Renal Cancer Treatment with Low Levels of Mixed Chimerism Induced by Nonmyeloablative Regimen Using Cyclophosphamide in Mice

Masahiko Harano,1 Masatoshi Eto,1 Toshiro Iwai,2 Katsunori Tatsugami,1 Keijiro Kiyoshima,3 Yoriyuki Kamiryo,1 Masazumi Tsuneyoshi,1 Yasunobu Yoshikai,1 and Seiji Naito1

Departments of 1Urology, 2Cardiovascular Surgery, and 3Anatomic Pathology, Graduate School of Medical Sciences; and 4Division of Host Defense, Research Center for Prevention of Infectious Diseases, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan

Abstract

Recently, much attention has been paid to nonmyeloablative allogeneic stem cell transplantation for the treatment of metastatic renal cancer. Mature donor T cells cause graft-versus-host disease (GVHD) although they are also the main mediators of the beneficial graft-versus-tumor activity associated with this treatment. Hence, the segregation of the graft-versus-tumor activity from GVHD is an important challenge in managing the clinical course of treatment. We previously reported a series of studies regarding the allograft tolerance induced by allogeneic spleen cells (with bone marrow cells) and cyclophosphamide in mice. Here, we show a modified cyclophosphamide-induced tolerance system for the treatment of murine renal cell carcinoma, RENCA, by shifting the equal balance between graft-versus-host and host-versus-graft reactions toward graft-versus-host reaction with donor lymphocyte infusion. Our results clearly show the antitumor activity against RENCA with only low levels of mixed chimerism in the periphery and the in vivo and in vitro acquired immunity against RENCA even when mixed chimerism is almost undetectable. Because the withdrawal of mixed chimerism reduces the risk of GVHD, the antitumor activity is thus sequentially segregated from the initial GVHD in our model. We believe that this is the first unique model system of nonmyeloablative allogeneic hematopoietic cell transplantation to ever be reported for the treatment of renal cancer. (Cancer Res 2005; 65(21): 10032–40)

Introduction

Since the first report of Child et al. (1), much attention has been paid to nonmyeloablative allogeneic stem cell transplantation for the treatment of metastatic renal cancer. 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, resulting in part from myeloablative chemoradiotherapy, still represent a barrier to successful bone marrow transplantation and continue to prevent the large-scale clinical application of stem cell transplantation by extending the indications for the treatment of a larger number of patients in need, including a variety of genetic diseases that may be exclusively correctable by bone marrow transplantation. As a result, a new concept to replace myeloablative conditioning with well-tolerated nonmyeloablative conditioning with fludarabine-based regimens has recently been introduced (1, 6–14). Even in nonmyeloablative conditioning, mature donor T cells cause graft-versus-host disease (GVHD) although they are the main mediators of the beneficial graft-versus-tumor activity of allogeneic bone marrow transplantation (5, 15–20). Therefore, reducing the incidence and severity of GVHD without a loss of graft-versus-tumor activity remains the ultimate goal of such tumor treatment.

We have reported a series of studies regarding the cyclophosphamide-induced tolerance system that comprises an i.v. injection of 1 × 10⁸ allogeneic spleen cells and 2 × 10⁷ 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, without any signs of GVHD in H-2 identical strain combinations in mice (21, 22). Namely, an equal balance between graft-versus-host and host-versus-graft reactions was thus established (22). Furthermore, we have recently overcome skin tolerance across H-2 barriers by adding T cell–depleted donor bone marrow cells 1 day after the cyclophosphamide treatment (23). We hypothesized that donor lymphocyte infusion to the tolerant mice may be able to induce antitumor activity in our method. Here, we describe our novel model system of nonmyeloablative allogeneic cell transplantation for the treatment of renal cancer by adding whole donor lymph node cells 1 day after cyclophosphamide treatment based on our conventional cyclophosphamide-induced tolerance system. The present findings show the antitumor activity against RENCA with only low levels of mixed chimerism in the periphery.

Materials and Methods

Animals. Female BALB/c (H-2b) 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 were then 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.

Tumors. RENCA, a murine carcinoma-induced renal cell carcinoma, was used in this study. Colon 26, a murine adenocarcinoma, was used as a control. Both tumor cell lines are of BALB/c origin and were 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 gentamicin (Schering Corporation, Kenilworth, NJ), and 0.2% sodium bicarbonate, as 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 was thus recorded as the tumor area (mm²). In some experiments, tumor acceptance was finally determined on day 31.

Cancer treatment protocol. To evaluate the in vivo antitumor activity, the 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 1640 containing a set quantity of a mixture of 1 × 10⁸ spleen cells and 2.0 × 10⁷ 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 × 10⁷) were injected i.v. to BALB/c mice 1 day after cyclophosphamide treatment.

Systemic and histopathologic analysis of graft-versus-host disease. The degree of clinical GVHD was assessed every 3 or 4 days based on weight loss, posture, 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 or day 120 after the inoculation of DBA/2 spleen cells and bone marrow cells 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 were then examined systematically by a single pathologist (K. Kiyoshima).

Histopathologic and immunofluorescent histologic analysis of tumors. The tumors were removed 3 weeks after the s.c. inoculation and were then fixed in 10% buffered formalin, embedded in paraffin, and cut into 5 µm slices. The sections were stained with H&E. For an immunofluorescent histologic analysis, frozen tumor samples were cut

### Table 1. Establishment of a model system for renal cancer treatment by using nonmyeloablative allogeneic hematopoietic cell transplantation in combination with cyclophosphamide in mice

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Group</th>
<th>Treatment*</th>
<th>Tumor size (mm²)</th>
<th>Mice with tumor/all mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SC + BMC</td>
<td>CP</td>
<td>LNC</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Abbreviations: SC, spleen cells; BMC, bone marrow cells; CP, cyclophosphamide; LNC, lymph node cells.

*BALB/c mice were injected s.c. with RENCA cells. After establishing the injected tumors, BALB/c mice were injected with 1 × 10⁶ spleen cells and 2 × 10⁷ bone marrow cells from DBA/2 mice on day 0, given 200 mg/kg cyclophosphamide on day 2, and injected with 1 × 10⁷ lymph node cells from DBA/2 (donor) or BALB/c (recipient) mice on day 3.

†Tumor size 31 days after the tumor inoculation.

‡Tumor acceptance was determined 31 days after tumor inoculation.

§P < 0.01 compared with groups 1 and 3 in experiment 1 and groups 1 in experiments 2 and 3.

∥Not specific compared with group 1 in experiment 4.
into 5 μm slices and were then placed on ready-to-use adhesive glass slides. After rinsing with PBS, the sections of each tumor tissue specimen were investigated for the presence of any infiltrate of Lyt-1.1+ donor lymphocytes by direct immunofluorescence using FITC-labeled mouse monoclonal antibody against Lyt-1.1 (anti-CD5, Meiji Institute of Health Science, Tokyo, Japan) at a dilution of 1:20 in PBS for 1 hour of incubation at room temperature. After the antibody reaction, the tissue slides were then rinsed twice with PBS. All images were processed by Axiophot microscope (ZEISS, Oberkochen, Germany) equipped with epifluorescence optics.

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. The labeled cells were analyzed by FACScan. Fluorescence histograms were accumulated on a logarithmic scale.

ELISA for detecting cytokines. Spleen cells (5 × 10^5 per well) were cultured with mitomycin C (MMC)–treated RENCA cells (1 × 10^5 per well) or Colon 26 cells (1 × 10^5 per well) in 96-well dishes (Coster, Cambridge, MA) in a volume of 1 mL for 72 hours. At the end of the culture period, the supernatant fluid was harvested from each culture by centrifugation and 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 by using the unpaired two-tailed Student’s t test. P < 0.05 was considered to be statistically significant.

Results

Donor lymphocyte infusion is a prerequisite for antitumor effects against RENCA. Considering the clinical application, we started the cancer treatment after establishing the s.c. injected RENCA tumors. Tumor growth was obviously suppressed in the
BALB/c mice that had been treated with DBA/2 spleen cells and bone marrow cells on day 0, cyclophosphamide on day 2, and DBA/2 lymph node cells on day 3 (Fig. 1). In addition, 40% to 60% of RENCA tumors disappeared only in the same group with donor lymphocyte infusion (Table 1). In the BALB/c mice treated with either cyclophosphamide alone (Fig. 1) or DBA/2 spleen cells and bone marrow cells on day 0 and cyclophosphamide on day 2, tumor growth was slightly suppressed probably because of the cytotoxic effects of cyclophosphamide. No tumor disappeared in either of the groups (Table 1). The requirement of donor lymphocyte infusion was further supported by the results that recipient lymphocyte infusion instead of donor lymphocyte infusion had no effects on tumor growth (experiment 4; Table 1). These results clearly indicate the important role that donor lymphocyte infusion plays in the antitumor mechanism against RENCA in line with the findings of previous clinical reports (1, 9, 11, 12, 14).

Mild graft-versus-host disease is observed in the BALB/c mice treated with donor lymphocyte infusion. We next investigated the degree of GVHD in this system by assessing weight loss, diarrhea, skin lesions, and histology of small intestine. The weight of the BALB/c mice treated with donor lymphocyte infusion initially decreased with mild diarrhea and a hunched posture for a while, but was not significantly different from those without donor lymphocyte infusion on day 6 after the inoculation of DBA/2 spleen cells and bone marrow cells (Fig. 2A). The initial GVHD was also confirmed by the histologic findings of the small intestine in the BALB/c mice treated with donor lymphocyte infusion (Fig. 2B). Namely, more apoptotic crypt cells were initially observed in the BALB/c mice treated with donor lymphocyte infusion than those in control mice (Fig. 2D). Interestingly, the weight of the BALB/c mice with donor lymphocyte infusion gradually increased after day 6 compared with the other controls having larger tumors (Figs. 1 and 2A), thus reflecting the decrease of tumor size in that group. Furthermore, apoptotic crypt cells decreased on day 120 in the BALB/c mice with donor lymphocyte infusion that survived for a long time after the rejection of RENCA tumors (Figs. 2C and D). Other signs of GVHD, such as alopecia, dermatitis, and death, were not observed in the BALB/c mice treated with donor lymphocyte infusion (data not shown).

Donor-derived lymphocytes infiltrate in the RENCA tumors from the BALB/c mice treated with donor lymphocyte infusion. To further investigate the antitumor mechanism in our model, we investigated the histopathologic findings of the tumors 3 weeks after tumor inoculation in each group. Representative histopathologic findings are shown in Fig. 3. In the RENCA tumors from the BALB/c mice treated with DBA/2 donor lymphocyte infusion, a diffuse infiltration of mononuclear cells was observed around the tumor cells (Fig. 3A). In addition, the immunofluorescent histologic findings showed the infiltrate of Ly-1.1+ donor-derived lymphocytes in the RENCA tumors from the BALB/c mice treated with DBA/2 donor lymphocyte infusion (Fig. 4A and D). However, a comparison of the histologic findings (Fig. 3A) with the immunofluorescent findings (Fig. 4A) of the RENCA tumors from the BALB/c mice treated with DBA/2 donor lymphocyte infusion revealed most of the infiltrating lymphocytes to be host-derived cells. In the tumors from the BALB/c mice without donor lymphocyte infusion, only a mild infiltrate of mononuclear cells was observed (Fig. 3B) and fewer Ly-1.1+ cells were detected than those from the BALB/c mice with donor lymphocyte infusion (Fig. 4B and D). In addition, no Ly-1.1+ cells were detected in the RENCA tumors from the untreated BALB/c mice (Fig. 4C and D).

Mixed chimerism is induced at the higher degree with donor lymphocyte infusion than without donor lymphocyte infusion but then subsides over time in the periphery. 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 donor lymphocyte infusion. Mixed chimerism was clearly detected in two groups, namely the BALB/c mice treated with DBA/2 spleen cells + bone marrow cells and cyclophosphamide, and BALB/c mice treated with DBA/2 spleen cells + bone marrow cells, cyclophosphamide, and DBA/2 donor lymphocyte infusion (Fig. 5A). Furthermore, the level of donor-derived cells was higher in the group with donor lymphocyte infusion than in that without donor lymphocyte infusion (Fig. 5A and B). However, even in the group with donor lymphocyte infusion, the degree of mixed chimerism was not so high and no complete chimerism was ever induced (Fig. 5A and B). The level of mixed chimerism was sequentially assessed only in the BALB/c mice with donor lymphocyte infusion that survived for a long time after the rejection of RENCA tumors because all the mice in other groups died of tumor progression. The level of donor-derived lymphocytes gradually decreased over time and was undetectable 120 days after...
donor lymphocyte infusion (Fig. 5C and D). The gradual decrease in the number of donor-derived cells was also seen in our previous report of the transplantation model using the same strain combinations without donor lymphocyte infusion (26).

Acquired immunity against RENCA is detected in the BALB/c mice treated with donor lymphocyte infusion. To assess the establishment of acquired immunity against RENCA in the BALB/c mice that had been treated with DBA/2 spleen cells + bone marrow cells, cyclophosphamide, and DBA/2 donor lymphocyte infusion and rejected the RENCA tumors, these mice were rechallenged with RENCA tumors on day 120 when the mixed chimerism was almost undetectable. As shown in Fig. 6A, all RENCA tumors were rejected, whereas the growth of the control Colon 26 was normal in all mice. The spleen cells from such mice were also assessed regarding the production of IFN-γ against RENCA. The RENCA-specific production of IFN-γ was clearly detected in the mice that had been treated with DBA/2 spleen cells + bone marrow cells, cyclophosphamide, and DBA/2 donor lymphocyte infusion and that had thus rejected the RENCA tumors (Fig. 6B).

**Discussion**

In this study, we established a novel model system of nonmyeloablative allogeneic cell transplantation for the treatment of renal cell carcinoma by modifying our tolerance-inducing method that uses cyclophosphamide (21). In our previous study, we were able to induce a long-lasting skin allograft tolerance in many H-2 identical strain combinations in mice by using our cyclophosphamide-induced tolerance system (21). In that 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 degree of mixed chimerism was induced with a tolerance to skin allografts without any signs of GVHD (22). By adding donor but not recipient lymph node cells 1 day after cyclophosphamide treatment, the degree of mixed chimerism of donor cells increased with the transient mild GVHD (Fig. 2A, B, and D) compared with the conventional cyclophosphamide-induced tolerance system without donor lymphocyte infusion (Fig. 5A and B), which was associated with a clear antitumor activity (Fig. 1; Table 1). Furthermore, the antitumor activity was strong enough to eliminate 40% to 60% of the established RENCA tumors (Table 1). Although the clinical antitumor effects of nonmyeloablative allogeneic hemopoietic cell transplantation have been reported for the treatment of metastatic renal cancer (1, 7–12, 14), we believe that this is the first experimental model system of nonmyeloablative allogeneic hemopoietic cell transplantation ever reported for the treatment of renal cancer.

![Figure 4. Donor-derived lymphocytes infiltrate in the RENCA tumors from the BALB/c mice treated with donor lymphocyte infusion. Immunofluorescent histologic findings of the RENCA tumors from the BALB/c mice treated with either DBA/2 spleen cells and bone marrow cells on day 0, cyclophosphamide on day 2, and DBA/2 lymph node cells on day 3 (A), or DBA/2 spleen cells and bone marrow cells on day 0 and cyclophosphamide on day 2 (B). As a control for the immunofluorescent histologic analysis, RENCA tumors from untreated BALB/c mice (C) were used. In all groups, RENCA tumors were removed 3 weeks after the s.c. inoculation. To identify the infiltrate of Lyt-1.1+ donor lymphocytes, a direct immunofluorescent analysis using FITC-labeled mouse monoclonal antibody against Lyt-1.1 was done. Columns, mean of Lyt-1.1+ donor lymphocytes in five different fields in each group (D); bars, SD. *, P < 0.01.](image)
Another remarkable feature of the present study is that the antitumor activity was clearly induced with only low levels of mixed chimerism of donor cells. In the clinical course of nonmyeloablative allogeneic hemopoietic cell transplantation for the treatment of malignancy, it is widely believed that the graft-versus-tumor activity is associated with GVHD and complete chimerism (1, 11, 27, 28). Indeed, mild GVHD was transiently observed in the group with donor lymphocyte infusion (Fig. 2A, B, and D). In the results of the present study, the important role that donor lymph node cells play in the antitumor activity was clearly shown as follows: First, only donor but not recipient lymphocyte infusion induced tumor regression (Fig. 1; Table 1); second, immunofluorescent histologic findings showed the infiltrate of Ly 1.1+ donor-derived lymphocytes in the RENCA tumors from the BALB/c mice treated with DBA/2 donor lymphocyte infusion (Fig. 4A); finally, the level of donor-derived cells was higher in the group with donor lymphocyte infusion than in that without donor lymphocyte infusion (Fig. 5A and B). However, even in the group with donor lymphocyte infusion, the degree of mixed chimerism was low and no complete chimerism was ever induced (Fig. 5A and B). In addition, the histologic (Fig. 3A) and immunofluorescent findings (Fig. 4A) of the RENCA tumors from the BALB/c mice treated with donor lymphocyte infusion showed that most of the infiltrating lymphocytes were host-derived cells, thus indicating that the antitumor activity may be attributed to the cooperation of donor lymph node cells and recipient lymph node cells. The level of donor-derived lymphocytes gradually decreased over time and was undetectable 120 days after the donor lymphocyte infusion.

**Figure 5.** Mixed chimerism is induced with donor lymphocyte infusion but it thereafter subsides over time in the periphery. A and B, after establishing the s.c. RENCA tumors, the BALB/c mice were treated with DBA/2 spleen cells and bone marrow cells alone (spleen cells + bone marrow cells alone), cyclophosphamide alone, DBA/2 spleen cells and bone marrow cells on day 0 and cyclophosphamide on day 2 (bone marrow cells + spleen cells/cyclophosphamide); or DBA/2 spleen cells and bone marrow cells on day 0, cyclophosphamide on day 2, and DBA/2 lymph node cells on day 3 (bone marrow cells + spleen cells/cyclophosphamide/donor lymphocyte infusion). Ten days after the donor lymphocyte infusion, chimerism was assessed in each group by a flow cytometric analysis of donor (Ly 1.1+) cells in the peripheral blood. As a positive and a negative control, peripheral blood cells from untreated BALB/c (control BALB/c) and untreated DBA/2 (control DBA/2) mice were examined. Representative fluorescence-activated cell sorting (FACS) data (A) and all data (B). Columns, mean; bars, SD. *, P < 0.001. C and D, the level of mixed chimerism was sequentially assessed only in the BALB/c mice with donor lymphocyte infusion that survived for a long time after the rejection of RENCA tumors. Chimerism was assessed at 10, 24, 72, and 120 days after the donor lymphocyte infusion. Representative FACS data in the BALB/c mouse that had rejected RENCA with donor lymphocyte infusion (C) and all data (D). Points, mean; bars, SD. **, not specific compared with untreated BALB/c.
The powerful antitumor effects of donor lymphocyte infusion to mixed chimeras were also recently reported (29). By using a model of B6 mice and EL-4 T-cell lymphoma, Mapara et al. (29) showed that donor lymphocyte infusion administration to mixed chimeras produced dramatically improved leukemia-free survival compared with administration of donor lymphocyte infusion to full donor chimeras, indicating a critical role of host antigen-presenting cells. In this sense, our study is compatible with them because low levels of mixed chimerism of donor cells suggest the existence of host antigen-presenting cells (Fig. 5A and B). In their system, however, donor lymphocyte infusion converted mixed chimeras to full chimeras without causing GVHD (29) in contrast to our study (Fig. 5C and D). The difference between the two systems may be attributable to the levels of mixed chimerism before donor lymphocyte infusion. In their system, 50% to 60% of mixed chimerism of donor-derived cells had been already established before donor lymphocyte infusion in contrast to only low levels of mixed chimerism in our studies (Fig. 5A; ref. 21). The conversion from mixed chimeras to full chimeras in their system is similar to the clinical course of donor lymphocyte infusion after the nonmyeloablative allogeneic stem cell transplantation for malignancies (1, 13). However, the chimeric state required for the graft-versus-tumor activity may be different between solid tumors (renal cancer) and hematologic malignancies (T-cell lymphoma).

In considering the ultimate goal, namely to separate the GVHD from the antitumor activity from GVHD has been sequentially established. Although GVHD was initially and transiently observed (Fig. 2A, B, and D), the acquired immunity against RENCA could be detected both in vivo (Fig. 6A) and in vitro (Fig. 6B) even on day 120 when the degree of mixed chimerism was almost undetectable (Fig. 5C and D). A low level of mixed chimerism is considered to reduce the risk of GVHD (27, 28). Indeed, the histologic findings of the small intestine showed no signs of GVHD on day 120 (Fig. 2C). The acquired immunity against RENCA observed on day 120 is considered to be mainly attributable to recipient lymphocytes because no donor-derived lymphocytes were detectable at that time. Regarding this point, we hypothesize as follows: Donor lymphocytes with graft-versus-host reaction secrete Th1 cytokines after donor lymphocyte infusion. Such cytokines may elicit both donor-derived and recipient-derived lymphocytes that are responsible for the antitumor activity. Even after the disappearance of mixed chimerism of donor cells, the acquired immunity against RENCA may be maintained by recipient lymphocytes with the antitumor activity. 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 (30). Furthermore, the critical role of recipient lymphocytes has also been recently reported in a murine model (31). Rubio et al. (31) showed that recipient lymphocyte infusion induced an antitumor effect in the mixed chimeras prepared with nonmyeloablative conditioning. They also showed the importance of recipient-derived IFN-γ (31). Regarding this point, their model was thus considered to be compatible with our study because we could also show the RENCA-specific production of IFN-γ in the spleen cells from the BALB/c mice without any detectable donor-derived lymphocytes. However, the big difference between the two studies is that only the donor but not the recipient lymphocyte infusion could induce tumor regression in our model (Table 1).
We consider the reason why we could induce cancer regression with only low levels of mixed chimerism in our system to be as follows: As shown in our previous study (21, 22), the destruction of both donor-reactive T cells of host origin and host-reactive T cells of donor origin was induced by cyclophosphamide in the periphery of the recipient mice in the induction phase. The destruction of donor-reactive T cells of host origin makes a room specific for donor cells in the recipient mice, thus resulting in the establishment of mixed chimerism (21). On the other hand, the destruction of host-reactive T cells of donor origin prevents the recipient from developing an onset of GVHD (22). Hence, neither GVHD nor graft-versus-tumor activity was observed without donor lymphocyte infusion (Fig. 1). When donor lymphocyte infusion was done 1 day after cyclophosphamide treatment, the room specific for donor cells may have facilitated the expansion of host-reactive T cells, thus resulting in tumor regression. One limitation of this study is that we could not directly show the expansion of host-reactive T cells in the current murine strain combination because of the low levels of mixed chimerism (Fig. 5A and B). However, similar findings have recently been reported in a clinical trial by Dudley et al. (32) in which they transferred highly selected tumor-reactive T cells directed against overexpressed self-derived differentiation antigens after a nonmyeloablative conditioning regimen. Although they used T cells of self-origin but no allogeneic lymphocytes, other reports have also shown that lymphodepletion can have a marked effect on the efficacy of T-cell transfer therapy in murine models (33–37). The destruction of regulatory cells, disruption of homeostatic T-cell regulation, or an abrogation of other normal tolerance mechanisms may help to explain the effects of such lymphodepletion (38). On the other hand, CD4+CD25+ regulatory T cells have recently been reported to preserve graft-versus-tumor activity while inhibiting GVHD after bone marrow transplantation (39). Hence, further studies are required to clarify the role of nonmyeloablative conditioning regimens.

Summary

We have herein established a novel model system for renal cancer treatment with low levels of mixed chimerism by means of nonmyeloablative allogeneic hematopoietic cell transplantation by using cyclophosphamide in mice. Our results clearly show the antitumor activity against RENCA with only low levels of mixed chimerism in the periphery and the in vivo and in vitro acquired immunity against RENCA even when the degree of mixed chimerism was almost undetectable. Because the disappearance of mixed chimerism decreases the risk of GVHD, our model is thus considered to be ideal in terms of segregating the antitumor activity from the initial GVHD. We hope that the concept of our model system can in the future be applied to clinical nonmyeloablative allogeneic stem cell transplantation for the treatment of solid tumors.

Acknowledgments

Received 2/9/2005; revised 8/12/2005; accepted 8/31/2005.

Grant support: Ministry of Education, Science and Culture of Japan grant 16659440 (M. Eto), a grant-in-aid for cancer research from the Fukuoka Cancer Society (Fukuoka, Japan; M. Eto), and a grant from Suzuki Foundation for Urological Medicine (M. Eto).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Takanori Teshima for his invaluable discussions.

References


Renal Cancer Treatment with Low Levels of Mixed Chimerism Induced by Nonmyeloablative Regimen Using Cyclophosphamide in Mice

Masahiko Harano, Masatoshi Eto, Toshiro Iwai, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/21/10032

Cited articles
This article cites 37 articles, 24 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/21/10032.full#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/21/10032.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.