Cyclophosphamide-Using Nonmyeloablative Allogeneic Cell Therapy against Renal Cancer with a Reduced Risk of Graft-versus-Host Disease

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Abstract

Purpose: Much attention has been paid to nonmyeloablative allogeneic stem cell transplantation for the treatment of solid cancer after the first report by Childs et al. (1). This therapy is based on the use of allogeneic bone marrow transplantation for the treatment of several hematologic malignancies in experimental animal models and in humans (2–5). However, the treatment-related toxicity and mortality hamper this type of treatment to be applied widely. To solve this issue, a new concept to replace myeloablative conditioning with well-tolerated nonmyeloablative conditioning with fludarabine-based regimens has recently been introduced (1, 6–14). Nevertheless, graft-versus-host disease (GVHD) is still considered inevitable in any type of nonmyeloablative conditionings because mature donor T cells are the main mediators of the graft-versus-tumor (GVT) activity of this type of therapy (5, 15–20). Therefore, new treatment modalities reducing the incidence and severity of GVHD without a loss of GVT activity are thus considered to be required.

We previously reported a series of studies regarding the cyclophosphamide-induced tolerance system that comprises an i.v. injection of $10^8$ allogeneic spleen cells (and $2 \times 10^7$ bone marrow cells) followed, usually 2 days later, by an i.p. injection of 200 mg/kg cyclophosphamide (21, 22). In this system, because the destruction of both donor-reactive T cells of host origin and host-reactive T cells of donor origin occurred in the induction phase, a stable mixed chimerism was induced with a tolerance to skin allografts in H-2 identical strain combinations in mice (21, 22). That is, the destruction of donor-reactive T cells of host origin makes a room for donor cells in the recipient mice, thus resulting in the establishment of mixed chimerism (21). In addition, we recently proposed a cyclophosphamide-using nonmyeloablative cell therapy in which donor lymphocyte infusion (DLI) was carried out 1 day after the cyclophosphamide treatment of that tolerance-inducing system (23). The cyclophosphamide-using cell therapy was able to induce a significant antitumor effect against...
Materials and Methods

Animals. Female BALB/c (H-2d) recipient mice and female DBA/2 (H-2b) donor mice were obtained from Japan Charles River (Yokohama, Japan) at 8 weeks of age. All mice were kept in specific pathogen-free conditions and then were used for experiments at 10 weeks of age. All animal protocols were approved by the University Committee on the Use and Care of Animals at Kyushu University. RENCA is a murine carcinogen-induced RCC of BALB/c origin, and it is maintained in vitro in a complete medium. RPMI 1640 (Life Technologies, Grand Island, NY), supplemented with 10% heat-inactivated FCS (Hyclone, Logan, UT), 5 × 10⁻⁵ mol/L 2-mercaptoethanol, 20 mmol/L HEPES, 30 μg/mL Gentamycin (Schering Corp., Kenilworth, NJ), and 0.2% sodium bicarbonate, was used as the complete medium.

Measurement of tumor growth in vivo. After s.c. tumor inoculation, tumor growth was inspected every 3 or 4 days by measuring the largest perpendicular diameters with a caliper, which thus was recorded as the tumor area (mm²).

Cancer treatment protocol. To evaluate the in vivo antitumor activity, BALB/c mice were injected s.c. with 2 × 10⁵ RENCA cells. Considering the clinical application, we started the cancer treatment after establishing the injected tumors (usually 7 days after tumor inoculation). Initially, 1.0 mL of RPMI containing a set quantity of a mixture of 1 × 10⁵ spleen cells and 2.0 × 10⁴ bone marrow cells originated from donor DBA/2 mice was injected i.v. into the tail vein of BALB/c mice. Cyclophosphamide (Endoxan, Shionogi, Osaka, Japan) dissolved in PBS (20 mg/mL) was injected i.p. at a dose of 200 mg/kg 2 days later. DBA/2 lymphocytes (1 × 10⁵) were injected i.v. to BALB/c mice 1 day or 5 days after the cyclophosphamide treatment.

Measurement of immune cells in the periphery of cyclophosphamide-treated mice. To determine when immune cells can recover from the cyclophosphamide treatment, we first counted the immune cells in the periphery of the cyclophosphamide-treated BALB/c mice. We mainly focused on lymph node cells in this study. The changes in the cell number in mesenteric lymph node cells were kinetically examined after the cyclophosphamide treatment.

Systemic and histopathologic analysis of GVHD. The degree of clinical GVHD was assessed every 3 or 4 days based on weight loss, posture, the presence of diarrhea, and skin lesions, such as alopecia and dermatitis. Acute GVHD was also assessed by a detailed histopathologic analysis of the small intestine, one of the GVHD target organs (24, 25). The small intestine was removed on day 6 after the cyclophosphamide treatment and was fixed in 10% buffered formalin, embedded in paraffin, and cut into 5-μm slices. The sections were then stained with H&E. The slides were coded without any reference to the prior treatment status and then were examined systematically by a single pathologist (K. Kiyoshima). In some experiments, the small intestine was further assessed by terminal deoxynucleotidyl transferase–mediated nick-end labeling staining, using the In situ Apoptosis Detection kit, MK500 (Takara, Otsu, Japan) according to the manufacturer’s instructions.

Flow cytometric analysis. The expression of the lymphocyte origin from BALB/c mice or DBA/2 mice was analyzed by two-color flow cytometry using a FACScan cytometer (Becton Dickinson, Mountain View, CA). Phycoerythrin-conjugated anti-mouse CD5 (Ly-1) monoclonal antibody (PharMingen International, Tokyo, Japan) and FITC-conjugated mouse anti-mouse CD5.1 (Ly-1.1) monoclonal antibody (PharMingen International) were used for the analysis of the lymphocyte origin from either BALB/c mice or DBA/2 mice. In some experiments, FITC-conjugated anti-CD4 and phycoerythrin-conjugated anti-CD8 monoclonal antibodies (PharMingen International) were used for a phenotype analysis of the lymph node cells. The labeled cells were analyzed by FACSscan, and fluorescence histograms were accumulated on a logarithmic scale.

ELISA for detecting cytokines. Spleen cells (5 × 10⁵ per well) were cultured with mitomycin C–treated RENCA (1 × 10⁵ per well) or Colon 26 (1 × 10⁵ per well) in 96-well dishes (Costar, Cambridge, MA) in a volume of 1 mL for 72 h. At the end of the culture period, the supernatant fluid was harvested from each culture by centrifugation, and then it was stored at −80 °C until a cytokine assay was done. The culture supernatant fluids were assayed for IFN-γ protein by means of a sandwich ELISA. A mouse IFN-γ ELISA kit (TECHNE, Minneapolis, MN) was used to perform the analysis.

Statistics. The statistical significance of the data was determined using the unpaired two-tailed Student’s t test. P < 0.05 was considered statistically significant.

Results

Recovery from nonspecific immunologic suppression 5 days after the cyclophosphamide treatment. To determine when immunosuppression can recover after the cyclophosphamide treatment, we first counted the immune cells in the periphery of the cyclophosphamide-treated BALB/c mice. Because we already reported the recovery of immune cells in the spleen, bone marrow, and peripheral blood after the cyclophosphamide treatment in our previous study (26), we mainly focused on the lymph node cells in this study. The changes in the cell number in mesenteric lymph node cells were kinetically examined after the cyclophosphamide treatment. Although lymph node cells obviously decreased 2 days after the cyclophosphamide treatment, they recovered to some extent by day 5 (Fig. 1), indicating the recovery from cyclophosphamide-induced nonspecific immunosuppression. In addition, cyclophosphamide did not cause any significant changes in the expression of CD4/CD8 in the lymph node cells (data not shown). These results suggest that DLI on day 5 after the cyclophosphamide treatment might, therefore, reduce the risk of GVHD.

Antitumor activity of cyclophosphamide-using cell therapy. We next determined whether or not the DLI 5 days after the cyclophosphamide treatment could preserve the antitumor activity against RCC. Considering the clinical application, we started the cancer treatment after establishing the s.c. injected RENCA tumors. Tumor growth was obviously suppressed even in the BALB/c mice that had been treated with spleen cells and bone marrow cells on day −2, cyclophosphamide on day 0, and DLI on day 5 (5 days after the cyclophosphamide treatment protocol).
treatment), similarly to those treated with spleen cells and bone marrow cells on day –2, cyclophosphamide on day 0, and DLI on day 1 (1 day after the cyclophosphamide treatment; Fig. 2). In addition, 40% to 60% of the RENCA tumors disappeared in these two groups, accompanied by a significantly prolonged survival in comparison with the control groups (data not shown). In the BALB/c mice treated with either cyclophosphamide alone or spleen cells and bone marrow cells on day –2 and cyclophosphamide on day 0, tumor growth was slightly suppressed in comparison to the untreated group probably due to the cytotoxic effects by cyclophosphamide, but no tumor disappeared in either of the two groups. These results indicate that the cyclophosphamide-using cell therapy with the DLI on day 5 can induce antitumor effects as well as that with the DLI on day 1.

**Mixed chimerism in the BALB/c mice treated with the cyclophosphamide-using cell therapy.** Chimerism was assessed in BALB/c recipients by a flow cytometric analysis of the donor (Ly 1.1+) cells in the peripheral blood 10 days after the cyclophosphamide treatment, irrespective of the timing of DLI treatment (23). Thus, all RENCA tumors were rejected, whereas the growth of the control Colon 26 was normal in all mice (Fig. 6). In addition, the RENCA-specific production of IFN-γ was lower than that in the BALB/c mice treated with DLI on day 5 (Fig. 5F and G). No other signs of GVHD, such as alopecia, dermatitis, and death, were observed in the BALB/c mice treated with DLI on day 1 or day 5 (data not shown).

**Acquired immunity against the RENCA was similarly induced in the BALB/c mice treated with the cyclophosphamide-using cell therapy with the DLI on day 5.** To assess the establishment of acquired immunity against RENCA in the RENCA-rejected BALB/c mice that had been treated with the cyclophosphamide-using cell therapy with DLI on day 5, these mice were rechallenged with RENCA tumors on day 120, as we did in the BALB/c mice treated with DLI on day 1 in our previous study (23). Thus, all RENCA tumors were rejected, whereas the growth of the control Colon 26 was normal in all mice (Fig. 6). In addition, the RENCA-specific production of IFN-γ was lower than that in the BALB/c mice treated with DLI on day 5 (Fig. 5F and G). No other signs of GVHD, such as alopecia, dermatitis, and death, were observed in the BALB/c mice treated with DLI on day 1 or day 5 (data not shown).

**A reduction of GVHD was observed in the BALB/c mice treated with the DLI on day 5.** We next evaluated the degree of GVHD in this system by assessing weight loss, diarrhea, skin lesions, and histology of small intestine. The weight of both BALB/c mice treated with DLI on day 1 and those on day 5 initially decreased with mild diarrhea and a hunched posture for a while but, thereafter, recovered (Fig. 4). However, no difference in the body weight was observed between the BALB/c mice treated with the cyclophosphamide-using cell therapy with DLI on day 1 and those with DLI on day 5 (Fig. 4). The initial GVHD was also confirmed by the histologic findings of the small intestine in the BALB/c mice treated with DLI (Fig. 5). Although apoptotic crypt cells were observed in the BALB/c mice treated with the DLI on day 1 (Fig. 5B) or day 5 (Fig. 5C), the number of apoptotic crypt cells was smaller in the mice treated with the cyclophosphamide-using cell therapy with DLI on day 5 compared with those with DLI on day 1 (Fig. 5D). The difference in apoptosis was further supported by terminal deoxynucleotidyl transferase–mediated nick-end labeling staining. That is, more apoptotic cells were observed in the mice treated with cyclophosphamide-using cell therapy with DLI on day 1 in comparison with those with DLI on day 5 (Fig. 5F and G). No other signs of GVHD, such as alopecia, dermatitis, and death, were observed in the BALB/c mice treated with DLI on day 1 or day 5 (data not shown).

**Fig. 1.** Recovery of immune cells in the periphery after cyclophosphamide treatment. The number of mesenteric lymph node cells was kinetically examined after BALB/c mice were treated with spleen cells and bone marrow cells on day –2 and 200 mg/kg cyclophosphamide on day 0. The number of those in untreated BALB/c mice (C). Points, mean of three mice examined; bars, SD. Representative findings of two separate experiments. The other experiment showed similar results.

**Fig. 2.** No difference in the antitumor activity of the cyclophosphamide-using cell therapy with the DLI on days 1 and 5. After establishing the injected tumors (usually 7 days after tumor inoculation), the BALB/c mice were treated as follows (n = 5 per group): ■, cyclophosphamide alone; □, spleen cells and bone marrow cells on day –2 and cyclophosphamide on day 0; ▲, spleen cells and bone marrow cells on day –2, cyclophosphamide on day 0, and DLI on day 1; ○, spleen cells and bone marrow cells on day –2, cyclophosphamide on day 0, and DLI on day 5; △, untreated. Tumor growth was inspected every 3 or 4 d. Points, mean of five mice examined; bars, SD. Representative findings among four separate experiments. The other three experiments showed similar results. *P < 0.01.
was clearly detected in the spleen cells of the same mice with DLI on day 5 after in vitro stimulation with inactivated RENCA cells (data not shown), and these findings were similar to those in our previous report (23).

**Discussion**

We recently proposed a novel model system of non-myeloablative allogeneic cell transplantation for the treatment of RCC by modifying our tolerance-inducing method using cyclophosphamide (21, 22). The cyclophosphamide-induced tolerance system comprises an i.v. injection of $1 \times 10^8$ allogeneic spleen cells (and $2 \times 10^7$ bone marrow cells) followed, usually 2 days later, by an i.p. injection of 200 mg/kg cyclophosphamide (21, 22). In that tolerance-inducing system, a stable degree of mixed chimerism as a result of the destruction of both donor-reactive T cells of host origin and host-reactive T cells of donor origin can be induced (21, 22). By adding donor lymphocytes 1 day after the cyclophosphamide treatment, the degree of mixed chimerism of donor cells increases with the transient mild GVHD, which is associated with antitumor effects, probably because the DLI was carried out after the tolerance induction to donor cells (23). We then hypothesized that a delay of DLI in the cyclophosphamide-using cell therapy could reduce a risk of GVHD and tested the possibility in the present study. Thus, we revealed that a delay of DLI in the cyclophosphamide-using cell therapy could decrease a risk of GVHD while preserving GVT activity in this study. Regarding the in vivo antitumor effect, there was no difference between DLI on day 1 and day 5 (Fig. 2). The histologic findings of the small intestine showed that the cyclophosphamide-using cell therapy with the DLI on day 5 decreased the risk of GVHD (Fig. 5). Considering that the ultimate goal in nonmyeloablative allogeneic hemopoietic cell transplantation for the treatment of malignancy is to separate...
GVHD from GVT activity, our modified cyclophosphamide-using cell therapy system therefore seems to be optimal.

In addressing the reason why a delay in performing DLI from day 1 to day 5 reduced a risk of GVHD while preserving GVT activity, we suppose that the following scenario takes place. Although lymph node cells in the recipients obviously decreased 2 days after the cyclophosphamide treatment, they recovered to some extent on day 5 (Fig. 1). The percentage of chimerism in the BALB/c mice treated with DLI on day 5 was lower than that in the BALB/c mice treated with DLI on day 1 (Fig. 3B), reflecting less room for donor cells on day 5 after the recovery of cyclophosphamide-induced nonspecific immunosuppression in the recipients than that on day 1. Thus, the histologic findings of the small intestine showed a reduction of GVHD in the BALB/c mice treated with DLI on day 5 (Fig. 5) because the lower levels of mixed chimerism can reduce the risk of GVHD (27). However, although the delay in DLI decreased the chimerism of donor-derived lymphocytes in the treated mice, the level of donor-derived cells in the BALB/c mice treated with DLI on day 5 was still significantly higher than that in the mice treated with donor spleen cells and bone marrow cells and cyclophosphamide without DLI (Fig. 3A and B), thus indicating that there still existed a donor-specific room on day 5 after the recovery of cyclophosphamide-induced nonspecific immunosuppression. In addition, such donor-derived lymphocytes were sufficiently effective to elicit an in vivo antitumor effect (Fig. 2). Regarding this point, further studies, including an analysis of tumor-infiltrating lymphocytes, are needed to clarify the underlying mechanisms of this cyclophosphamide-using cell therapy.

A remarkable feature of the cyclophosphamide-using cell therapy is that the antitumor activity is induced with low body weight, reflecting the efficacy of the treatment. Although there was no difference in body weight between the BALB/c mice treated by the cyclophosphamide-using cell therapy with the DLI on day 1 and those with the DLI on day 5, BALB/c mice treated as follows (n = 5 per group): ♦, spleen cells and bone marrow cells on day 0; cyclophosphamide on day 0, and DLI on day 1; ■, spleen cells and bone marrow cells on day 0; cyclophosphamide on day 0, and DLI on day 5. Changes in the body weight were assessed. Points, mean of five mice examined; bars, SD. Representative findings among three separate experiments. The other two experiments showed similar results.

Fig. 4. No difference was observed in the body weight between the BALB/c mice treated by the cyclophosphamide-using cell therapy with the DLI on day 1 and those with the DLI on day 5. BALB/c mice were treated as follows (n = 5 per group): ♦, spleen cells and bone marrow cells on day 0, and DLI on day 1; ■, spleen cells and bone marrow cells on day 0, cyclophosphamide on day 0, and DLI on day 5. Changes in the body weight were assessed. Points, mean of five mice examined; bars, SD. Representative findings among three separate experiments. The other two experiments showed similar results.

Fig. 5. Apoptotic crypt cells in the small intestine of the BALB/c mice treated by the cyclophosphamide-using cell therapy. Histopathology (A–C) and terminal deoxynucleotidyl transferase–mediated nick-end labeling staining (E–G) of the small intestine from the untreated BALB/c mice (A and E), BALB/c treated with the cyclophosphamide-using cell therapy with the DLI on day 1 (B and F), or BALB/c treated with the cyclophosphamide-using cell therapy with the DLI on day 5 (C and G). The small intestine was assessed on day 6 after the cyclophosphamide treatment. Apoptotic crypt cells (arrows). Columns, mean number of apoptotic crypt cells in five different fields in each group; bars, SD (D). Representative findings among three separate experiments. The other two experiments showed similar results. *, P < 0.05.
levels of mixed chimerism of donor cells. However, in the clinical course of nonmyeloablative allogeneic hematopoietic cell transplantation for the treatment of malignancy, it is widely believed that the GVT activity is associated with GVHD and complete chimerism (1, 11, 28, 29). The powerful antitumor effects of DLI to mixed chimeras have also been recently reported (30). Using a model of B6 mice and EL-4 T-cell lymphoma, the DLI administration to mixed chimeras has been reported to produce a dramatically improved leukemia-free survival in comparison with the administration of DLI to full donor chimeras (30). In this sense, our results are compatible with theirs. In their system, however, DLI converted mixed chimeras to full chimeras without causing GVHD (30), in contrast to our study. The conversion from mixed chimeras to full chimeras in their system is similar to the clinical course of DLI after the nonmyeloablative allogeneic stem cell transplantation for malignancies (1, 13). We suppose that the chimeric state required for the GVT activity may thus be different between solid tumors (renal cancer) and hematologic malignancies (T-cell lymphoma). That is, in the treatment of hematologic malignancies, the establishment of complete chimerism in the peripheral blood therefore seems to be required to completely eliminate leukemic cells in the whole body, and incomplete chimerism may also suggest a possibility of residual leukemic cells. On the other hand, complete chimerism in the peripheral blood may not necessarily be required in the treatment of solid tumors because effector cells basically need to work only in the metastatic sites. The cyclophosphamide-using cell therapy could induce a transient mixed chimerism (Fig. 3), and a protective immunity against RENCA tumors was elicited even after the disappearance of donor-derived cells (Fig. 6). Therefore, the cyclophosphamide-using cell therapy is considered an optimal treatment modality for nonmyeloablative allogeneic cell transplantation for the treatment of solid cancers, including renal cancer.

Importantly, RENCA-rejected mice that had been treated with DLI on day 5 showed RENCA-specific in vivo protective immunity after the disappearance of donor-derived lymphocytes (Fig. 6), similar to the findings of previous report in which DLI was done on day 1 (23). This means that the acquired immunity against RENCA observed on day 120 is considered to be mainly attributable to recipient lymphocytes. After DLI, donor lymphocytes with GVH response are considered to secrete Th1 cytokines, which may elicit both donor-derived and recipient-derived lymphocytes with an antitumor activity. This may be the reason why the acquired immunity against RENCA is maintained by the recipient lymphocytes, even after the disappearance of mixed chimerism of donor cells. Interestingly, the antitumor response despite loss of donor chimerism has also been recently reported in patients treated with nonmyeloablative conditioning and allogeneic stem cell transplantation for advanced hematologic malignancies (31). In addition, both the critical role of recipient lymphocytes and the importance of recipient-derived IFN-γ in an antitumor effect have been recently reported in the mixed chimeras prepared with nonmyeloablative conditioning in a murine model (32). Although their model is considered compatible with our study regarding the fact of RENCA-specific production of IFN-γ in the spleen cells from the BALB/c mice without any detectable donor-derived lymphocytes, there is a big difference in the transferred cells between the two models [i.e., recipient lymphocyte infusion in their study (32) and DLI in our study].

Recently, significant advances in understanding the molecular mechanisms underlying RCC have led to the development of rationally designed therapies, which are now being clinically tested. To date, the vascular endothelial growth factor receptor pathway has been the most promising target, and two agents (BAY 43-9006 and SU11248) that inhibit not only vascular endothelial growth factor receptor, but also other receptors, including the platelet-derived growth factor receptor, FMS-like tyrosine kinase 3, KIT, or Raf kinase, have been recently approved by Food and Drug Administration for advanced RCC (33, 34). Thus, much attention is now being paid to such molecular targeting therapies. However, the underlying mechanisms for the antitumor effects are different between nonmyeloablative allogeneic stem cell transplantation and molecular targeting therapy. Therefore, nonmyeloablative allogeneic stem cell transplantation should be done especially for patients with advanced RCC who are resistant to molecular targeting therapies. In such patients with a poor performance status because of advanced metastatic tumors, the risk of GVHD should be particularly minimized in the process of nonmyeloablative allogeneic stem cell transplantation. We hope that the application of the concept in our current study will help reduce the risk of GVHD during the course of nonmyeloablative allogeneic stem cell transplantation for the treatment of patients with advanced RCC.

![Fig. 6. Protective immunity against the RENCA in the BALB/c mice treated with the cyclophosphamide-using cell therapy with the DLI on day 5: BALB/c mice that had been treated with spleen cells and bone marrow cells, cyclophosphamide, and DLI on day 5 and rejected the RENCA tumors; ▲, Colon 26 tumors in the untreated BALB/c mice; ◆, Colon 26 tumors in the BALB/c mice that had been treated with spleen cells on day −2, cyclophosphamide on day 0, and DLI on day 5 and rejected the RENCA tumors; ▲, Colon 26 tumors in the untreated BALB/c mice; ◆, Colon 26 tumors in the BALB/c mice that had been treated with spleen cells on day −2, cyclophosphamide on day 0, and DLI on day 5 and rejected the RENCA tumors. Points, mean of five mice examined; bars, SD. Representative findings among three separate experiments. The other two experiments showed similar results. * P < 0.001, in comparison with the untreated mice; ** , not specific in comparison with the untreated mice.](cancertherapypreclinical.org)
References

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