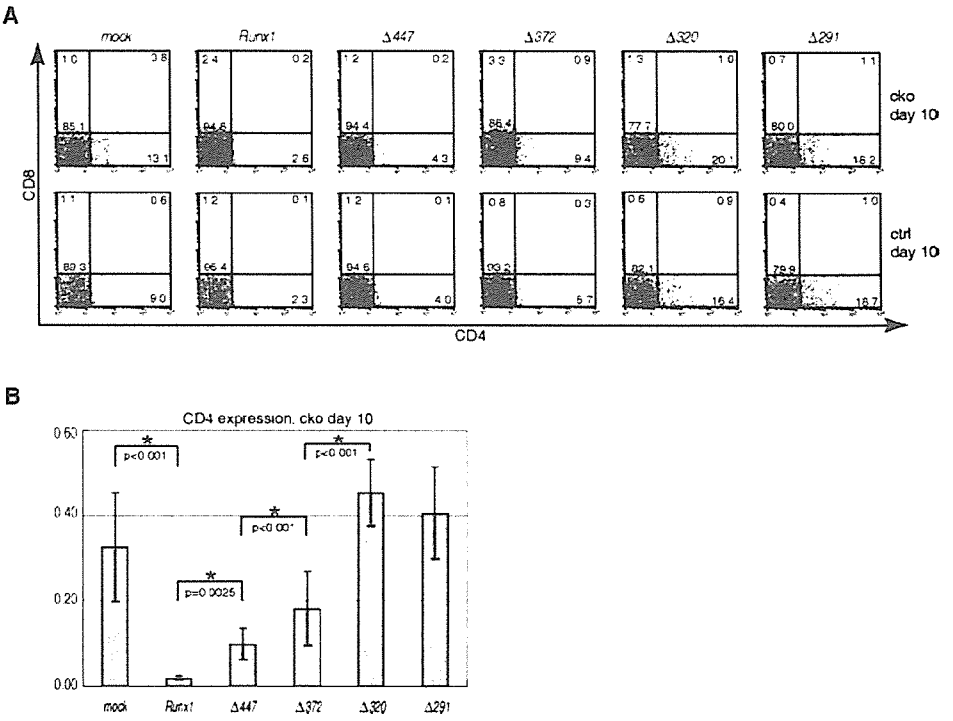

FIGURE 5. CD4 repression in the FL-derived cells transduced with Runx1 or its C-terminal deletion mutants. *A*, $CD4^{-}CD8$ expression profiles of cko (*top panels*) and ctrl (*bottom panels*) FL cells on day 10 of culture. Transduced Runx1 mutants are shown above. Cells were stained with anti-CD4 PE, anti-CD8 PerCP, and anti-CD45.2 allophycocyanin. GFP-positive and allophycocyanin-positive cells were analyzed for CD4/8 expression. The percentage of cells in each quadrant is indicated. *B*, Proportions (%) of $CD4^{int}$ cells among CD8-negative cells in nine independent experiments were averaged and are shown with ±1× SE. ANOVA and post hoc comparison (Fisher test) were performed using StatView software (SAS Institute). Asterisks indicate the statistically significant differences, and *p* values were indicated.

mock-infected cells (Fig. 4, *bottom*), and those TCRβ-expressing cells corresponded with CD44-negative (DN3 or DN4) cells (data not shown). Although it is yet to be determined whether decreased expression of TCRβ was the cause or the result of impaired thymocyte differentiation, the fact that the *TCRβ* gene has canonical binding sites for Runx1 within its enhancer region (34) and is transcriptionally up-regulated by Runx1 (8) supports the notion that Runx1 promotes thymocyte maturation at least partly by up-regulating TCRβ expression. Our results also indicate that the activation domain, but not the VWRPY motif, is critical for Runx1-mediated TCRβ up-regulation.

C-terminal VWRPY motif of Runx1 is necessary for CD4 repression

As shown in Fig. 5*A*, the $CD4^{int}CD8^{-}$ subsets in day 10 culture of cko FL cells disappeared upon the reintroduction of *Runx1* (Fig. 5*A*), which was again consistent with the established role of Runx1 in *CD4* repression (14). This observation also demonstrates that the aberrant expression of CD4 observed in DN subsets of cko FL-derived cells can be ascribed to *Runx1* depletion. To determine the domains of Runx1 that are relevant for CD4 repression, a series of C-terminal deletion mutants of Runx1 were transduced into cko or ctrl FL cells, and the proportion of $CD4^{int}CD8^{-}$ cells was evaluated on day 10 of culture (Fig. 5). Whereas full-length Runx1 almost completely repressed aberrant CD4 expression, only partial repression was seen with Δ447 or Δ372 mutants. These results suggest that CD4 repression by Runx1 requires some C terminus-mediated interaction with other molecules such as TLE. The extent of CD4 repression by Δ447 is greater than that by Δ372, which might reflect the existence of an additional repression domain in the C terminus other than the VWRPY motif (23).

Δ320 and Δ291 each failed to repress CD4 expression, resulting in an increase in the $CD4^{int}$ population compared with the mock-infected cko FL cells. Because *Runx1* depletion is incomplete in the DN subsets of cko FL-derived cells on day 10 (Fig. 2*D*), the increase in the $CD4^{int}$ population is probably due to a dominant-negative effect of Δ320 and Δ291 on remaining endogenous Runx1. This notion is supported by the observation that *Δ320*- or *Δ291*-transduced control FL cells produced a significant number of $CD4^{int}CD8^{-}$ cells, which were barely detected in mock-infected ctrl FL-derived cells (Fig. 5*A*, *bottom*).

Discussion

In the current study, we demonstrated that Runx1 was important for thymocyte development using the FL/OP9-DL1 coculture system. This system is superior to conventional FTOC in that a sufficient number of cells for extensive analyses can be easily obtained, especially DN thymocytes. Another advantage of this system is the highly efficient transfer of the genes of interest. In this study, we were able to introduce various mutants of Runx1 by retroviral infection with an efficiency of ~80% (data not shown), which is higher than that obtained with FTOC. In contrast, terminal maturation of SP cells cannot be achieved in this culture system, which makes it difficult to analyze more mature stages of thymocytes.

The absolute need for Runx1 in thymocyte development in vivo has been unequivocally demonstrated using conditionally *Runx1*-targeted mice. When Runx1-deficient bone marrow cells are transplanted to lethally irradiated mice, the development of thymocytes is severely blocked at the DN2–3 transition (35), whereas the deletion of *Runx1* in later stages of DN thymocytes using the *Lck-Cre* tg results in a profound defect in the DN3–4 transition.[4] Together, these findings suggest that Runx1 is necessary for normal thymocyte development at multiple steps during the DN-DP transition. Despite the DN3–4 block in T lymphocyte-specific *Runx1*-targeted mice, thymocyte development of the cognate FL cells was arrested at the DN2–3 transition in this culture system. The difference in the DN stage at which the developmental block occurs may be due to earlier Cre-mediated *Runx1* deletion in vitro rather than in vivo. In the FL culture system, deletion of the floxed *Runx1* allele occurs predominantly at the DN2–3 transition, which leaves few, if any, DN3 cells with an intact *Runx1* allele (Fig. 3*D*, *lane 3*). *Lck-Cre* tg mice harbor a transgenic gene encoding Cre recombinase driven by the $p56^{lck}$ proximal promoter (32, 36). The *Lck*

encodes a lymphocyte-specific protein tyrosine kinase, which mediates β-chain-dependent signaling during β-selection, is associated with allelic exclusion of β locus (37), and is transcribed from two developmentally regulated, independently functioning promoters. The proximal promoter is used exclusively in thymocytes, but not in peripheral T lymphocytes (38), and Cre-mediated gene deletions are expected to be activated by $p56^{lck}$ proximal promoter at the DN2 and DN3 stages when Vβ gene rearrangement and subsequent β-selection occurs. However, even if the same $p56^{lck}$ proximal promoter is used, exact timing of gene expression differs depending on the transgenic mice lines, and different lines of *Lck-Cre* tg mice are used to target a gene at different developmental stages (39).

The function of the VWRPY motif in hematopoiesis has been examined in embryonic stem cell culture (26) and in para-aortic splanchnopleural culture (40). Because Runx1 mutants that lack the VWRPY motif could fully restore hematopoiesis in Runx1-deficient cells in these two studies, the VWRPY motif does not seem to be necessary for hematopoiesis. On the contrary, because mice in which cDNA for the VWRPY-deficient Runx1 mutant had been homozygously knocked-in to the *Runx1* alleles exhibited a reduced number of thymocytes and deviant CD4 expression during thymocyte ontogeny (27), the VWRPY motif seems to play a role in thymocyte development, although the precise molecular mechanism is unclear. In the present study, although the VWRPY-deficient Runx1 mutant (Δ447) could restore not only maturation to the DN4 subset but also TCRβ expression in cko FL-derived thymocytes as efficiently as wild-type Runx1 (Fig. 4), it had only a limited capacity to repress aberrant CD4 expression (Fig. 5). These different requirements for the VWRPY motif indicate that Runx1 functions in both TLE-dependent and TLE-independent manners during early thymocyte development. In fact, the context-dependent need for interaction with a transcriptional corepressor has been reported for Runt and Groucho, *Drosophila* homologues of Runx and TLE, respectively (41). One possible explanation for TLE-dependent *CD4* repression is that TLE actively converts Runx1 to a transcriptional repressor by recruiting histone deacetylase, as seen in *Drosophila* (41). Another possibility is that TLE displaces some coactivators from Runx1 under particular conditions, which prevents Runx1 from up-regulating *CD4* expression. A similar mechanism has been proposed for transcription by lymphoid enhancer binding protein 1/T cell factor, which is repressed until TLE is replaced by β-catenin (42). Further analyses are needed to clarify the role of the VWRPY motif in the regulation of *CD4* transcription.

The introduction of Runx1 mutants into cko FL cells has shown that the activation domain makes a critical contribution to various functions of Runx1 in thymocyte development, including *CD4* repression, the DN2–3 transition, and the expression of TCRβ. Significantly, Δ320 and Δ291, both of which lack the activation domain, dominantly suppress *CD4* repression and the DN2–3 transition but do not interfere with TCRβ expression. This may be due to a higher affinity of Runx1 for the *TCRβ* enhancer compared with Δ320 and Δ291. Although this speculation is not supported by experimental evidence, a potential mechanism that accounts for this finding is that the interaction of Runx1 with other transcription factors may confer on Runx1 a higher affinity for specific gene promoters. Otherwise, Δ320 and Δ291 may retain a marginal potential to up-regulate TCRβ, which would prevent the total loss of TCRβ when they are forcibly expressed.

In conclusion, we have successfully reproduced the phenotype of Runx1-deficient thymocytes in vitro using the FL/OP9-DL1 coculture system and have evaluated the function of Runx1 and its mutants by retroviral gene transduction. The activation domain is essential for the function of Runx1 in *CD4* repression, the DN2–3 transition, and the expression of TCRβ, whereas the VWRPY motif does not contribute to the DN2–3 transition or the expression of TCRβ, but it is partially involved in *CD4* repression. Further studies are needed to understand how the VWRPY motif of Runx1 regulates *CD4* transcription and how Runx1 functions at multiple steps in thymocyte development.

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Disclosures

The authors have no financial conflict of interest

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In Vivo Alemtuzumab Enables Haploidentical Human Leukocyte Antigen-Mismatched Hematopoietic Stem-Cell Transplantation Without Ex Vivo Graft Manipulation

Yoshinobu Kanda, Kumi Oshima, Yuki Asano-Mori, Koji Kandabashi, Masahiro Nakagawa, Mamiko Sakata-Yanagimoto, Koji Izutsu, Akira Hangaishi, Shiho Tsujino, Seishi Ogawa, Toru Motokura, Shigeru Chiba, and Hisamaru Hirai

Background. Alemtuzumab, a humanized monoclonal antibody directed against human CD52, has a strong lympholytic effect. This study evaluates the safety of unmanipulated peripheral blood stem-cell transplantation from two or three loci-mismatched related donors using alemtuzumab in vivo.

Methods. A total body irradiation-based regimen was used in young patients, whereas those 50 years or older received fludarabine-based conditioning. Alemtuzumab was added to these regimens by intravenous infusion at 0.2 mg/kg per day for 6 days (days −8 to −3).

Results. We treated 12 patients with a median age of 49.5 years. Eight patients demonstrated active disease, and four patients demonstrated acute leukemia in high-risk remission. All achieved neutrophil engraftment a median of 17.5 days after transplantation with complete donor-type chimerism. The cumulative incidence of grades III to IV acute graft-versus-host disease was only 9%. Infection-related deaths were not observed. CD3+/CD4+ and CD3+/CD8+ T cells were strongly suppressed within 2 months after transplantation, but recovered on day 90. Relapse was observed in five of eight patients who underwent transplantation for active disease, whereas none of the three patients who underwent transplantation in first remission had a relapse.

Conclusions. We conclude that in vivo alemtuzumab enables haploidentical hematopoietic stem-cell transplantation without ex vivo graft manipulation.

Keywords: Alemtuzumab, T-cell depletion, HLA mismatch, Allogeneic hematopoietic stem-cell transplantation, Graft-versus-host disease.

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Allogeneic hematopoietic stem-cell transplantation from a human leukocyte antigen (HLA)-identical sibling donor is an established treatment for hematologic malignancies. However, such a donor is available in only approximately 30% of patients in most developed countries (*1*, *2*). Therefore, alternative donor transplantation, including partially mismatched related donor transplantation, matched unrelated donor transplantation, and cord blood transplantation, has been investigated. Although transplantation from a one locus-mismatched related donor or a matched unrelated donor produces outcomes similar to those of transplantation from an HLA-identical sibling donor in high-risk patients (*3*), there is little chance of finding a one locus-mismatched related donor. In addition, it can sometimes be too time-consuming to coordinate a matched unrelated donor for patients with high-risk diseases. On the other hand, there is an excellent chance of identifying a family member who shares one haplotype with the patient and has two or three mismatched antigens in the second haplotype. Cord blood transplantation is also a possible alternative, but it is difficult to find a cord blood graft that contains enough nucleated cells for adult patients. Furthermore, it is impossible to obtain additional donor cells for immunotherapy after cord blood transplantation.

HLA incompatibility between the donor and recipient increases the risk of both graft rejection and severe graft-versus-host disease (GVHD). The outcome of two or three loci-mismatched transplantation without graft manipulation has been extremely poor (*3*, *4*), and thus it has been believed that ex vivo T-cell depletion from the graft is necessary to prevent severe GVHD. Although thorough T-cell depletion by CD34-positive cell selection has almost prevented GVHD (*5*), the incidences of graft rejection and infection increase after T-cell–depleted transplantation.

Campath-1 series of monoclonal antibodies is directed against human CD52, an antigen expressed on T, B, natural killer (NK), and dendritic cells, but not on hematopoietic stem cells (*6*, *7*). The original rat immunoglobulin (Ig)M and IgG monoclonal antibodies, Campath-1 M and Campath-1G, were used for ex vivo and in vivo T-cell depletion, respectively. The incidence of GVHD was significantly decreased by the use of these antibodies ex vivo only or both ex vivo and in vivo (*8–10*). Subsequently, Campath-1G was reshaped into a humanized form, alemtuzumab (Campath-1H), by genetic

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Department of Cell Therapy and Transplantation Medicine, University of Tokyo Hospital, Tokyo, Japan.

Address correspondence to: Yoshinobu Kanda, M.D., Ph.D., Department of Cell Therapy and Transplantation Medicine, University of Tokyo Hospital, 7–3–1 Hongo, Bunkyo-ku, Tokyo 113–8655, Japan.

E-mail: ycanda-tky@umin.ac.jp.

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engineering (*11*). It has a longer terminal half-life (15–21 days) than Campath-1G (<1 day) (*12*). The addition of in vivo alemtuzumab to a conditioning regimen decreases graft rejection by depleting host T cells. In addition, it prevents GVHD because the alemtuzumab concentration is higher than that required to kill donor T cells at the time of graft infusion and remains at a potentially lympholytic level for approximately 2 months after transplantation (*13*). In fact, Mackinnon and coworkers showed that in vivo alemtuzumab has excellent prophylactic action against GVHD in a reduced-intensity conditioning regimen using fludarabine, melphalan, and alemtuzumab followed by stem-cell infusion mainly from HLA-matched donors (*14, 15*). However, there have been no reports on the application of in vivo alemtuzumab in two or three loci-mismatched transplantation. This study evaluates the safety of unmanipulated stem-cell transplantation from haploidentical two or three loci-mismatched related donors using alemtuzumab only in vivo.

PATIENTS AND METHODS

Patients

This study was approved by the ethical committee of the University of Tokyo Hospital, and all of the patients were seen and underwent transplantation at this hospital. Adult patients less than 65 years old who demonstrated high-risk acute leukemia, chemorefractory non-Hodgkin lymphoma, chronic myelogenous leukemia (CML) in blast crisis, myelodysplastic syndrome (MDS), or aplastic anemia with refractory severe neutropenia ($<500/mm^3$) were eligible for the study. The definition of high-risk acute leukemia included acute leukemia not in remission, in second or later remission, and in first remission with poor prognostic features such as positive Philadelphia chromosome (Ph^+), requiring more than two courses to achieve remission, and so on. Patients who had an available HLA-A/B/DR-matched or one locus-mismatched donor among family members were excluded. Patients who had an HLA-matched unrelated donor were also excluded unless the disease status precluded time-consuming donor coordination. Patients had to have a two or three loci-mismatched haploidentical related donor in good physical condition. Written informed consent was obtained from all patients and donors.

Stem-Cell Collection

Donors received granulocyte colony-stimulating factor at 200 $\mu g/m^2$ subcutaneously twice daily starting 3 days before the first collection of peripheral blood stem cells until the end of collection. Leukapheresis was performed daily until more than 5.0×10^6 CD34+ cells/kg of the recipient body weight were collected. Collected cells were then cryopreserved using standard techniques without ex vivo manipulation. The target cell dose was not achieved in three donors, but the minimum requirement dose had been set at 3.0×10^6 CD34+ cells/kg, and thus transplantation was performed using these grafts.

Conditioning Regimens

The conditioning regimen consisted of total body irradiation (TBI) at 2 Gy twice daily for 3 days (from days −7 to −5) and cyclophosphamide at 60 mg/kg per day for 2 days (from days −3 to −2). The dose of cyclophosphamide was decreased to 20 mg/kg per day for 2 days and etoposide at 40 mg/kg per day was added instead on day −4 in a patient with impaired cardiac function caused by anthracycline. For patients 50 years old or older, a non-TBI regimen consisting of fludarabine at 30 mg/kg per day for 6 days (days −8 to −3) and busulfan 1 mg/kg four times daily for 4 days (days −6 to −3) was applied. However, after we observed frequent relapse of lymphoid malignancies following this regimen, we added TBI at 2 Gy twice daily on day −1 and decreased the dose of busulfan to 4 mg/kg per day for 2 days (days −6 and −5) in the last two patients.

Alemtuzumab was added to these regimens at 0.2 mg/kg per day for 6 days (days −8 to −3). We adjusted the dose of alemtuzumab by body weight, because the body weight greatly differs among Japanese adult patients. The dose of daily alemtuzumab was determined by considering the total dose of alemtuzumab in previous studies (*14, 15*), the average body weight of white patients, and the daily dose of alemtuzumab in pediatric studies (*16*). To prevent acute infusion-related reactions to alemtuzumab, patients were pretreated with 1 mg/kg of methylprednisolone. Alemtuzumab was infused over 4 hr. On the first day of alemtuzumab infusion, 3 mg of alemtuzumab was infused over 2 hr and, after confirming that no severe infusion-related toxicities were observed, we infused the remaining alemtuzumab over the next 2 hr.

Other Transplantation Procedures

On day 0, the cryopreserved donor cells were thawed and infused. Prophylaxis against GVHD was performed with cyclosporine A (CsA) and short-term methotrexate. CsA was started on day −1 at a dose of 3 mg/kg per day by continuous infusion, and the dose was adjusted to maintain a blood concentration between 250 and 350 ng/mL. CsA was changed to an oral form when it could be tolerated by the patient. Methotrexate was administered at 15 mg/m^2 on day 1 and 10 mg/m^2 on days 3, 6, and 11. For patients without acute GVHD, we started to taper CsA from day 30 by 10% per week and discontinued CsA on day 100.

Prophylaxis against bacterial, fungal, and Pneumocystis carinii infection consisted of tosufloxacin, fluconazole, and sulfamethoxazole/trimethoprim. Some of the patients who had active or recent aspergillosis received antifungal prophylaxis with micafungin instead of fluconazole. As prophylaxis against herpes simplex virus infection, acyclovir was given 500 mg/day intravenously or 1,000 mg/day orally from days −7 to 35, followed by long-term, low-dose (200 mg/day) oral administration (*17*). Patients without myeloid malignancies received granulocyte colony-stimulating factor (filgrastim) at 300 μg/day by 3-hr infusion beginning on day 10 until the neutrophil count recovered to $500/mm^3$. Cytomegalovirus (CMV) antigenemia assay using C10/C11 antibody was performed at least once per week after engraftment. Ganciclovir was started when more than two positive cells were detected on two slides (*18*). Acute GVHD was graded as previously described (*19*). Patients who developed grades II to IV acute GVHD were treated with 1 mg/kg of intravenous methylprednisolone.

Host/donor cell chimerism after transplantation was analyzed by sex-chromosome FISH or the short tandem re-

TABLE 1. Patient characteristics

Median age	49.5 yr (range 27–60)	
Sex	Male 8/Female 4	
Diagnosis	ALL 5 (Ph^+ ALL 3), AML 2, MDS 2, CML-BC 1, NHL 2	
Disease status	Active disease	8
	High-risk remission	4
Comorbidities	Active/recent invasive aspergillosis	5
	Infective endocarditis, mitral valve replacement	1
	Anthracycline-induced cardiac failure	1
	Interstitial pneumonitis caused by radiation for breast cancer	1
	Diffuse lung infiltration of lymphoma	1
	Obstructive lung disease	1
	History of autologous transplantation	1
Donor	Sibling	4
	Son/daughter	6
	Uncle	1
	Cousin	1
No. of mismatched loci	Graft-versus-host direction 3 loci	7
	2 loci	5
	Host-versus-graft direction 3 loci	7
	2 loci	5
Conditioning regimen	Total body irradiation-based	6
	Fludarabine-based	6
Number of CD34+ cells in the graft	5.1×10^6 cells/kg (range 4.3–7.7)	
Number of CD3+ cells in the graft	2.6×10^8 cells/kg (range 1.8–7.1)	

ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; MDS, myelodysplastic syndrome; CML-BC, chronic myeloid leukemia-blast crisis; NHL, non-Hodgkin lymphoma.

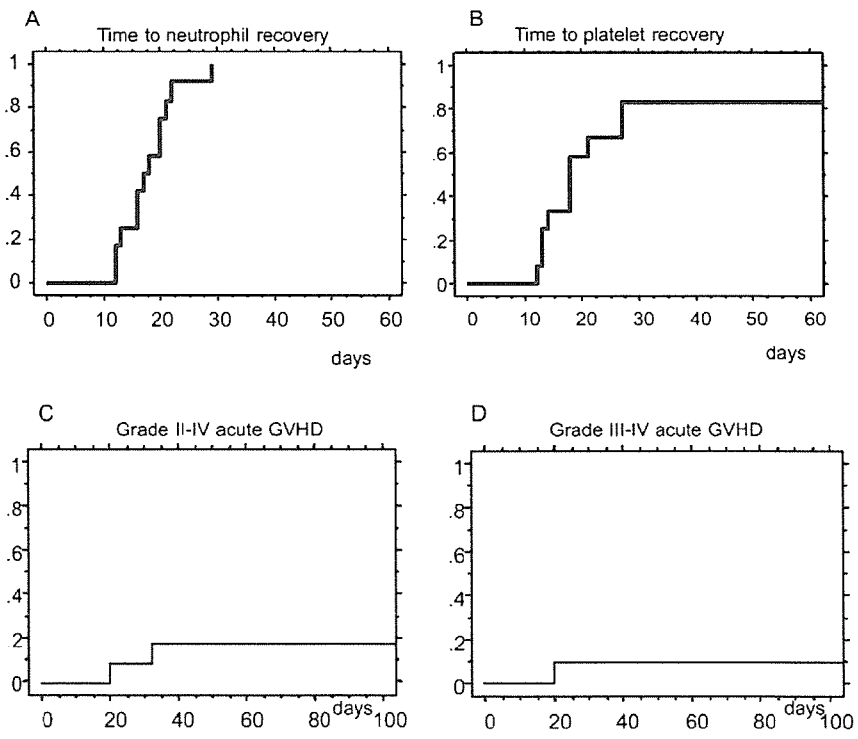

FIGURE 1. Days to neutrophil (A) and platelet (B) recovery and cumulative incidence of grades II to IV (C) and grades III and IV (D) acute GVHD after transplantation.

peat method monthly after transplantation (*20*). Immune reconstitution was evaluated by the quantification of CD3+/CD4+, CD3+/CD8+, CD3−/CD19+, and CD3−/CD56+ cells by flow cytometry. CMV-specific T-cell reconstitution was evaluated using fluorescent HLA-peptide tetramers in patients who were HLA-A*0201- or HLA-A*2402-positive

(*21*, *22*). As a functional assay, a phytohemagglutinin (PHA) stimulation test was performed as previously described (*23*).

Statistical Considerations

The primary endpoint of this study was the incidence of nonrelapse mortality within 100 days after transplantation. We defined success as the absence of early nonrelapse mortality and planned 7 and 9 patients in the first and second stages of this study, with target and lower success rates of 80% and 50% and alpha and beta errors of 10% and 10%, respectively (*24*). Nonrelapse mortality was observed in only one of the seven patients in the first stage, and thus the study was continued to the second stage. This was an interim analysis performed in February 2004. Overall survival and the incidences of GVHD and CMV reactivation were calculated using the Kaplan-Meier method. The data were compared with those who underwent allogeneic hematopoietic stem-cell transplantation from an HLA-identical sibling donor or a matched unrelated donor in the same period. Overall survival and the incidence of CMV reactivation were compared using the log-rank test. The recovery of immunologic parameters was compared using the Mann-Whitney *U* test.

RESULTS

Characteristics of the Patients

Twelve patients were included in the study (Table 1). There were eight males and four females with a median age of 49.5 years (range 27–60 years). The underlying disease was acute lymphoblastic leukemia (ALL) in five patients, acute myeloblastic leukemia in two patients, MDS in two patients, CML in blast crisis in one patient, and non-Hodgkin lymphoma in two patients. Eight patients demonstrated active disease at transplantation. The other four patients underwent transplantation for ALL in remission. Of these, two demonstrated Ph^+ ALL in first remission, one demonstrated ALL in second remission, and one demonstrated ALL in first remission and required more than 3 months to achieve remission. Most patients demonstrated comorbidities before transplantation including recent or active invasive aspergillosis in five, anthracycline-induced cardiac failure, interstitial pneumonitis caused by radiation for breast cancer, obstructive lung disease, and so on. Six patients who were more than 50 years old received a fludarabine-based regimen, whereas the other six received a TBI-based regimen.

Recovery of Donor Cells

The median number of CD34+ and CD3+ cells in the graft was 5.1×10^6 cells/kg (range 4.3–7.7) and 2.6×10^8 cells/kg (range 1.8–7.1), respectively. The median duration to the neutrophil recovery greater than 500/mm^3 and platelet recovery greater than 20,000/mm^3 without transfusion was 17.5 days (range 12–29 days) and 16 days (range 12–27 days), respectively (Fig. 1A and B). Complete donor-type chimerism was achieved on day 28 in all patients and was sustained thereafter, except for one patient who underwent transplantation for MDS (chronic myelomonocytic leukemia) using a fludarabine-based regimen and developed mixed chimerism (8.5% host cells) on day 60, and then relapsed with acute myeloblastic leukemia on day 90.

Graft-Versus-Host Disease

Grades II to IV acute GVHD was observed in two patients. One of the two patients developed grade II acute GVHD of the gut on day 32, which responded to methylprednisolone. The other patient developed grade III acute GVHD of the skin and gut on day 20, which was refractory to steroids, and eventually died of thrombotic microangiopathy on day 66. This patient received a three loci-mismatched graft from a cousin. He developed early hemorrhagic cystitis followed by postrenal azotemia and could not receive CsA at a therapeutic concentration. The cumulative incidence of grades II to IV and III to IV acute GVHD was 18% and 9%, respectively (Fig. 1C and D).

Of the eight evaluable patients who survived more than 100 days after transplantation, limited chronic GVHD that did not require treatment was observed in two patients. Notably, all five patients who are alive more than 100 days after transplantation as of this analysis are free from immunosuppressants.

Infectious Complications

Of the five patients who had recent or active invasive pulmonary aspergillosis before transplantation, one had a recurrence of aspergillosis during the neutropenic period after

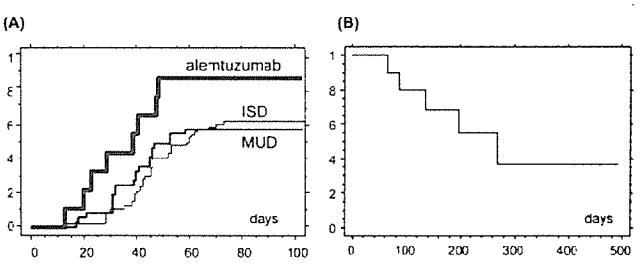

FIGURE 2. Cumulative incidence of cytomegalovirus reactivation detected by antigenemia assay, grouped according to the donor type (A). Overall survival of all patients (B). ISD, human leukocyte antigen (HLA)-identical sibling donors; MUD, matched unrelated donors.

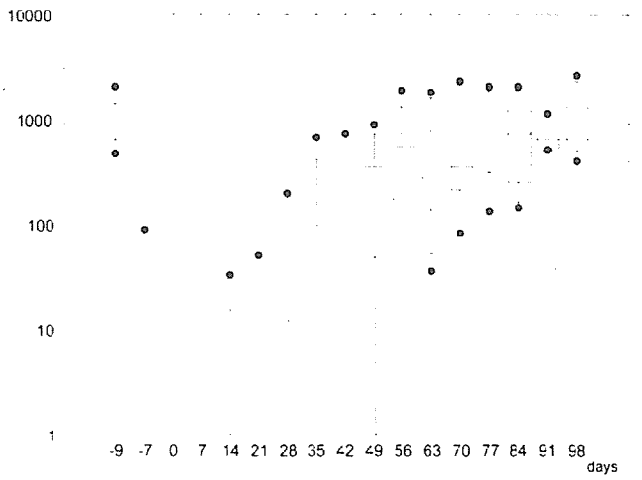

FIGURE 3. Recovery of peripheral blood lymphocytes after transplantation; 10, 25, 50, 75, and 90 percentile values (*box-and-whisker plot*). Outliers (*dots*).

transplantation, which was improved with neutrophil recovery. Otherwise, severe bacterial or fungal infection was not observed throughout the entire period after transplantation.

Of the 11 patients who were seropositive for CMV or who had a donor who was seropositive for CMV before transplantation, CMV reactivation was detected in 10 by antigenemia assay. The incidence of CMV reactivation was significantly higher than that after transplantation from an HLA-identical sibling donor or a matched unrelated donor (P=0.032, Fig. 2A). However, there was no death or severe disease related to CMV infection. Two patients developed asymptomatic CMV retinitis on days 149 and 160, respectively, and another patient developed hemorrhagic cystitis with CMV viruria on day 45, all of which were successfully treated with ganciclovir.

Relapse, Nonrelapse Mortality, and Survival

As a primary endpoint of the study, early nonrelapse mortality before day 100 was observed in one patient, who died of thrombotic microangiopathy and gut hemorrhage on day 66. Nonrelapse mortality was observed in another patient, who died of worsening of interstitial pneumonitis on day 197. This patient had received 60 Gy of local radiation to the right upper lung lobe for breast cancer and had already had local interstitial pneumonitis before transplantation. Six patients had a relapse of the underlying hematologic malignancy at a median of 111 days (range 49–223 days) after transplantation, and five of these had active disease before transplantation. Three of them died, and two are alive with disease. The remaining patient, who had undergone transplantation for ALL in second remission, received donor lymphocyte infusion after relapse of ALL and is alive in remission. None of the three patients who underwent transplantation for acute leukemia in first remission have relapsed thus far. Of these, two patients who had Ph^+ ALL were in molecular remission after transplantation. Overall survival is shown in Figure 2B.

Immune Reconstitution

The peripheral lymphocyte count dramatically decreased on the day after the first infusion of alemtuzumab and then gradually increased after day 28 (Fig. 3). Immune recon-

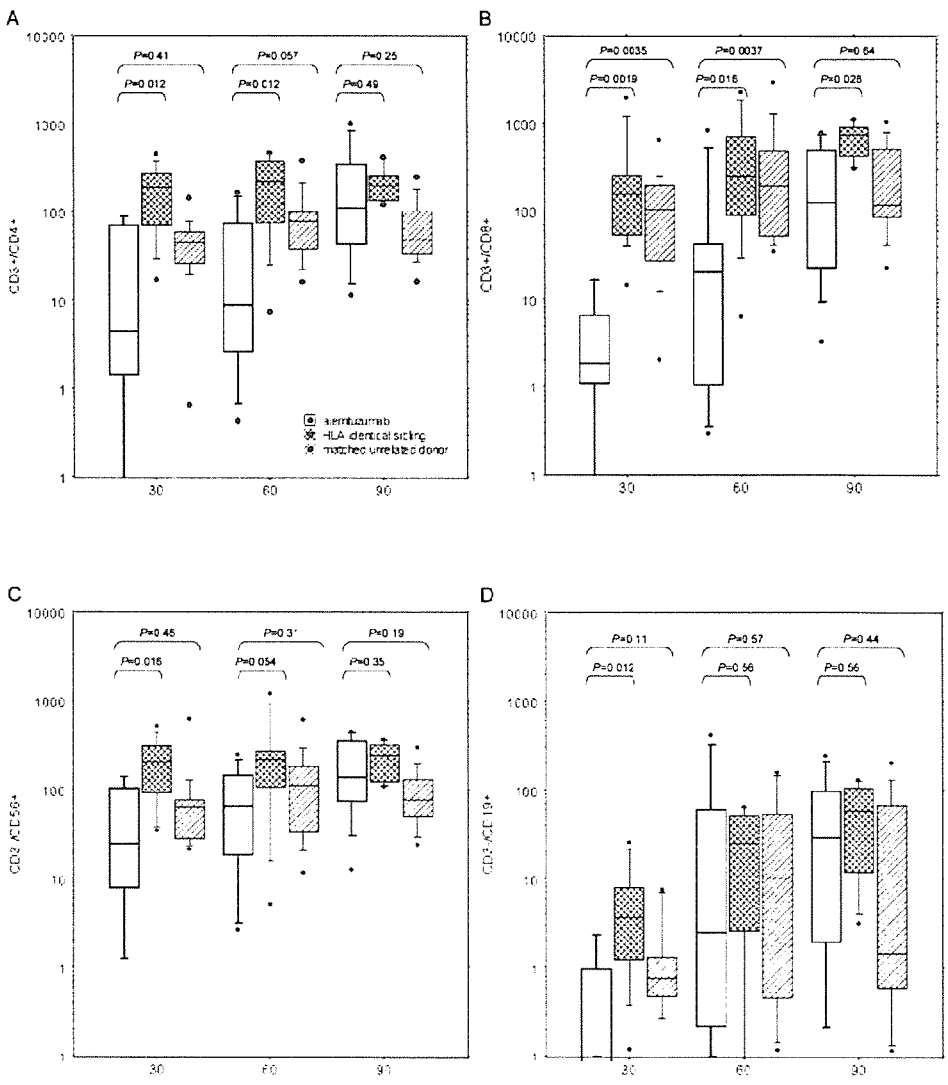

FIGURE 4. Recovery of CD3+/CD4+ (A), CD3+/CD8+ (B), CD3−/CD56+ (C), and CD3−/CD19(+) (D) cells on days 30, 60, and 90 after transplantation, grouped by the donor type; 10, 25, 50, 75, and 90 percentile values (*box-and-whisker plot*). Outliers (*dots*).

TABLE 2. Summary of outcome

Complications		
Infections	Possible invasive pulmonary aspergillosis	1
	Cytomegalovirus reactivation	10
	Cytomegalovirus retinitis	2
	Adenovirus hemorrhagic cystitis	2
Acute GVHD	0	6
	I	4
	II	1
	III	1
	IV	0
Chronic GVHD	None	6
	Limited	2
	Extensive	0
	Not evaluable	4
Outcome		
Status before transplantation	Current status	
Remission 4	Alive in remission	3
	Died in remission	1 (radiation pneumonitis)
Active disease 8	Alive in remission	2
	Alive after relapse	2
	Died after relapse	3
	Died in remission	1 (thrombotic microangiopathy)

GVHD, graft-versus-host disease.

stitution after transplantation was evaluated in greater detail by the quantification of CD3+/CD4+, CD3+/CD8+, CD3−/CD19+, and CD3−/CD56+ cells, and compared with that after transplantation from an HLA-identical sibling donor or a matched unrelated donor (Fig. 4). The numbers of CD3+/CD4+ and CD3+/CD8+ cells were significantly lower than those after transplantation from an HLA-identical sibling donor or a matched unrelated donor during the first 2 months after transplantation. However, the numbers of CD3+/CD4+ and CD3+/CD8+ cells caught up with those after matched unrelated transplantation on day 90. CD3−/CD56+ and CD3−/CD19+ cells recovered earlier than T cells.

CMV-specific T-cell recovery was evaluated by tetramer assay in six patients who had HLA-A*0201 or HLA-A*2402. CMV-specific cytotoxic T lymphocytes were detected on day 90 after transplantation in two patients, at 0.03% and 0.25% of CD8+ T cells, respectively. Both patients had CMV reactivation before the detection of CMV-specific cytotoxic T lymphocytes. As a functional assay, a PHA stimulation test was performed using peripheral lymphocytes in three patients on days 120, 377, and 509, respectively, after transplantation. The stimulation index was 415.2 and 391.0, respectively, in the two patients who were tested more than 1 year after transplantation. Considering that the 95% confidence interval for the stimulation index in the normal population is 74 and 508, peripheral T cells in these patients have a normal proliferative response to PHA stimulation. Although the stimulation index was only 10.7 in the patient who was tested on day 120, at that time only 6% of the peripheral lymphocytes were T cells, and thus the response was within the normal range when corrected for the percentage of T cells.

DISCUSSION

We evaluated the safety of haploidentical peripheral blood stem-cell transplantation from a two or three loci-mismatched family member using in vivo alemtuzumab. There was no graft rejection, and the incidence of grades III to IV acute GVHD was only 9%, almost equivalent to that after transplantation from an HLA-matched sibling donor (*3*). The median age of the 12 patients was 49.5 years, and 9 and 6 patients were older than 40 and 50 years, respectively. Thus, the patients in this study were much older than those in previous reports on haploidentical stem-cell transplantation (*5*, *25*, *26*). Nevertheless, nonrelapse mortality was observed in only two patients.

A major concern with this strategy was infectious complications caused by prolonged immunosuppression. In fact, CD3+/CD4+ and CD3+/CD8+ T cells were strongly suppressed within 2 months after transplantation, which was reflected by the high incidence of CMV reactivation. However, this did not translate into the development of severe infections. None of the patients died of infectious causes. Another concern was relapse after transplantation. Five of the eight patients who underwent transplantation for active disease had a relapse, whereas this was not seen in any of the three patients who underwent transplantation in first remission. Therefore, the dose of alemtuzumab is appropriate for patients with early disease, whereas it may be better to reduce

the dose of alemtuzumab for patients with active disease, considering the low incidence of severe GVHD in this study.

The transplantation procedure was simplified and the cost was reduced by the omission of ex vivo graft manipulation. Antithymocyte globulin (ATG) could also be used for in vivo T-cell depletion. However, considering the results of HLA-matched transplantation, the prophylactic effect of alemtuzumab against GVHD seemed to be much stronger than that of ATG (*14, 15, 20, 27*). The unique pharmacokinetic profile, in which a lympholytic concentration remains for approximately 2 months after transplantation, may contribute to the potent effect against GVHD (*13*). Depletion of host dendritic cells, which also express CD52, could be another mechanism to prevent GVHD, because host antigen-presenting cells have been shown to be important for the development of GVHD in mouse models (*28*). Alemtuzumab may be more appropriate for clinical use than ATG, because alemtuzumab is a recombinant monoclonal antibody with a consistent quality, whereas lot-to-lot variability of ATG cannot be avoided, because ATG is prepared by immunizing horses or rabbits with human lymphoid cells. Another advantage of alemtuzumab is that it kills not only T cells but also B cells, and thus there may be a lower risk of posttransplant lymphoproliferative disorders. A possible disadvantage of alemtuzumab is that alemtuzumab may kill NK cells, which may be important for a graft-versus-leukemia/lymphoma effect (*29*). However, the recovery of NK cells was observed early after transplantation in this study and was equivalent to that after matched unrelated donor transplantation. As further evidence that the lympholytic effect of alemtuzumab on NK cells is weaker than that on T cells, alemtuzumab "in the bag" resulted in the 99.8% and 94% depletion of CD4+ and CD8+ T cells, respectively, whereas 30% of NK cells were conserved in the graft (*30*).

CONCLUSION

Unmanipulated hematopoietic stem-cell transplantation was safely performed from a two or three loci-mismatched family member using in vivo alemtuzumab. This novel therapeutic approach could be applied to patients aged more than 50 years without the need for an HLA-matched donor or any specific devices. [AU: Please cite Table 2 in text.]

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Graft-versus-Tumor Effect Against Advanced Pancreatic Cancer after Allogeneic Reduced-Intensity Stem Cell Transplantation

Yoshinobu Kanda,[1,5] Yutaka Komatsu,[2] Masaaki Akahane,[3] Shigeyuki Kojima,[4] Yuki Asano-Mori,[1] Minoru Tada,[2] Kumi Oshima,[1] Hiroyuki Isayama,[2] Seishi Ogawa,[1] Toru Motokura,[1] Shigeru Chiba,[1] Kuni Ohtomo,[3] Masao Omata,[2] and Hisamaru Hirai[1]

Background. The prognosis of advanced pancreatic cancer is extremely poor and therefore a novel treatment strategy is desired. The authors thus started a prospective study of allogeneic reduced-intensity hematopoietic stem cell transplantation (RIST) for patients with advanced pancreatic cancer to evaluate the feasibility and efficacy of this approach for such patients.

Methods. Only patients with pathologically proven pancreatic cancer that was locally advanced or metastatic and not amenable to curative resection were included. The conditioning regimen consisted of gemcitabine, fludarabine, and busulfan.

Results. In the first stage of this study, the authors treated seven patients. Treatment-related mortality before day 100 was observed in one patient. The median survival after RIST was 229 days. An objective response on computed tomographic scan was observed in two patients and another had a tumor marker response. Marked tumor shrinkage was observed in one of the remaining patients after donor lymphocyte infusion. These antitumor effects appeared after the effect of the conditioning regimen had disappeared. In addition, some of these responses were associated with an increase in the serum anticarcinoembryonic antigen antibody level.

Conclusions. Pancreatic cancer appeared to be sensitive to a graft-versus-tumor effect; therefore, a larger clinical study with a refined strategy is warranted.

Keywords: Reduced-intensity stem cell transplantation, Minitransplantation, Pancreatic cancer, Graft-versus-tumor effect, Graft-versus-host disease.

(*Transplantation* 2005;79: 821–827)

Pancreatic cancer is the fifth most common cause of cancer-related mortality in Japan and the United States. The median duration of survival in advanced pancreatic cancer is less than 6 months, even when treated with gemcitabine (*1*), and therefore a novel treatment strategy is desired. Allogeneic nonmyeloablative or reduced-intensity hematopoietic stem cell transplantation (RIST) is a recently developed treatment approach for obtaining a graft-versus-tumor (GVT) effect without the toxicity associated with a myeloablative conditioning regimen (*2–6*). This treatment strategy is suitable for patients with solid tumors, because patients with advanced solid tumors are generally clinically infirm, and also a strong antitumor effect cannot be expected with an intensification of chemotherapy. In addition, a GVT effect has been noted in several solid tumors after conventional hematopoietic stem-cell transplantation (*7, 8*). Based on this background, RIST has been investigated for use against solid cancers since the late 1990s and its feasibility has already been demonstrated in several studies (*9–14*). However, there is still little information available regarding its efficacy against individual solid cancers. Childs et al. showed an excellent response rate of 53% after RIST against metastatic renal cell cancer (*9*). A GVT effect against renal cell cancer was confirmed in trials by other centers. In contrast, RIST against metastatic melanoma, which has been considered to be a good candidate for immunotherapy, resulted in a miserable outcome (*15*). Therefore, it is difficult to predict whether a GVT effect can be achieved against an individual tumor. We started a prospective study to evaluate the feasibility and efficacy of RIST against advanced pancreatic cancer after ethical approval in April 2002.

PATIENTS AND METHODS

Patients

Eligible patients were younger than 70 years of age and had pathologically proven pancreatic adenocarcinoma, which was locally advanced or metastatic and not amenable to curative resection. Patients had a human leukocyte antigen (HLA)-matched sibling or a family donor with a single mismatched HLA antigen. Patients with a poor performance status (Eastern Cooperative Oncology Group 2–4) and those with severely impaired organ function were excluded. Patients and donors gave their written informed consent to participate in this study.

Twelve patients with pancreatic cancer fulfilled the inclusion criteria but lacked a suitable donor. They were considered control patients. Four of them had metastatic lesions, whereas eight had locally advanced disease. Nine received

[1] Department of Cell Therapy & Transplantation Medicine, University of Tokyo, Tokyo, Japan.
[2] Department of Gastroenterology, University of Tokyo, Tokyo, Japan.
[3] Department of Radiology, University of Tokyo, Tokyo, Japan.
[4] R & D Center, BML, Inc., Kawagoe, Japan.
[5] Address correspondence to: Yoshinobu Kanda, M.D., Department of Cell Therapy & Transplantation Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: ycanda-tky@umin.ac.jp.
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chemotherapy with gemcitabine, whereas three were observed without chemotherapy.

Transplantation Procedure

Donors received granulocyte colony-stimulating factor at 200 $\mu g/m^2$ administered subcutaneously twice daily starting 3 days before the first collection of peripheral blood stem cells until the end of collection. Leukapheresis was performed daily until more than 2.0×10^6 $CD34^+$ cells/kg of the recipient's body weight were collected. Collected cells were then cryopreserved using standard techniques without ex vivo manipulation.

The conditioning regimen consisted of gemcitabine (1,000 mg/m^2/day as a 90-min infusion on days −16, −9, and −2) (*16*), fludarabine (30 mg/m^2/day as a 30-min infusion on days −8 to −3), and busulfan (4 mg/kg/day administered orally in four divided doses on days −6 and −5) (*4*, *17*). Graft-versus-host disease (GVHD) prophylaxis was performed with cyclosporine (CsA) (3 mg/kg/day as a continuous infusion) and short-term methotrexate (10 mg/m^2 on day 1 and 7 mg/m^2 on days 3 and 6). Frozen peripheral blood stem cells were thawed and infused on day 0. CsA was decreased at 10% per week from day 30 and discontinued by day 100 unless the patient developed acute GVHD. In patients with progressive disease without any evidence of acute GVHD, CsA was rapidly tapered over a 4-week period. Acute GVHD was graded as previously described (*18*). Grade II to IV acute GVHD was treated with methylprednisolone at 1 mg/kg per day, except for grade II GVHD limited to the skin, which was treated with topical steroid ointment.

Prophylaxis against bacterial, fungal, and *Pneumocystis carinii* infection consisted of tosufloxacin, fluconazole, and sulfamethoxazole-trimethoprim. As prophylaxis against herpes simplex virus infection, acyclovir was administered at 500 mg per day intravenously or 1,000 mg per day orally from days −7 to 35, followed by long-term low-dose (200 mg/day) oral administration (*19*). Patients received granulocyte colony-stimulating factor (filgrastim) at 300 μg per day by 3-hr infusion beginning on day 7 until the neutrophil count recovered to $500/mm^3$. Cytomegalovirus antigenemia assay using C10/C11 antibody was performed at least once per week after engraftment. Ganciclovir was started when more than two positive cells were detected on two slides (*20*, *21*).

Donor lymphocyte infusion (DLI) was performed for patients who had progressive disease and did not develop GVHD even after CsA was discontinued. The initial $CD3^+$ cell dose was 1 to 3×10^7 cells/kg and the dose was escalated every 4 weeks when the patient did not develop tumor response or GVHD.

Chimerism and Immunologic Analyses

Host-donor cell chimerism after transplantation was analyzed monthly by sex-chromosome fluorescent in situ hybridization or the short tandem repeat method after transplantation (*22*). The serum anti-carcinoembryonic antigen (CEA) antibody level was determined by enzyme-linked immunosorbent assay as previously described (*23*). Briefly, 96-well microplates were coated overnight at 4°C with a 5-μg/mL CEA preparation. The plates were washed and blocked for 2 hr at room temperature with 200 μL/well of a 0.1% Tween20, 5% nonfat dry milk, phosphate-buffered saline solution to prevent nonspecific binding. After the plates were washed further, 50 μL of 1:100 diluted patient sera was added per well and incubated for 2 hr at room temperature. After the plates were washed five times, 50 μL of horseradish peroxidase-labeled anti-human immunoglobulin G antiserum at 0.1 μg/mL in blocking buffer was added per well. The plates were incubated for 90 min at room temperature and then washed five times. The conjugated anti-CEA antibody was detected by adding 100 μL of tetramethylbenzidine substrate per well, incubating for 30 min at room temperature, adding 50 μL of 2N H_2SO_4 per well to terminate the reaction, and measuring the absorbance at 450 nm. The net anti-CEA antibody absorbance was determined by subtracting the absorbance of a noncoated well from the gross absorbance.

Outcome Measures

The primary endpoint of this study was transplant-related mortality within 100 days after transplantation. The secondary endpoint was the tumor response within 6 months after transplantation. Toxicities associated with the conditioning regimen were graded according to the criteria of Bearman et al. (*24*). Objective tumor response was evaluated by an independent radiologist using a monthly computed tomographic (CT) scan. Complete response was defined as disappearance of all clinical evidence of tumor for a minimum of 4 weeks. Minor and partial responses were defined as decreases of 25% to 50% and greater than 50%, respectively, in the sum of the products of the maximum diameter and its perpendicular diameter of all measurable lesions for a minimum of 4 weeks (*1*). The tumor marker response was evaluated by biweekly measurement of the serum CA19-9 level, because imaging modalities including ultrasonography and CT scan are

TABLE 1. Characteristics of the patients

Patient	Age/sex	Prior treatment	Duration from Dx to transplant (mo)	Meta	HLA	No. of $CD34^+$ cells
1	48/M	Gem	3	—	6/6	4.8×10^6/kg
2	40/M	Gem+RT, Gem	9	—	6/6	5.1×10^6/kg
3	57/F	Gem+CDDP	4	Liver	6/6	4.0×10^6/kg
4	36/F	Gem	3	Liver	6/6	2.9×10^6/kg
5	59/M	Gem	2	—	6/6	5.6×10^6/kg
6	66/F	Gem	2	Peritonitis	6/6	2.0×10^6/kg
7	61/M	Gem+RT	12	Liver	6/6	3.0×10^6/kg

Dx, diagnosis; Meta, metastatic lesion; Gem, gemcitabine; RT, local radiation; CDDP, cisplatin.

TABLE 2. Outcome after RIST

Patient	Donor chimerism (%)	aGVHD	Objective response	Final outcome
1	100	III	MR	Died as a result of bacteremia on day 192
2	100	II	SD	Died as a result of PD on day 293
3	100	II[a]	MR[d]	Died as a result of PD on day 262
4	100	II[a]	PD	Died as a result of PD on day 72
5	100	II	SD[c,d]	Died as a result of PD on day 587
6	100	III[b]	PD	Died as a result of PD on day 229
7	100	III[a]	SD	Died as a result of pneumonia on day 83

[a] GVHD that occurred after the rapid tapering of immunosuppressants.
[b] GVHD that occurred after DLI.
[c] Partial response on tumor marker evaluation.
[d] Morphine was discontinued.
aGVHD, acute graft-versus-host disease; MR, minor response; SD, stable disease; PD, progressive disease; DLI, donor lymphocyte infusion.

not sufficient to determine the accurate tumor size of pancreatic cancer (*25*). For patients with a normal value of CA19-9 before RIST, CEA, Dupan-II, or Span-I was measured instead. A complete marker response was defined as normalization of the tumor marker for a minimum of 4 weeks. Minor and partial responses were defined as decreases of 25% to 50% and greater than 50%, respectively, in the tumor marker for a minimum of 4 weeks.

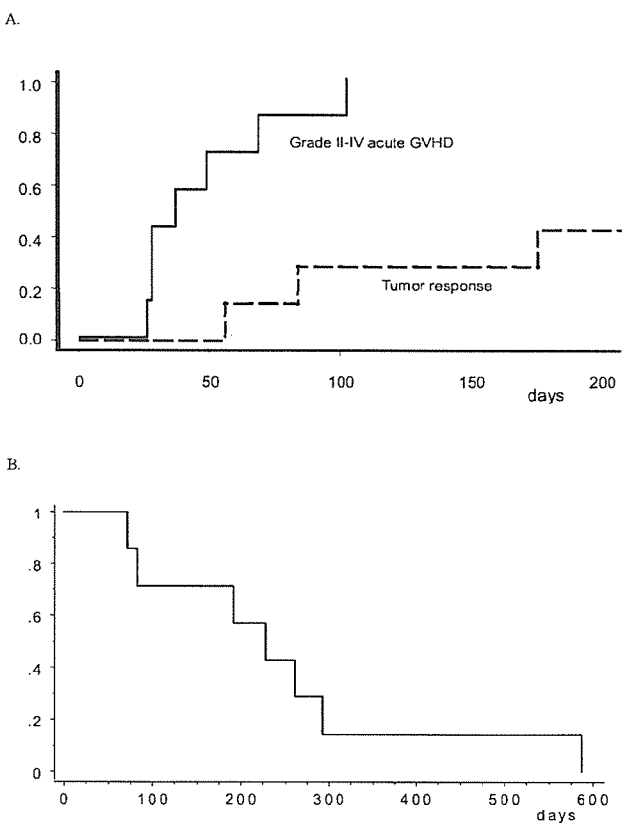

FIGURE 1. (A) Cumulative incidence of grade II to IV acute GVHD (*solid line*) and tumor response including both the objective response and the tumor marker response (*broken line*). (B) Overall survival after RIST against advanced pancreatic cancer.

Statistical Considerations

We defined success as the absence of early transplant-related mortality and planned seven and nine patients in the first and second stages of the study, with target and lower success rates of 80% and 50% and α and β errors of 10% and 10%, respectively (*26*). This is an analysis of the seven patients in the first stage. The cumulative incidence of tumor response was calculated by Gray's method considering death without response as a competing risk (*27*).

RESULTS

Patients

In the first stage of this study, seven patients with a median age of 57 years (range, 36–66 years) underwent RIST (Table 1). The duration from diagnosis to transplantation was 2 to 12 months. Four had metastatic disease and three had locally advanced disease. Prior treatment consisted of chemotherapy mainly with gemcitabine without an objective response, except for one patient (patient 2) who achieved a transient partial response after gemcitabine combined with local irradiation. All of the patients had progressive disease just before the conditioning regimen. All received a peripheral blood stem-cell graft from an HLA-identical sibling donor. The median number of $CD34^+$ cells in the graft was 4.0×10^6 cells/kg recipient body weight (range, 2.0–5.6 cells/kg). Three patients (patients 2, 5, and 6) underwent DLI for tumor progression 221, 336, and 69 days after transplantation, respectively. The dose of infused $CD3^+$ cells ranged between 2.7×10^7 and 1.8×10^8 cells/kg.

Regimen-Related Toxicity, Engraftment, and GVHD

Regimen-related toxicities were generally mild and well tolerated. Grade II to IV toxicity according to Bearman's grade was observed in two patients. One developed mild hepatic veno-occlusive disease, which recovered spontaneously. Another developed ileus caused by the pancreatic head tumor during the neutropenic period, which required nasogastric suction.

The median number of infused $CD34^+$ cells was 4.0×10^6 cells/kg (range, $2.0–5.6\times10^6$ cells/kg). Neutrophil engraftment was obtained within 12 days (range, 11–12 days)

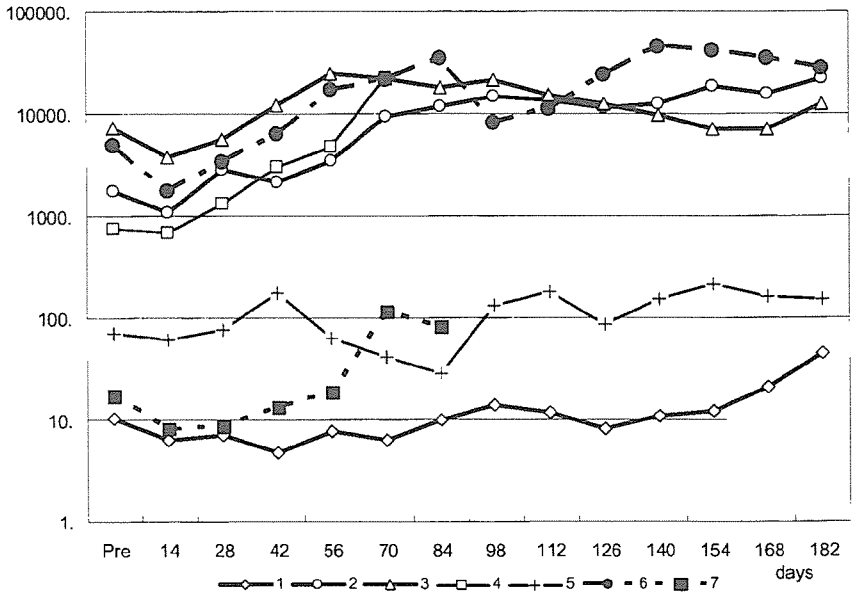

FIGURE 2. Serial changes in the values of tumor markers. CA19-9 was used all except patients 1 and 7, in whom CEA was measured instead because the CA19-9 level was within normal limits before transplantation. The units for the y-axis are units per milliliter for CA19-9 and nanograms per milliliter for CEA. A log scale was used for the y-axis to show serial data of all patients in one figure. Pre, pretransplant level.

after transplantation. Complete donor chimerism (>95%) was achieved in all patients by day 28 and maintained thereafter (Table 2). Grade II to III acute GVHD was observed in three patients (patients 1, 2, and 5) without rapid tapering of CsA, in three (patients 3, 4, and 7) after rapid tapering of CsA, and in one after DLI (Fig. 1A). Acute GVHD limited to the skin was cured with topical steroid only, whereas gut GVHD was successfully treated with systemic steroid.

Transplant-Related Mortality and Survival

Transplant-related mortality within 100 days after transplantation was observed in 1 patient (patient 7), who died as a result of pneumonia on day 83. Another patient (patient 1) died with bacteremia on day 192 caused by bacterial cholangitis. The other five patients died as a result of progressive disease. Median survival after RIST was 229 days (Fig. 1B), which was longer than the median survival of control patients after they were referred to our hospital (125 days), but this difference was not statistically significant.

Tumor Response

An objective minor response on CT scan was seen in two patients (patients 1 and 3) (Table 2 and Fig. 1A). Another patient (patient 5) achieved a partial tumor marker response. Two (patients 3 and 5) of the responders became free from all analgesics after achieving tumor regression.

As shown in Figure 2, the serum CA19-9 or CEA level generally increased within 6 to 8 weeks after transplantation after a transient decline associated with the conditioning chemotherapy. However, it stabilized (n=1) or began to decrease (n=3) thereafter, associated with the discontinuation of CsA or the development of GVHD. This suggests that the antitumor effect was caused by a GVT effect, not a chemotherapy effect. In particular, the serum CA19-9 level decreased from 25,180 U/mL on day 56 to 7,100 U/mL on day 154, with a tumor shrinkage on CT scan in patient 3 after the development of gut GVHD on day 69 (Fig. 3A). Evidence of a GVT effect against pancreatic cancer was also clearly seen in patient 6, who underwent DLI on day 69 for a progressive peritoneal metastatic lesion. The serum CA19-9 level decreased from 35,160 U/mL to 8,140 U/mL in 1 month, with an improvement of the peritoneal lesion on abdominal CT scan (Fig. 3B). However, these tumor responses were not durable and the response duration was between 28 and 98 days.

Serum Anti-CEA Antibody Level

Serum anti-CEA antibody levels were sequentially measured before and after RIST. The serum anti-CEA antibody level before RIST was higher than that in a normal population (n=3), with borderline significance (optical density, 0.109 ± 0.065 vs. 0.028 ± 0.030; $P=0.076$). It generally decreased 1 month after RIST with conditioning chemotherapy. Thereafter, an increase in the serum anti-CEA antibody level was observed in three patients. All three of these patients showed a tumor response, including one after DLI, whereas only one of the four patients without an increase in the serum anti-CEA antibody level showed a response. As shown in Figure 4, the increase in the serum anti-CEA antibody level was simultaneously associated with, or followed by, a decrease in the tumor marker level. However, this response was suppressed by the administration of high-dose steroid (Fig. 4A).

The increase in the serum anti-CEA level did not reflect nonspecific immune recovery, because we did not observe a significant relationship between serum anti-CEA antibody levels and antibody levels against other viral antigens (data not shown). In addition, the increase in the serum anti-CEA level did not result from a decrease in the serum CEA antigen that may absorb anti-CEA antibody, because we did not observe a negative relationship between them (data not shown).

DISCUSSION

In this study, we showed that RIST is a feasible treatment for patients with advanced pancreatic cancer. In addition, an objective response and a tumor marker response were observed in two and one of seven patients, respectively, who

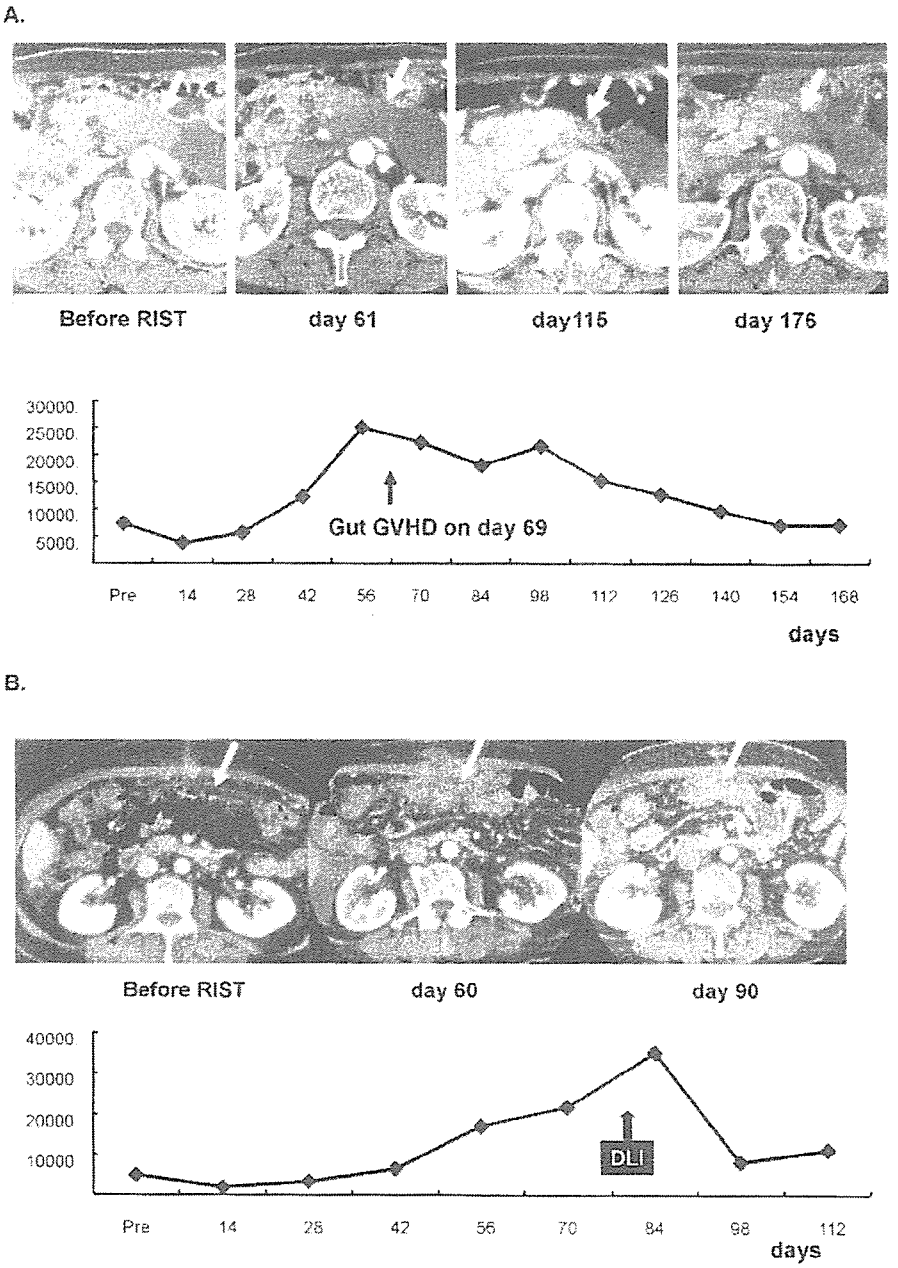

FIGURE 3. (A) Serial abdominal CT scans and serum CA19-9 levels of a patient (patient 3) who achieved an objective response after transplantation. The pancreatic body tumor shrunk and the serum CA19-9 level decreased after the development of gut GVHD. (B) Serial abdominal CT scans and serum CA19-9 levels of a patient (patient 6) who achieved an objective response after DLI. The peritoneal lesion worsened in the first 2 months after transplantation. However, the tumor began to shrink and the serum CA19-9 level rapidly decreased after DLI.

had not responded to conventional treatments. A response to DLI was seen in another patient. Although it is too early to evaluate survival after RIST, the median survival of 229 days might be better than that after conventional treatments. However, the tumor response was not durable, and all of the responding patients eventually died as a result of tumor progression.

It has been shown that at least 2 months are required to obtain a GVT effect after RIST against solid cancers. Childs et al. reported that tumor growth was frequently observed during the first few months after RIST for renal cell cancer (9). Therefore, we added gemcitabine to the combination of fludarabine and busulfan, a widely used conditioning regimen in RIST (4, 17), to suppress tumor progression before the emergence of a GVT effect, because pancreatic cancer progresses much faster than renal cell cancer. A synergistic antitumor effect with the combination of gemcitabine and fludarabine has been demonstrated in in vitro studies (28).

It can be difficult to distinguish between a chemotherapy effect and a GVT effect after RIST. In this study, however, a transient chemotherapy effect attributable to the conditioning regimen was observed as tumor marker regression during the first 2 weeks after RIST. Thereafter, tumor marker began to increase, which suggested that the chemotherapy effect did not persist for longer than 2 weeks. The second regression of tumor marker was observed at least 2 months after RIST, associated with the discontinuation of CsA, the development of GVHD, or DLI. An objective tumor response was also observed at least 2 months after RIST. Therefore, the GVT effect

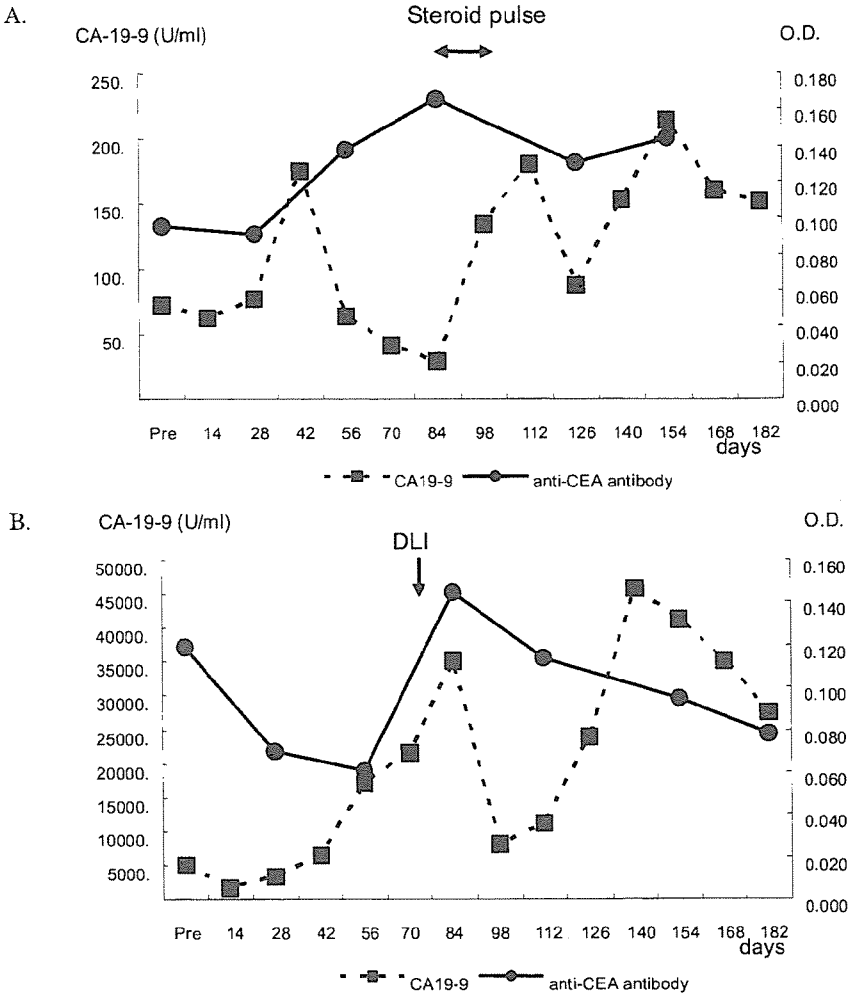

FIGURE 4. Serial changes in the serum CA19-9 level and anti-CEA antibody level. (A) Patient 5, who achieved a partial response on tumor marker evaluation. (B) Patient 6, who showed a tumor response after DLI.

against pancreatic cancer was clearly distinguished from the chemotherapy effect in this study.

Although a GVT effect against pancreatic cancer was seen in this study, this effect did not persist for more than 3 months. In addition, the development of GVHD was required to achieve a GVT effect. Therefore, we need a strategy for maintaining the GVT effect for a longer period without aggravating GVHD. To accomplish this, a specific immunotherapy against pancreatic cancer is required. Candidate target antigens include CA19-9, CA242, CEA, Her-2, mutated K-ras, and MUC-1 (*29*). Among these, CEA is attractive, because it is expressed in 85% to 90% of pancreatic cancer (*29*), and a specific immunotherapy against CEA could also be applied to other gastrointestinal cancers. Therefore, we retrospectively measured serial serum anti-CEA antibody levels in these patients. An increase in the serum anti-CEA antibody level after a transient decline just after RIST was associated with a tumor response. An elevation of serum anti-CEA antibody levels has already been demonstrated after a vaccine therapy targeting CEA (*23, 30*). Also, the presence of anti-CEA antibody at diagnosis was associated with better survival in patients with colon cancer, and thus anti-CEA antibody was suggested to have biologic significance (*31*). Thus, the GVT effect against pancreatic cancer in this study might be in part attributable to the specific immunity against CEA antigen. We also tried to detect CEA-specific cytotoxic T cells by enzyme-linked immunospot assay, but failed, probably because of the use of frozen peripheral blood mononuclear cells.

A major difference between RIST for hematologic malignancies and that for pancreatic cancer is the complications after transplantation. One patient (patient 1) experienced repeated episodes of biliary stent obstruction and bacterial cholangitis. Another two patients (patients 2 and 7) developed intestinal obstruction, probably because of the pancreatic head tumor. Therefore, a clinical trial of RIST for pancreatic cancer should be performed in close cooperation with the transplantation staff and gastroenterologists.

CONCLUSION

RIST appeared to be a feasible treatment for patients with advanced pancreatic cancer. The existence of a GVT effect against pancreatic cancer was strongly suggested by this study, but this effect was not durable and required the development of GVHD. We need to refine the strategy, for example, by the combination of specific immunotherapy against CEA after RIST.

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Notch1 oncoprotein antagonizes TGF-β/Smad-mediated cell growth suppression via sequestration of coactivator p300

Shigeo Masuda,[1] Keiki Kumano,[1] Kiyoshi Shimizu,[1] Yoichi Imai,[1] Mineo Kurokawa,[1] Seishi Ogawa,[1,2] Makoto Miyagishi,[3] Kazunari Taira,[3] Hisamaru Hirai[1] and Shigeru Chiba[1,4]

[1]Departments of Hematology, Oncology and Cell Therapy, and Transplantation Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, [2]Department of Regeneration Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, and [3]Department of Chemistry and Biotechnology, School of Engineering, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

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The Notch proteins constitute a family of transmembrane receptors that play a pivotal role in cellular differentiation, proliferation and apoptosis. Although it has been recognized that excess Notch signaling is potentially tumorigenic, little is known about precise mechanisms through which dysregulated Notch signaling induces neoplastic transformation. Here we demonstrate that Notch signaling has a transcriptional cross-talk with transforming growth factor-β (TGF-β) signaling, which is well characterized by its antiproliferative effects. TGF-β-mediated transcriptional responses are suppressed by constitutively active Notch1, and this inhibitory effect is canceled by introduction of transcriptional coactivator p300. We further show that this blockade of TGF-β signaling is executed by the sequestration of p300 from Smad3. Moreover, in a human cervical carcinoma cell line, CaSki, in which Notch1 is spontaneously activated, suppression of Notch1 expression with small interfering RNA significantly restores the responsiveness to TGF-β. Taken together, we propose that Notch oncoproteins promote cell growth and cancer development partly by suppressing the growth inhibitory effects of TGF-β through sequestrating p300 from Smad3. (*Cancer Sci* 2005; 96: 274–282)

The Notch pathway constitutes an evolutionarily conserved signaling pathway that mediates critical cell fate decisions, such as differentiation, proliferation and apoptosis.[1,2] In addition to the fact that Notch signaling plays pivotal roles in embryonic development, and post-embryonic growth and differentiation in multiple systems such as the immune system,[3,4] substantial evidence indicates that the constitutively activated forms of Notch family proteins are involved in tumorigenesis:[5–17] *Notch1/TAN-1* was originally identified as a recurrent chromosomal translocation, t(7;9)(q34; q34.3), in a human acute T-cell lymphoblastic leukemia,[5] resulting in the expression of an extracellular region-truncated form of Notch1 that is known to be constitutively active. The N-terminal truncated forms of the Notch1 and Notch2 proteins have been implicated in the transformation of rat kidney cells in cooperation with an adenoviral oncoprotein, E1A.[10,15] Activated forms of the Notch1[8] and Notch3 proteins[13] are capable of generating T-cell leukemia when retrovirally introduced into bone marrow cells that are transplanted into irradiated recipient mice. Also, *Int-3*, which encodes a truncated form of Notch4, has been shown to contribute to the generation of mammary carcinoma in mice.[6,11] Interestingly, recent reports have suggested that Notch1 is upregulated in Ras-transformed cells in which activation of Notch1 signaling is necessary to maintain the neoplastic phenotype.[18] Notch activation that causes human neoplasms has been shown to result not only from the truncation, based on the genetic aberration, but also Notch ligand stimulation,[18,19] suggesting that Notch activation without its own genetic abnormalities could be frequently involved in tumorigenesis.[20,21]

Despite rapidly accumulating information about the Notch signaling system, little is known about the mechanism through which excess Notch signaling triggers cellular transformation. One of the clues to this issue is the fact that Notch serves as an adaptor for molecules involved in transcriptional machinery, among which we focus on p300,[22] one of the most common transcriptional coactivator proteins.

The p300 protein interacts with molecules functioning in multiple signaling pathways. Transforming growth factor-β (TGF-β) also uses p300 through activated Smad3.[23,24] TGF-β inhibits proliferation of a wide range of cells including epithelial, endothelial and hematopoietic cells. It plays an important role in controlling tumor development, and its signaling constitutes one of the tumor-suppressor pathways.[25–27] Smads are a class of proteins that function as intracellular signaling effectors for the TGF-β surperfamily, which includes TGF-β, activins and bone morphogenetic proteins (BMP).[28,29] Smad2 and Smad3 are directly phosphorylated by the type I TGF-β receptor in response to TGF-β, leading to formation of heteromeric complexes with Smad4, and are then translocated into the nucleus where they bind to the TGF-β-responsive regulatory sequences, either directly through the Smad-binding elements or in conjugation with other sequence-specific DNA-binding proteins.[30–32] It is suggested that p300 forms the bridge between the Smad complex and the transcriptional apparatus.

Here we show that constitutively active Notch1, consisting of the intracellular domain alone (ICN1), inhibits the antiproliferative activity of TGF-β via the sequestration of p300 from Smad3. We propose that conferring resistance to TGF-β signaling may, in part, be attributed to a mechanism of Notch-induced neoplastic transformation.

Materials and Methods

Plasmids. Expression vectors for Smad2-Flag, Smad3-Flag and Smad4-hemagglutinin A (Smad4-HA) were described previously (pCMV5/Smad2-Flag, pCMV5/Smad3-Flag and pCMV5/Smad4-HA).[33,34] p3TP-Lux, pcDNA3/TβRI(TD)-HA, pcDNA3/6Xmyc-Smad3 and pcDEF3/p300-Flag were kindly provided by K. Miyazono (University of Tokyo, Japan). pcDNA3/myc-ICN1 (amino acids 1747–2531 of mouse Notch1), pME18Sneo/myc-ICN, pTracerCMV/ICN1-Flag, pTracerCMV/ICN2-Flag and pTracerCMV/ICN3-Flag were described previously.[35,36] The C-terminal deletion constructs,

[4]To whom correspondence should be addressed. E-mail: schiba-tky@umin.ac.jp

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pcDNA3/myc-RAMICΔC (1747–2193) and pcDNA3/myc-RAM/ANK (1747–2097), were made by digestion of pcDNA3/myc-ICN1 with *Eco*RI and *Xba*I (RAMICΔC), and with *Eco*RI and *Eco*RV (RAM/ANK), respectively. The ΔEP in-frame deletion of ICN1 was constructed by digesting pcDNA3/myc-ICN1 with *Pvu*II and *Eco*RI, and religating the plasmid. The EP mutant construct corresponds to the 2102LDE/AAA2104 mutation in the EP domain of ICN1 and was made by using an *in vitro* mutagenesis system (Stratagene). The TP-1-Luc reporter plasmid pGa981–6 was a gift from L. Strobl and U. Zimber-Strobl (GSF Institute for Clinical Molecular Biology, Germany). pEF-BOSneo-RBP-J (R218H) was kindly provided by T. Honjo (Kyoto University, Japan).

Cell culture and establishment of stable clones. HepG2, Mv1Lu, C2C12 and COS-7 cells were cultured at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). CHO(r) cells were maintained in alpha-minimal essential medium containing 10% FCS. CaSki cells were cultured in RPMI 1640 containing 10% FCS. To generate stable Mv1Lu clones overexpressing ICN1, myc-ICN1 subcloned into the pME18Sneo vector was transfected using SuperFect (Qiagen) according to the manufacturer's instructions. These cells were selected in medium containing G418 (800 μg/mL). G418-resistant clones were screened for expression of myc-ICN1 by Western blotting. Two independent clones with comparable expression levels were used in further assays.

Growth inhibition assay. The stable clones derived from Mv1Lu cells were plated in duplicate at a density of 5×10^3 per well in 96-well culture plates. After 12 h, cells were treated with increasing concentrations of TGF-β1 (R and D Systems) for 48 h. During the last 4 h, the cells were labeled with 2.5 μCi/mL [^{3}H]thymidine (Amersham Pharmacia Biotech). Thereafter, the incorporation of [^{3}H]thymidine was determined by liquid scintillation counting.

For RNA interference, CaSki cells were seeded at a density of 5×10^3 per well in a 96-well culture plate 24 h after transfection with small interfering RNA (siRNA). At 12 h after seeding the cells were treated with increasing concentrations of TGF-β for 48 h, after which time the [^{3}H]thymidine incorporation assay was carried as described above.

RNA interference. The siRNA against human Notch1, 5′-AAGGUGUCUUCCAGAUCCUGA-3′, was produced by Qiagen-Xeragon (Germantown). A non-silencing siRNA, 5′-AAUUCUCCGAACGUGUCACGU-3′ (Qiagen-Xeragon), was used as a control. At 24 h after CaSki cells (1×10^5 per well) were seeded in six-well plates, the cells were transfected with siRNA at a final concentration of 50 nM using Oligofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions. The cells were lysed 48 h after transfection and were subjected to Western blotting or used for the thymidine incorporation assay as described above.

Luciferase assay. For analysis of luciferase activities, HepG2 cells were seeded in 12-well culture plates at a density of 4×10^4 per well. Cells were then transfected 12 h after seeding with various amounts of effector plasmids, together with the reporter plasmids, using SuperFect (Qiagen). As an internal control of transfection efficiency, a plasmid expressing β-galactosidase was cotransfected. The cells were harvested 48 h after transfection and assayed for luciferase activity. The data were normalized to β-galactosidase activity. Cells were treated with 1 ng/mL TGF-β1 for 48 h before harvesting.

Coimmunoprecipitation and Western blotting. COS-7 cells transiently transfected with the constructs were washed and lysed in TNE buffer (10 mM Tris-HCl [pH 7.8], 150 mM NaCl, 1 mM ethylenediaminetetracetic acid, 0.5% Nonidet P-40, 0.1% aprotinin, 1 mM phenylmethylsulfonylfluoride, 12.5 mM β-glycerophosphate, 1 mM Na_3VO_4 and protease inhibitor cocktail [Sigma; 1/50 volume]). For immunoprecipitation, total cell extracts were incubated with anti-p300 monoclonal antibody (Upstate Biotechnology) for 4 h at 4°C. The samples were then incubated with protein G Sepharose (Pharmacia Biotech) for 15 min at 4°C. Immunoprecipitates were washed five times with the TNE buffer, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Western blotting with anti-myc antibody (9E10). Western blotting to detect intramembranously cleaved Notch1 was carried out using anticleaved Notch1 (Val 1744) antibody (Cell Signaling Technology).

Results

Active Notch1 inhibits the antiproliferative effects of TGF-β. To determine whether constitutively active Notch1 could affect the antiproliferative effects of TGF-β, we established several Mv1Lu cell lines that express ICN1 stably (N1-1 and N1-4), along with control clones (M-1 and M-2) (Fig. 1a). We carried out [^{3}H]thymidine-incorporation assays in the presence of various concentrations of TGF-β. Results are expressed as percentages relative to values obtained from control cultures in the absence of TGF-β. For example, at 1 ng/mL TGF-β, the relative [^{3}H]thymidine incorporation by M-1 and M-2 was $6.5 \pm 1.0\%$ and $5.9 \pm 0.6\%$, respectively, while that by N1-1 and N1-4 was $21.6 \pm 0.2\%$ and $28.6 \pm 0.7\%$, respectively (Fig. 1b). These results demonstrate that the growth of the mock clones was effectively inhibited by TGF-β, whereas the Mv1Lu clones that overexpress ICN1 showed reduced responsiveness to TGF-β.

Knockdown of active Notch1 expression by siRNA restores the antiproliferative effects of TGF-β. To further investigate the role of active Notch1 on the antiproliferative effects of TGF-β, we used a human cervical carcinoma cell line, CaSki. This line of cells

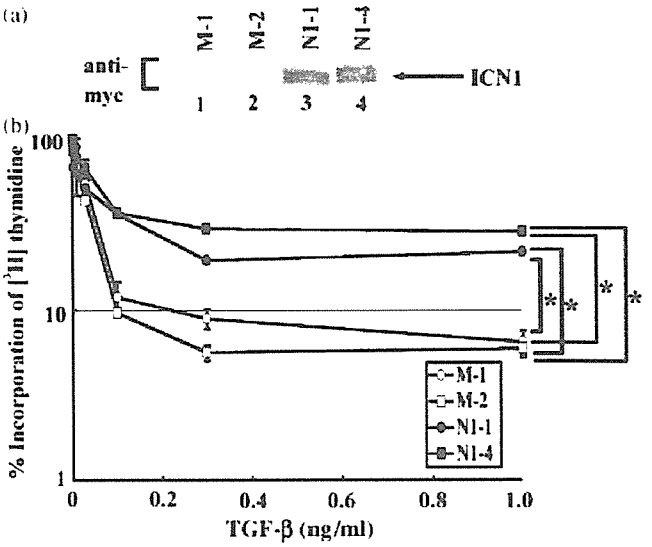

Fig. 1. Constitutive expression of active Notch1 (ICN1) in Mv1Lu cells overcomes transforming growth factor (TGF)-β-mediated growth inhibition. (a) Expression of ICN1 in stable Mv1Lu transfectants. Clones M-1 (lane 1) and M-2 (lane 2) are mock clones transfected with pME18Sneo empty vector followed by G418 selection. Clones N1-1 (lane 3) and N1-4 (lane 4) were established from cells transfected with pME18Sneo/myc-ICN1. The results of immunoblotting with anti-myc antibody are shown. (b) [^{3}H]thymidine incorporation into Mv1Lu clones was assayed in the presence of the indicated concentrations of TGF-β. Results are expressed as percentages relative to values obtained from control cultures in the absence of TGF-β. *$P < 0.01$.

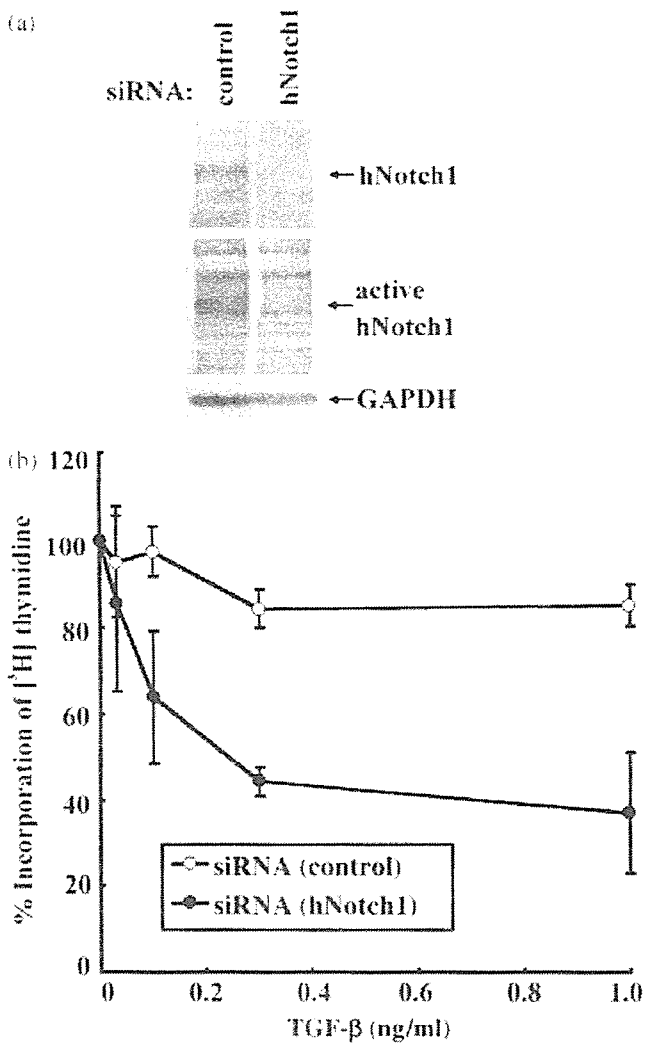

Fig. 2. Suppression of active Notch1 expression by small interfering RNA (siRNA) results in recovered responsiveness to transforming growth factor (TGF)-β. (a) Suppression of human Notch1 protein expression by siRNA. CaSki cells, spontaneously expressing truncated Notch1 protein, were transfected with control or human Notch1 siRNA, followed by immunoblotting. The upper panel, with antihuman Notch1 antibody (bTAN20); the middle panel, with anticleaved human Notch1 (Val 1744) antibody, specific to intramembranously truncated human Notch1; the lower panel, with anti-glyceraldehyde-3-phosphate dehydrogenase antibody as loading control. (b) [³H]Thymidine incorporation into CaSki cells, which had been transfected with control or human Notch1 (hNotch1) siRNA, was assayed in the presence of the indicated concentrations of TGF-β. Results are expressed as percentages relative to values obtained from control cultures in the absence of TGF-β.

has been shown to endogenously overexpress Notch1, leading to spontaneous activation of Notch1 (Fig. 2a).[18] Thus, this cell line could be useful for an approach based on siRNA. We transfected CaSki cells with siRNA targeted to human Notch1, which specifically and efficiently abrogated Notch1 protein expression (Fig. 2a). By using fluorescein-labeled control siRNA, we confirmed that siRNA could be introduced into CaSki cells with almost 100% efficiency (data not shown). Interestingly, an activated form of Notch1, which is detectable only by the antibody recognizing intramembranously cleaved Notch1, was undetectable if this siRNA was introduced (Fig. 2a). We found that wild-type and control siRNA-introduced CaSki cells had poor responsiveness to TGF-β, but after repression of active human Notch1 by siRNA. CaSki cells responded to TGF-β (Fig. 2b). Taken together, these results indicate that active Notch1 functions in CaSki cells to maintain their unresponsiveness to TGF-β.

Transcriptional responses mediated by TGF-β or Smad overexpression are suppressed by Notch signaling. Next, we examined the effects of ICN1 on TGF-β-mediated transcriptional responses in HepG2 cells with reporter assays using p3TP-Lux, a TGF-β-responsive reporter plasmid. Fold increase in the luciferase activity triggered by TGF-β was repressed to 20–30% when ICN1 was introduced (Fig. 3a).

To further investigate whether ligand-induced Notch signaling also represses TGF-β signaling, we used the C2C12 cell line, which is responsive to both Notch ligand[37] and TGF-β.[38] To stimulate cells with the Notch ligand Delta1, C2C12 cells were cocultivated with irradiated CHO(r) cells expressing full-length Delta1 (CHO-fD1).[39] The increase in luciferase activity of p3TP-Lux in the presence of TGF-β was repressed by Delta1 stimulation (Fig. 3b), suggesting that ligand-stimulated Notch signaling can antagonize TGF-β signaling.

We then examined whether ICN1 inhibits the transcriptional responses induced by Smad overexpression. Transcriptional activation of p3TP-Lux induced in HepG2 cells either by Smad3 alone or a combination of Smad2 or Smad3 with Smad4 was also suppressed by cotransfection with ICN1, in a dose-dependent manner (Fig. 3c,d). Similar repression was observed when we used other Smad-responsive reporter plasmids: p800neo-Luc, which contains the natural PAI-1 promoter, and p15P113-Luc, which contains the p15 promoter (Fig. 3e,f).

In vertebrates, Notch proteins comprise a family of four transmembrane receptors (Notch1 through Notch4).[1] To examine whether constitutively active Notch proteins other than ICN1 also inhibit TGF-β/Smad signaling, we compared the effect of ICN2 and ICN3 with that of ICN1 on the TGF-β-induced activation of the p3TP reporter. We found that ICN2 and ICN3 suppress TGF-β-induced transcriptional activation just as ICN1 did (Fig. 3g), indicating that suppression of TGF-β signaling is common to the constitutively active Notch proteins.

Overexpression of p300 partially overcomes the inhibitory effect of ICN1 on Smad3-mediated transactivation. Recent studies have indicated that both Smad proteins and ICN1 bind the general transcriptional coactivator p300 to mediate their transcriptional activities.[22–24] It is known that the binding regions of the p300 protein for the partner signaling molecules are variable and that both Smads and ICN1 bind the C-terminal region of p300. This information prompted us to examine whether p300 is involved in the Notch-mediated blockade of TGF-β/Smad signaling. When p300 was exogenously introduced into HepG2 cells, we observed that the ICN1-mediated suppression of Smad3-induced p3TP-Lux transactivation was reversed in a manner dependent on the dose of introduced p300 (Fig. 4).

These observations suggest that the availability of p300 by Smad3 may be limited and reduced when ICN1 is introduced. Therefore, we speculated that p300 may be sequestered from Smad3 by ICN1 when these molecules coexist. To see whether there is a reciprocal sequestration, we investigated whether ICN1-induced transcriptional activation of the TP-1 promoter, which is well characterized as a target of Notch signaling, is suppressed by the TGF-β/Smad activation. We observed a positive, although less remarkable, reciprocal repression of the ICN1-induced TP1 transactivation by overexpression of Smad3 and Smad4 (data not shown).

ICN1 mutants defective in p300 binding fail to repress the Smad-dependent transcriptional activation. Notch1 interacts with p300 through the 'EP domain' located at the C-terminal flanking region of the ankyrin repeats (ANK).[22] To further demonstrate that p300 is involved in the ICN1-mediated repression of TGF-β/Smad-induced transactivation, we used ICN1 mutants either

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