束

図3　尿道後面を利用した尖部の把握
尿道後面を理解することで，前立腺尖部をより確実に把握する。

尿道後面を通したベッセルテープ
内骨盤筋膜
DVCと周囲の脂肪
前立腺反転鈎

前立腺被膜と収束結紮されたDVCの深さに注意しながら処理を進める。DVCの切開の深さが被膜より深くなっている場合には当然，前立腺内に切り込んだことを意味する。DVCの切開では前立腺被膜の深さに到達する前に鋭的な切断に変更する。これは尿道筋層，あるいはその中に進展しているかもしれない前立腺組織の認識をより確実にするためである。

直視下で切断するDVCあるいはその先に何が存在するか，最大の注意を払いながら切断を進める。広汎全摘除術を行う場合にある程度の進行癌が予想されるか，患者が神経温存を希望しない場合に実施されるが，尖部では予想外の癌の尿道側への進展があることもあり，尿道と前立腺との間を剥離することはせず，そのまま先ほどの把握した尿道後面に向かい垂直に尿道を離断している。尿道側方の切離ではときにlateral pelvic fasciaと尿道筋層間に進展した前立腺組織を認めることがあり，側方の切開も順次「そこに何があるか」を，確認しながら進めることが肝要であると考えている（図4）。

尿道への吻合糸

近年，尿道カテーテルを早期に抜去することにより尿禁制の早期回復が確認されている。これはカテーテルを長期に留置することにより，本来閉鎖している尿道が開口した状態におかれるため，周囲の炎症なども相まって尿道閉鎖に悪影響を及ぼすものと推定される。したがって尿道吻合におけるポイントとしては尿道括約筋のダメージを最小限にし，かつ確実に尿道と膀胱を吻合することが大切であると考えている。

吻合のリークは吻合部の無用な炎症の原因となり，決して尿道機能に有利には働かない。ただ吻合部のリークがなければよいというものでもなく，尿道と膀胱が口径差のなく適切に密着することが肝要と考えている。このようなポイントを考慮しながら吻合糸を尿道に運針することが必要である。

実際の吻合にあたっては3-0 monocryl SH針10本で吻合している。10本もの吻合糸を使わなくても問題はないとの考えもあるが，前述の問題意識から尿道を大きく運針することはせず，比較的小さいbiteとpitchで運針するためこの程度の本数が必要なことと，尿道後面に対する吻合不全の問題を克服するため試行錯誤を繰り返しているうちに，結果的にこのような本数となったものである。

尿道の上半分を切開し尿道カテーテルが露出した段階で尿道前面への運針を開始する。尿道への運針ではすでに重要性が指摘されているようにまずlateral pelvic fasciaに運針を行う。10時から2時の位置ではlateral　pelvic fasciaの認識は容易であるが，症例によっては肛門挙筋内に埋没したような状況となることがある。

ときにlateral pelvic fasciaではなく肛門挙筋に運針しているようなことがあるが，直視下によく確認するとともにlateral pelvic fasciaを拾った後，少し緊張をかけてみると，きっちり運針されている場合には確実に運針されている緊張を感じる。次に尿道粘膜のみを拾うように運針し，針の先端を尿道カテーテルとの間に抜く（図5）。

この運針に際しては粘膜を拾うときに運針の角度を変える必要がある。尿道粘膜は非常に脆弱であり，運針した後，針を抜くときにも十分注意をする。尿道運針に関してはできるだけ無用な抵抗を避けるため，著者は血管持針器を使っている。

以前は尿道離断後に改めてブジーを挿入し運針を行っていたが，尿道が完全に離断されると尿道断端が肛門挙筋内に埋没してしまい，正確にlateral pelvic fasciaあるいは尿道粘膜を認識することに困難があることと，特に尿道後面の運針ではまったく不正確な部位に運針していること，あるいは切断され，埋没してしまった尿道後面への運針が技術的に困難があるからである。「そんなことをしなくても大丈夫である」という意見もあるかと思われるが，目標はこの手術を受けたすべての症例に対して，尿道カテーテルの早期抜去とともに，確実な尿禁制とスムーズな尿排出を可能とする方法の確立をめざしているのである。

順次，角度を調整しながら1時，3時，4時に運針を行う。この段階で尿道を少し切り足し尿道が4時から8時のみを残した状態となった段階でカテーテルを把持し，これを切断する。切断された尿道カテーテル末梢側は抜去せず，尿道断端に位置させるようにする。このことにより尿道が管状構造を維持し，後の運針を正確にすることが