disease-associated mutant of *CIAS1* in THP-1 cells resulted in rapid necrosis-like cell death in a cathepsin B–dependent manner.[27] The caspase-1 inhibitor YVAD-fmk failed to inhibit LPS-induced monocyte cell death, while it effectively suppressed LPS-induced IL-1β production. Nigericin, a potassium ionophore, induces not only caspase-1–dependent IL-1β/IL-18 release but also rapid necrosis in LPS-primed THP-1 cells.[45] Interestingly, as we observed, the cathepsin B inhibitor CA074-Me inhibited the nigericin-induced necrosis while the caspase-1 inhibitor YVAD-cmk did not.[45] This indicates that a common pathway inducing cathepsin B-dependent necrosis in monocytes exists. Cross-talk between the LPS-TLR4 signaling pathway and the cryopyrin inflammasome to cause monocyte cell death is another possibility.

It was recently reported that the cytoplasmic receptor Ipaf recognizes bacterial flagellin, and induces rapid necrosis of *Salmonella*-infected macrophages.[46,47] It has been proposed that cryopyrin functions as a pattern-recognition receptor[48-50] that mediates inflammation; thus, it is possible that cryopyrin-induced rapid necrotic cell death and subsequent release of various cellular components facilitates local inflammation and prevents intracellular bacterial proliferation. Additional experiments are needed to clarify the mechanism of monocyte cell death observed in *CIAS1*-mutant cells in response to LPS.

While LPS-induced monocyte cell death seemed to be a specific property of *CIAS1* disease-associated mutant cells, the clinical and physiologic relevance of this biologic activity is unknown. The primary etiology of CAPS is considered to be excessive IL-1β production by constitutively activated inflammasomes. Although this hypothesis is supported by the fact that the autoinflammatory symptoms of the syndrome are successfully treated with IL-1β–targeted therapy,[10,29,33-35] it remains unclear whether the unique articular and cartilage manifestations of CAPS can also be attributed to IL-1β overproduction. Histologic analysis of the growth cartilage of CINCA syndrome patients revealed necrosis and disorganized proliferation of chondrocytes, and focal calcification, while infiltration of inflammatory cells was not described.[51] Feldmann et al speculated that the characteristic growth cartilage burst and epiphyseal overgrowth observed among CINCA patients might be due to dysregulated apoptosis of chondrocytes, which express a high amount of *CIAS1*.[4] One possibility is that certain stimuli, probably other than LPS, cause destructive necrosis of chondrocytes, rather than apoptosis, resulting in a loss of regularity of growth cartilage and subsequent bizarre joint destruction. We observed that LPS induces monocyte cell death independently of anti-IL-1β therapy status; thus, careful observation of anakinra-treated patients will provide a more precise understanding of the involvement of mutant *CIAS1*-mediated cell death in CAPS symptoms.

While the strategy we used in the current study was good at detecting single nucleotide substitutions, we cannot exclude the possibility that there were other types of genetic abnormalities present that were not detected, such as mis-splicing and noncoding region mutations. However, it is also possible that mosaicism, with an unequal distribution of mutant cells, is prominent in nonhematopoietic cells or tissues, such as the skin or central nervous system. An analysis of nonhematopoietic tissues may therefore be necessary before concluding that the *CIAS1* mutation is not responsible for the disease symptoms. Importantly, because diagnosis of CAPS is primarily based on clinical symptoms, a reassessment of the patients' histories and a physical re-evaluation is necessary before reestablishing the disease entity of *CIAS1*-unrelated patients.

In summary, we found that monocytes bearing mutations in *CIAS1* rapidly undergo necrosis-like cell death when treated with LPS, enabling us to diagnose *CIAS1* mutation-negative patients as *CIAS1* mosaic patients. Our investigation revealed that for a majority of CAPS patients without detectable *CIAS1* mutations by ordinary genomic sequencing, disease development may be attributable to low-level mosaicism. Not all *CIAS1* mutation-negative patients have *CIAS1* mosaicism, presenting the opportunity to uncover genes other than *CIAS1* as causative genes for CAPS. Our findings also raise the possibility that low-level mosaicism in other hereditary autoinflammatory syndromes may play a role in disease development, in the absence of detectable gene mutations.

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Authorship

Contribution: M.S. performed research and wrote the paper. R.N. and N.K. designed the research, wrote the paper, and analyzed data. A.F. and H. Tanizaki performed research. K.T., T. Imagawa, T. Iehara, H. Takada, T.M., H. Tanaka, H.K., K.K., and S.K. treated the patients and analyzed data. I.O. and T.Y. performed research and discussed results. S.A. wrote the paper and discussed results. T.H., Y.M., and T.N designed the research.

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Prospective multicenter trial comparing repeated immunosuppressive therapy with stem-cell transplantation from an alternative donor as second-line treatment for children with severe and very severe aplastic anemia

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We conducted a prospective multicenter study to compare the efficacy of repeated immunosuppressive therapy (IST) with stem-cell transplantation (SCT) from an alternative donor in children with acquired aplastic anemia (AA) who failed to respond to an initial course of IST. Patients with severe (n = 86) and very severe disease (n = 119) received initial IST consisting of antithymocyte globulin (ATG) and cyclosporine. Sixty patients failed to respond to IST after 6 months from the initial IST and were eligible for second-line treatment. Among them, 21 patients lacking suitable donors received a second course of IST. Three patients developed an anaphylactoid reaction to ATG and could not complete the second IST. A trilineage response was seen in only 2 of 18 (11%) evaluable patients after 6 months. Thirty-one patients received SCT from an alternative donor. At 5 years from the initiation of second-line therapy, the estimated failure-free survival (FFS), defined as survival with response, was 83.9% (± 16.1%, SD) in the SCT group compared with 9.5% (± 9.0%) in the IST group ($P = .001$). These results suggest that SCT from an alternative donor offers a better chance of FFS than a second IST in patients not responding to an initial IST. (Blood. 2008;111: 1054-1059)

Introduction

Acquired aplastic anemia (AA) is a heterogeneous disorder characterized by pancytopenia of peripheral blood and hypocellular marrow. Currently 2 effective treatments are available for this disorder: hematopoietic stem cell transplantation (SCT) and immunosuppressive therapy (IST). There are several reports comparing bone marrow transplantation (BMT) and IST as first-line treatment for AA.[1-4] These studies indicate that allogeneic BMT from an HLA-matched sibling donor is the treatment of choice for young patients. IST consisting of antithymocyte globulin (ATG) and cyclosporine (CyA) with or without granulocyte-colony stimulating factor (G-CSF) has been successfully used for patients with AA who lack an HLA-matched sibling donor or who are not eligible for SCT. Several reports indicate that 2- to 5-year survival following IST is between 60% and 90%.[5-7] We reported results of a multicenter trial of IST for children younger than 18 years with AA (AA-92 trial).[8] In the AA-92 trial, 119 children with newly diagnosed AA were enrolled, and the response rate at 6 months was 71%, with the probability of survival at 4 years greater than 90%. However, approximately 30% of the patients did not respond to an initial course of IST. Moreover, a significant proportion of patients subsequently relapsed and required second-line therapy.[9] The optimal treatment for such patients has not been established.

A repeated course of IST has been used for patients who fail to respond to, or who have relapsed after an initial course of, IST. Tichelli et al reported the results of a Basel study that consisted of repeated courses of IST, using ATG from the same species (horse) for nonresponders.[10] In their study, repeated IST was well tolerated and the response rate was 63%. An Italian group reported the results of repeated IST using ATG from different species (horse to rabbit), where the response rate was also high.[11] Investigators at the National Institutes of Health (NIH) recently reported the results of retreatment with rabbit ATG and CyA in 22 patients refractory to horse ATG and CyA. Contrary to the reports from Europe, the overall response rate was only 27% and no patients achieved complete response.[12]

SCT from an alternative donor has also been used as salvage therapy for patients not responding to IST because recent progress

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in the management of patients who undergo SCT, and better selection of donors by DNA typing of HLA loci, has improved the outcome for these patients.[13,14] However, no prospective study has been performed to date comparing repeated IST versus SCT from an alternative donor as second-line therapy. Therefore, we conducted a prospective multicenter trial to compare these 2 treatment options for pediatric patients with severe and very severe AA who had failed to respond to initial IST.

Methods

Patients

This multicenter study was designed by the Japan Childhood Aplastic Anemia Study Group and involved 79 hospitals in Japan. The eligibility criteria were as follows: age younger than 18 years, diagnosis less than 180 days before registration, no specific prior treatment for AA, and severe to very severe disease. The definition of disease severity was determined according to currently used criteria.[15] The disease was considered severe if at least 2 of the following were noted: a neutrophil count less than 0.5×10^9/L, a platelet count less than 20×10^9/L, and a reticulocyte count less than 20×10^9/L with hypocellular bone marrow. AA was considered very severe if the criteria for severe disease were fulfilled and the neutrophil count was less than 0.2×10^9/L. Patients were excluded if they had congenital AA. Patients were screened for paroxysmal nocturnal hemoglobinuria (PNH) by flow cytometry using anti-CD55 and anti-CD59 antibodies. Bone marrow cytogenetic studies were performed in all patients. Allogeneic SCT was recommended for patients with severe or very severe disease who had an HLA-matched sibling: these patients were not included in AA-97 study.

Treatment protocol

Patients with very severe disease were treated with IST, which consisted of horse ATG (Lymphoglobulin; IMTIX-SANGSTAT, Lyon, France) 15 mg/kg per day on days 1 through 5; CyA 6 mg/kg per day from day 1 until at least day 180, with subsequent adjustment according to whole blood CyA concentration between 100 and 200 ng/mL; methylprednisolone (MePred) 2 mg/kg per day for 5 days, with subsequent halving of the dose every week until discontinuation on day 28 for prophylaxis of allergic reaction of ATG; and G-CSF (Filgrastim, Kirin, Tokyo, Japan) 400 $\mu g/m^2$ per day from day 1, with responding patients (neutrophil count $> 10^9$/L) receiving the same dose 3 times a week for 60 days (ATG/CyA/MePred/G-CSF). Patients with severe disease were given the same treatment regimen, with the exception that G-CSF was not given unless severe infection was documented (ATG/CyA/MePred).

The hematologic response was evaluated at 6 months after the initiation of therapy. A complete response (CR) was defined for all patients as a neutrophil count more than 1.5×10^9/L, a platelet count more than 100×10^9/L, and a hemoglobin level more than 11.0 g/dL.[8] A partial response (PR) was defined as a neutrophil count more than 0.5×10^9/L, a platelet count more than 20×10^9/L, a hemoglobin level more than 80 g/L (8.0 g/dL) and no requirement of blood transfusions. Patients with very severe or severe disease who failed to respond to initial IST underwent SCT if they had a serologically HLA-matched unrelated donor, HLA-one antigen mismatched family donor, or HLA-matched or HLA-one antigen mismatched unrelated cord blood donor at the time of evaluation. Those lacking a suitable donor received a second course of IST. A second course of IST consisted of the same regimen (horse ATG/CyA/MePred) used in the initial treatment of each patient. To reduce the risk of an anaphylactoid reaction to treatment with horse ATG, patients were initially given a 100-fold diluted dose of ATG as a test dose. An antihistamine was administered to all patients receiving a second course of IST to suppress allergic reactions.

The recommended conditioning regimen for SCT from an alternative donor consisted of cyclophosphamide (CY, 120 mg/kg), rabbit ATG (Thymoglobulin, IMTIX-SANGSTAT, 10 mg/kg), and total body irradiation (TBI, 10 Gy) or CY (3000 mg/m^2), rabbit ATG (10 mg/kg), fludarabine (100 mg/m^2), and local field irradiation (3 Gy).[16,17] Prophylaxis against graft versus host disease (GVHD) consisted of a combination of CyA (3mg/kg per day) or tacrolimus (0.02mg/kg per day) plus short-term methotrexate. CyA dose were adjusted to maintain whole blood concentration of 100 to 200 ng/mL and tacrolimus dose 5 to 10 ng/mL, respectively.

Informed written consent was obtained from all patients or their parents in accordance with the Declaration of Helsinki. The study was approved by the ethics committee of each participating hospital. The list of participating hospitals can be found in Document S1, (available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Table 1. Pretreatment characteristics

	SAA	VSAA
Registered	86	119
Evaluable	84	117
Sex (M/F)	48/36	65/52
Median age, y (range)	8 (0-17)	9 (0-15)
Cause of AA		
Idiopathic	73	91
Hepatitis	8	24
Viral infection	1	2
Drug	2	0
Median days from diagnosis to treatment (range)	13 (1-94)	19 (1-179)

SAA indicates severe aplastic anemia; VSAA, very severe aplastic anemia

Statistical analysis

The primary end point of this study was failure-free survival (FFS) after second-line therapy, which was defined as survival with response. Death, no response by 6 months, disease progression requiring clinical intervention, or relapse were considered treatment failures.[18] Overall survival and FFS were analyzed using the Kaplan-Meier method. Differences between the 2 arms of the study were evaluated by the log-rank test. P less than .05 was considered statistically significant.

Results

Patient characteristics

From October 1997 to April 2004, 205 patients with newly diagnosed severe (n = 86) and very severe AA (n = 119) were enrolled in the AA-97 study (Table 1). An interim analysis was performed in April 2005. Four patients were excluded from further analysis for the following reasons: IST without ATG (2 patients) or stem cell transplantation within 4 months of diagnosis (2 patients). Two patients without any granulocytes were not treated with ATG because of severe infections; both of them died of fungal pneumonia within 2 months of diagnosis. Both patients who underwent SCT within 4 months of diagnosis died of graft rejection or cardiac toxicity to the preconditioning regimen. There were 2 further deaths within 6 months of patient registration: hemolysis of unknown cause and aspiration pneumonia. None of the patients was diagnosed with PNH at the time of registration. Severe and very severe AA were associated with hepatitis in 32 patients, with other viral infection in 3 patients, and with medication use in 2 patients. The median days (range) from diagnosis to treatment of severe and very severe AA were 13 (1-94) days and 19 (1-179) days, respectively (Table 1).

Trilineage hematologic response

At 3 months after the initiation of therapy, 49 patients (58%) with severe AA and 46 patients (39%) with very severe AA had

Table 2. Response to treatment after initial treatment

	SAA	VSAA
3 months		
Evaluable	84	117
CR	9*	6†
PR	40*	40†
NR	35	71
Alive	34	71
Dead	1	0
6 months		
Evaluable	83	115
CR	17‡	20§
PR	38‡	63§
NR	28	32
Alive	27	31
Dead	1	1

Data are numbers (%) of responders.
NR indicates no response.
*These classes combined were 58% to the total number.
†These classes combined were 39% to the total number.
‡These classes combined were 66% to the total number.
§These classes combined were 72% to the total number

responded to the initial course of IST (Table 2). By 6 months, 55 patients (66%) with severe AA and 83 patients (72%) with very severe AA had evidence of a trilineage response and had become transfusion-independent. Three patients died between 3 and 6 months. Overall, of 198 evaluable patients receiving an initial course of IST, 37 patients (19%) had a complete response and 101 patients (51%) showed a partial response, for an overall response rate of 70% after 6 months. Sixty patients (30%), 28 (34%) with severe AA and 32 (28%) with very severe AA, did not attain CR or PR status at 6 months, and were therefore eligible for second-line therapy (Fig 1).

Repeated IST versus SCT as second-line therapy

Figure 1 shows the outcome of 201 patients with treatment assigned. Twenty-one patients lacking a suitable donor at the time of evaluation were assigned to receive a second course of IST. Three of these patients developed an anaphylactoid reaction to ATG and thus could not complete their second course of treatment. Anaphylactoid reactions were not observed during the first course of IST in these 3 patients. These patients were subsequently treated with corticosteroids, which rapidly resolved their symptoms. Among them, 1 patient died from complications of severe pancytopenia and 2 patients are alive with a late hematologic response.

Table 3. Characteristics of 52 patients who underwent second-line therapy

	SAA	VSAA
Patients	21	31
Sex, M/F	14/7	14/17
Median age, y (range)	9 (2-17)	8 (0-17)
Cause of AA		
Idiopathic	17	29
Hepatitis	4	2
Severity of disease		
SAA	7	21
VSAA	14	10
Median months from diagnosis to second-line therapy (range)	7 (5-25)	8 (5-20)

Data are numbers except where indicated.

Thirty-one patients received SCT from an alternative donor as follows: BMT from an HLA-matched unrelated donor (UBMT; n = 25), cord blood transplantation from an unrelated donor (UCBT; n = 2), and BMT from an HLA-mismatched family donor (n = 4). Twenty patients were conditioned with a CY, ATG, and TBI regimen and 4 received CY, Flu, ATG, and local field irradiation. Others received other types of conditioning regimen. Methotrexate and CyA were given for the prevention of GVHD in 5 patients. Tacrolimus was used instead of CyA in other patients. Five patients who had transformed to myelodysplastic syndrome (MDS) and 3 patients who were searching for an alternative donor still were excluded from the analysis.

In all, 52 patients were evaluated for response to second-line therapy. Characteristics of both groups are shown in Table 3. The median interval between the first course of IST and a second-line treatment was 7 months for the IST group and 8 months for the SCT group. At 6 months after the initiation of second IST, a trilineage response was seen in only 2 of 18 evaluable patients (11%). Among 16 nonresponders, 8 patients received UBMT as a third-line therapy and all of them are alive. They could not find a suitable donor at 6 months after initial therapy and received a second IST, but failed to respond.

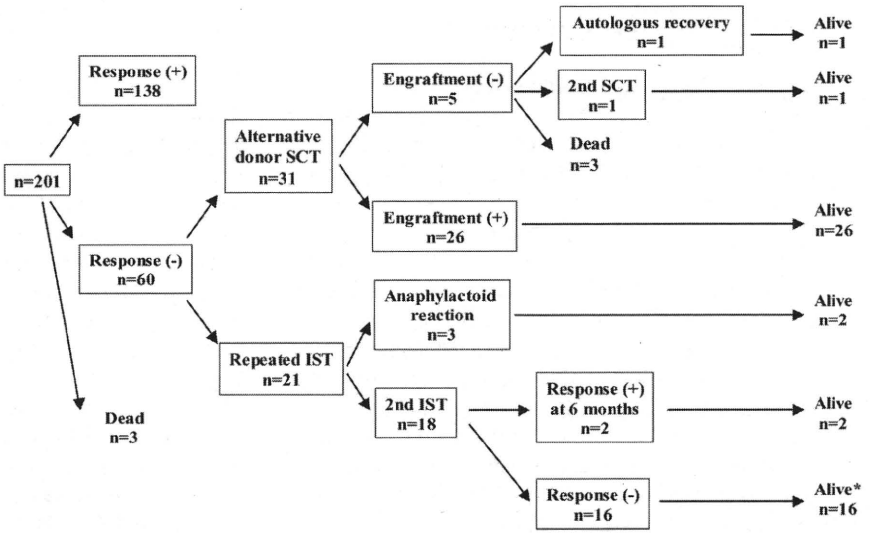

Figure 1. Overall outcome of 201 patients assigned to second-line therapy. *Among 16 patients who failed to respond to second IST, 8 patients received SCT and were alive. Four of the remaining 8 patients attained a late hematologic response and were alive. The other 4 patients were alive without response.

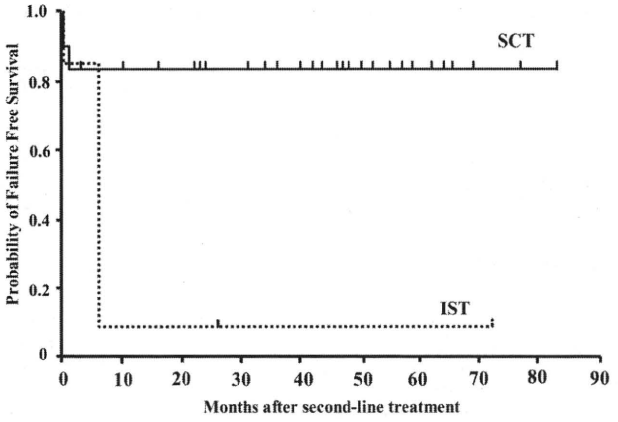

Figure 2. Actuarial probability of failure-free survival after second-line treatments with immunosuppressive therapy (n = 21) or stem-cell transplantation from an alternative donor (n = 31). FFS is defined as survival with response. Death, nonresponse by 6 months, disease progression requiring a second-line therapies, and relapse were considered as treatment failure.

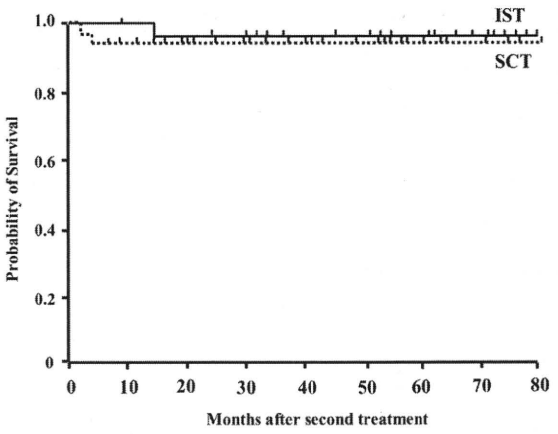

Figure 3. Actuarial probability of survival after second-line treatments with immunosuppressive therapy (n = 21) or stem cell transplantation from an alternative donor (n = 31).

Marrow donors included HLA-one antigen mismatched unrelated donor (n = 3) and HLA serologically 6/6 matched unrelated donor (n = 5). Four patients attained late response and another 4 patients are alive with regular blood transfusions. Overall, 20 of 21 patients are alive with a median follow-up period of 66 months from the start of second IST (range: 9-80 months).

In the SCT group, 5 patients did not engraft. Bacterial or fungal infections resulted in the death of 2 patients at an early phase of SCT. One patient who received UCBT had recovery of autologous bone marrow function and is alive 68 months after the transplant. One patient transplanted from an HLA-mismatched sibling had a successful second transplant from an unrelated donor. Another patient who failed to engraft after UBMT was rescued by second transplant from an HLA-2 antigen mismatched mother. Blood count normalized in the remaining 26 patients and they are all alive. Four evaluable patients developed grade II to IV acute GVHD, and chronic GVHD was observed in 4 patients. Twenty-nine of 31 patients are alive with a median follow up period of 35 months from the alternative donor transplantation (range: 4-83 months). The probability of FFS was calculated after excluding deaths and patients failing to respond to a second-line treatment by 6 months and requiring further treatment, that is, including only patients who were alive with hematologic response. The estimated FFS at 5 years from the beginning of second-line therapy was 83.9% (± 16.1% SD) in the SCT group compared with 9.5% (± 9.0%) in the IST group (P = .001) (Fig 2). The overall survival rate was not different between the IST group (95.2 ± 6.7%) and the SCT group (93.5 ± 4.2%) after second-line treatment (Fig 3).

Cytogenetic analysis and clonal disease

At the time of diagnosis, a clonal cytogenetic abnormality (monosomy 7, trisomy 8) was detected in 2 patients, who had morphologically typical AA. The disappearance of monosomy 7 was observed in 1 patient,[19] but trisomy 8 remained for 52 months after IST in another patient. New clonal cytogenetic abnormalities appeared in 10 patients after IST: monosomy 7 (5 patients), trisomy 8 (2 patients), trisomy 8 and del(7) (1 patient), monosomy X (1 patient), and t (3;3)(q21;q26) (1 patient). Eight patients underwent SCT from alternative donors and 6 of them are still alive.

Stem-cell transplantation

SCT was attempted in 52 patients in whom the initial IST failed (n = 31), the second IST failed (n = 8), who had relapse after initial response (n = 5), or who developed MDS and leukemia (n = 8). Alternative donors included unrelated bone marrow donors (n = 40), HLA-mismatched family donors (n = 6), and unrelated cord blood donors (n = 6). Five patients died: 3 received UCBT and 2 received UBMT. Causes of death were bacterial or fungal infections (n = 3), relapse of leukemia (n = 1), and venooclussive disease (n = 1).

Survival

We analyzed the actuarial survival of 201 enrolled patients according to the severity of their disease. The actuarial survival of all enrolled patients was 94.5% (± 1.7%) with a median follow-up period of 48 months (range: 12-90 months). The actuarial survival was 92.6% (± 2.8%) in the 117 patients with very severe AA and 96.8% (± 2.1%) in the 84 patients with severe AA. There were 6 deaths in the very severe AA group and 3 in the severe AA group. The causes of death were SCT-related toxicities (n = 5), MDS/acute myelogenous leukemia (AML) (n = 1), bacteremia (n = 1), hemolysis of unknown causes (n = 1), and aspiration pneumonia (n = 1).

Discussion

The introduction of intensive IST with ATG and CyA has dramatically improved the outcome of patients with severe and very severe AA.[5-8] However, 30% to 40% of patients still fail to respond to IST and require second-line therapy. The treatment options for patients not responding to IST include further treatment with immunosuppressive agents or SCT from an alternative donor. At present, however, there is no consensus as to the best therapy for these patients. Recent studies have reported a high response rate and a favorable outcome after repeated ATG therapy in these patients, suggesting that SCT from an alternative donor should perhaps be considered third-line therapy.[10,11] However, the majority of patients in these studies were adults. Because the outcome after alternative donor transplantation is better in children than in adults,[20] the treatment choice may be different in children from in adults. Our prospective study showed that SCT from an alternative donor is superior to the repeated IST for FFS. At 6 months, the response rate to a second course of IST was only 11% (2/18), which increased to 33% (6/18) at

12 months, much lower than figures reported by others. The overall response was 63% in the Basel[10] and 77% in the Italian[11] studies. In addition, none of our patients achieved CR, whereas CR was achieved in 42% of patients in the Basel,[10] and 30% in the Italian study.[11] In the recent study from the National Institutes of Health, the overall response rate was 30%, although no one achieved a CR.[12] The reasons for the discrepancy in the response rates among these studies are not known. However, there are a number of differences between our study (AA-97) and the others. First, our study group consisted of only pediatric patients, whereas other studies included both pediatric and adult patients. The median age of the patients was 9, 15, 18, and 31 years, in the AA-97, Basel, Italian, and National Institutes of Health studies, respectively. Until now, there have been no reports of repeated IST restricted to children with AA.

In the majority of patients with acquired AA, bone marrow failure is believed to result from immunologically mediated destruction of the hematopoietic progenitor cells.[21] Whereas in some patients, a single course of ATG is not sufficient to achieve the degree of immunosuppression required to restore bone marrow function, necessitating further ATG therapy, the results of our study may indicate that pediatric patients are more susceptible than adult patients to the intensive IST currently used and that a single course of IST is adequate to discern their response to these immunosuppressive agents. Our results may also suggest that the efficacy of any immunosuppressive therapy for children with AA should be evaluated separately from adult patients.[22]

In the Italian study,[11] the assessment of response to the first course of IST was carried out at 120 days and some patients received a second course of treatment as early as 2 months after initiation of immunosuppressive therapy. In our previous study (AA-92), we observed no further patient response to an initial course of IST after 6 months, thus making the time of assessment at 6 months.[8] The timing of the evaluation of response to an initial course of IST is an important factor in determining the need for further treatment, making it difficult to compare the response rates to a second course of IST between our study and other studies.

In the AA-97 study, 31 severe and very severe patients who did not respond to immunosuppressive therapy received SCT from an alternative donor. Twenty-nine of these patients are alive with their bone marrow function restored. Importantly, all 26 engrafted patients are alive without failure. Of 2 patients who received UCBT, 1 died of fungal infection before engraftment, and the other reconstituted autologous bone marrow function. In a recent analysis of a large series of UCBT from the New York Blood Center, only 8 of 19 patients with severe AA engrafted after UCBT. The cohort of AA patients was among the group with the highest incidence of transplant-related mortality.[23] Because of discouraging results in the early period, we thereafter recommend that UCBT not be used as a second-line therapy. In contrast, in our study, results after BMT from an unrelated donor were excellent. Twenty-four of the 25 patients are alive and well. The National Marrow Donor Program in the United States reported on the results of UBMT for IST-resistant AA patients.[14] Fifty-one of 131 patients (39%) were alive at 11 to 94 months (median: 36 months) after transplantation. The major causes of death were graft failure and treatment-related events including GVHD and infections. Fifty-five patients were matched with donors using both serology and allele-level DRB1 typing; these patients had a survival rate of 56%. In a recent report from the Japan Marrow Donor Program, the overall survival rate for AA patients receiving HLA-matched unrelated BMT was 56%, with 81% survival in patients younger than 15 years and 32% survival in patients aged 16 and older.[20] Therefore, younger patients clearly have a survival advantage after UBMT. Similarly, in our AA-92 study, 13 of 15 patients who failed IST and who were subsequently treated with UBMT are alive and well, with a median follow-up of 36 months.[8] The duration of FFS of these pediatric patients with AA appeared to plateau at 2 years after SCT. Recently, 2 novel transplant regimens were reported: one from the United States and another from Europe.[24,25] The first tested de-escalating doses of radiation from 6 Gy to 2 Gy. The best results were achieved with 2 Gy TBI. The European group designed a radiation-free preparative regimen consisted of fludarabine, cyclophosphamide, and ATG. The Japan Marrow Donor Program is now performing high-resolution HLA typing at the DNA level at loci A and B as well as DRB1. It is expected that more precise HLA matching between patient and donor will further improve the outcome for UBMT recipients.

On the other hand, IST appears to be associated with an increased risk of evolution of clonal diseases such as MDS and PNH.[26,27] In our previous study, 11 of 50 children with AA that were treated with IST developed MDS/AML. None of the 48 patients who underwent SCT developed a clonal disorder.[28] In the Basel study, clonal disease developed in 53% of patients who received multiple courses of IST.[10] In the current study, there were 5 patients (8.3%) with MDS among 60 patients in whom initial IST was not effective, whereas no patient developed MDS/AML after SCT from an alternative donor treated as second-line therapy. This issue must be taken into consideration in the discussion of the appropriate second-line therapy for patients with AA.

Our study clearly demonstrates that SCT from an alternative donor provides a better chance of FFS than a second course of IST for children with AA who have failed to respond to an initial course of IST. Thus, we recommend SCT, and in particular BMT from an alternative donor, rather than a second course of IST as salvage therapy for these patients.

Authorship

Contribution: F.B., T.N., I.T., and S. K. designed the study. Y.K., H.Y., K.S., and S.K. analyzed results and wrote the manuscript. R.K., H.A., T.K., H.Y., M.T., H.M., A.O., A.M., Y. O., and S.O. enrolled the patients.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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LETTER TO THE EDITOR

Successful treatment of a patient with Klinefelter's syndrome complicated by mediastinal germ cell tumor and AML(M7)

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The association of mediastinal germ cell tumors (GCTs) and hematological malignancy has been documented for more than 20 years.[1,2] This rare association is known to be extremely refractory to modern therapy. Here we report the successful treatment of a boy with Klinefelter's syndrome complicated by this disease.

A 13-year-old boy with no significant past medical history was referred to our hospital due to persistent fever and nasal bleeding. Physical examination demonstrated extensive ecchymosis, hepatosplenomegaly and reduced breath sounds on the right chest. Chest X-ray showed an abnormal mass in the anterior mediastinum (Figure 1). The mass contained dense calcification, which was suggestive of a teratoma. Complete blood count showed a white blood cell count of 8.0×10^9/l with 21% blasts, hemoglobin of 9.8 g per 100 ml, and platelets of 33×10^9/l. Blood chemistry showed a normal level of aspartate and alanine aminotransferase and elevated lactate dehydrogenase level of 2253 IU/l. Serum α-fetoprotein (AFP) and human chorionic gonadotropin (hCG) were elevated to 995 ng/ml (normal <15 ng/ml) and 15.3 mIU/ml (normal <0.5 mIU/ml), respectively. Bone marrow (BM) aspiration demonstrated 26.4% leukemic blast cells that were morphologically acute myeloid leukemia (AML (M7)). Fluorescence-activated cell sorting analysis showed that these blasts were positive for CD41a and CD61. Computed tomography and magnetic resonance imaging of chest showed a tumor measuring $11 \times 8 \times 7$ cm in the anterior right mediastinum, which demonstrated a complex pattern of signals. Fine needle biopsy of the anterior mediastinal mass was subsequently performed. Histological examination demonstrated neuroepithelial components with mature teratomatous tissue, indicating germ cell tumor. The patient had the characteristics of Klinefelter's syndrome including long extremities and gynecomastia. Chromosome analysis demonstrated 47, XXY, confirming the diagnosis. According to the cases reported to date, almost all cases of mediastinal GCT complicated by hematological malignancy have died of leukemia. Therefore, we decided to treat the leukemia first. The patient was treated with the AML99 protocol,[3] which consists of induction chemotherapy (cytarabine, etoposide and mitoxantrone) and five courses of intensive chemotherapy (high-dose cytarabine, etoposide, idarubicine and mitoxantrone). After induction chemotherapy, the patient achieved complete remission (CR). We added cisplatinum to each course of intensive chemotherapy to augment the antitumor effect against GCT. Intensive chemotherapy plus cisplatinum was administered every 4 weeks without significant toxicity other than severe pancytopenia. During chemotherapy, the size of the mediastinal tumor remained almost the same, while serum AFP and the hCG levels declined to near-normal levels. About 7 months after the initiation of chemotherapy, the patient underwent cord blood transplantation (CBT), because there were no potential donors in either his relatives or the Japan Marrow Donor Program. The cord blood was serologically full matched, genetically mismatched in one of the HLA-DRB1 locus only in the graft-versus-host disease (GvHD) direction. The conditioning regimen consisted of 16 mg/m2 busulfan, 120 mg/m2 cyclophosphamide and 50 mg/kg etoposide. The transplanted cell dose was 2.25×10^7/kg ($CD34^+$ cells; 6×10^4/kg). For GvHD prophylaxis, cyclosporine (3 mg/kg per day) and short-term methotrexate (10 mg/kg on day 1 and 7 mg/kg on days 3 and 6) were administered. Mild engraftment syndrome was observed on day 12, which responded well to 1 mg/kg prednisolone. Engraftment of neutrophils was observed on day 20 ($>5 \times 10^8$/l), platelets on day 35 ($>20 \times 10^9$/l) and red blood cells on day 35 (reticulocytes >10‰). Grade I GvHD (skin, stage 2) was observed about 4 weeks after transplantation, which responded well to the standard steroid therapy. BM aspiration at 1 month demonstrated complete chimerism of donor origin. On day 56, surgical removal of the mediastinal tumor was performed. The tumor measured $16 \times 14 \times 6$ cm, consisted of fibrous tissue intermingled with mucous cysts, brain-like structure, teeth and hair. The tumor did not invade the adjacent organs but a small area of lung tissue was firmly attached to it. It was completely resected with adequate margins. Microscopic examination demonstrated atypical cells compatible with adenocarcinoma or choroid plexus carcinoma as well as mature tissues such as squamous epithelium, cartilage, bone, gut, brain and thyroid gland. These findings are compatible with a diagnosis of malignant teratoma with nongerminal malignant tumor (carcinoma). Although the patient developed respiratory failure due to pneumonia shortly after surgery, he recovered fully with antibiotic therapy and mechanical ventilation. He has now remained free from disease $3\frac{1}{2}$ years after the onset of disease.

Cases of mediastinal GCT associated with hematological abnormalities have been reported since the 1980s. In 1990, Nichols *et al.*[4] described 16 cases of GCT, including two cases, which seemed to have Klinefelter's syndrome. All patients were relatively young males and had nonseminomatous mediastinal germ cell tumors. AML (M7) and

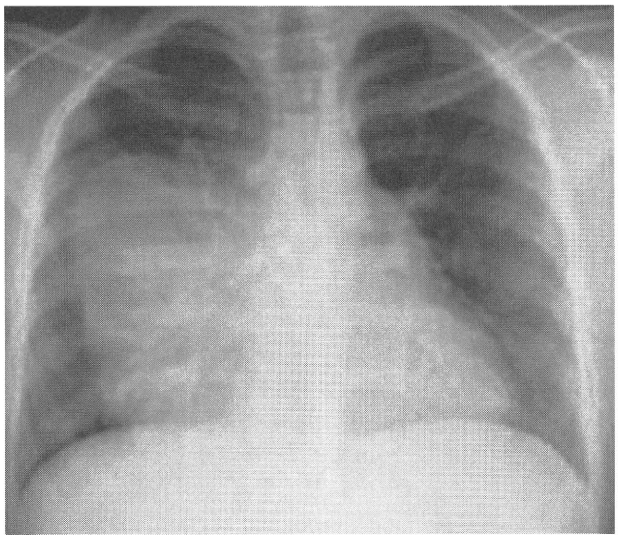

Figure 1 Chest roentgenogram on admission.

Table 1 Chromosomal analysis of germ cell tumor (GCT) and M7

Cells analyzed	*Karyotype*	*Frequency*
GCT	50, XY, +X, +21, +21, +22	17/20
M7	50, XY, +X, +1, −5, −19, +21, +22, +r1, +mar1	13/20
	50, idem, add(Y)(q12), −21,−r1, +mar2, +mar3	3/20

malignant histiocytosis were the most common hematological abnormalities. In one patient, i(12p) isochromosome was identified in both the GCT and leukemic blasts. Woodruff *et al.*[5] also identified i(12p) in both the mediastinal GCT and associated hematologic malignancy. Orazi *et al.*[6] reported hematopoietic precursors within the mediastinal GCT in patients with this association. With these observations, the hypothesis that the leukemic clone originates from GCTs has been suggested. Although histological examination did not demonstrate any hematopoiesis in GCT in our patient, chromosome analysis showed marked similarities between GCT cells and leukemic blasts (Table 1), which supports this hypothesis. Considering previous findings that patients with this syndrome have an extremely poor prognosis due to leukemia, we administered chemotherapy before surgical removal of the mediastinal cell tumor. Some reports argue against intensive consolidation therapy for AML.[7,8] However, in view of the fact that current protocols of major clinical trials for childhood AML contain consolidation therapy, and that four courses of bleomycin, etoposide and cisplatin are the standard therapy for extragonadal germ cell tumors,[9] we concluded that AML99, standard treatment for childhood AML in Japan, which involved the use of cisplatin, would be a reasonable combination. With this therapy, our patient achieved CR and stabilization of mediastinal GCT. The reports that allogeneic HSCT for childhood AML with a poor prognosis can be a powerful therapeutic modality[10,11] and the efficacy of CBT for childhood AML in the first CR in Japan is as good as unrelated BM transplantation convinced us to undertake CBT using well-matched cord blood. Also, importantly, our patient did not experience severe GvHD, making surgery uncomplicated.

In summary, we present a therapeutic strategy for the successful management of mediastinal GCTs associated with hematological malignancy. Hopefully, this experience will contribute to improving the prognosis of this intractable disease.

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Successful autologous peripheral blood stem cell transplantation with a double-conditioning regimen for recurrent hepatoblastoma after liver transplantation

Niwa A, Umeda K, Awaya T, Yui Y, Matsubara H, Hiramatsu H, Watanabe K-I, Adachi S, Itoh T, Uemoto S, Nakahata T. Successful autologous peripheral blood stem cell transplantation with a double-conditioning regimen for recurrent hepatoblastoma after liver transplantation.
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Abstract: A four-yr-old boy developed a solitary metastasis nine months after living-related liver transplantation for unresectable hepatoblastoma. After resection of the metastatic lesion, he received an auto-PBSCT with a double-conditioning regimen consisting of melphalan and thiotepa. Auto-PBSCT could be safely performed without any serious regimen-related toxicity or infection. However, transient cessation of tacrolimus during myelosuppression resulted in graft rejection of the liver just after hematological engraftment, but rejection was resolved by tacrolimus and methylprednisolone. The patient is alive and free from disease two yr after auto-PBSCT without any signs of graft rejection. High-dose chemotherapy using this conditioning regimen may be feasible for recurrent hepatoblastoma after liver transplantation in terms of safety and anti-tumor activity.

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Key words: hepatoblastoma – high-dose chemotherapy – double-conditioning regimen – liver transplantation

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Hepatoblastoma is a common malignant tumor in childhood. Combination of multidrug chemotherapy and surgical resection has improved the survival rates up to 70% (1, 2). Furthermore, LT has recently contributed to the elevation of cure rates for patients with an unresectable tumor. The prognosis of relapse cases after LT, however, remains dismal and the treatment of such cases has not been established (3, 4). Here, we report a case that underwent auto-PBSCT with high-dose chemotherapy for recurrent hepatoblastoma after LT and discuss the role of high-dose chemotherapy for such cases.

Abbreviations: AFP, alpha-fetoprotein; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; auto-PBSCT, autologous peripheral blood stem cell transplant; CBDCA, carboplatin; CDDP, cisplatin; CMV, cytomegalovirus; CT, computer tomography; EBV, Epstein–Barr virus; G-CSF, granulocyte-stimulating factor; GGT, gamma glutamyl transferase; HLA, human leukocyte antigen; IFO, ifosfamide; JPLT, Japanese Study Group for Pediatric Liver Tumor; LDH, lactate dehydrogenase; L-PAM, melpharan; LRLT, living-related liver transplantation; LT, liver transplantation; mPSL, methylprednisolone; PBSC, peripheral blood stem cell; TEPA, thiotepa; THP-ADR, tetrahydropyranyl-adriamycin; VOD, veno-occlusive disease.

Case report

A four-yr-old boy with an abdominal mass was diagnosed with embryonic hepatoblastoma by liver biopsy. At the time of diagnosis, the AFP level was 1 880 000 ng/mL. Abdominal CT showed that the tumor involved both lobes, which was categorized as pretreatment extent of disease system (PRETEXT) III (5). There was no metastatic disease detected by bone scintigraphy or chest CT scan. He was treated with two courses of CDDP 80 mg/m^2 on day one and THP-ADR 30 mg/m^2 on days one and two, and three courses of IFO 3 g/m^2 on days one and two, CBDCA 400 mg/m^2 on day three,

THP-ADR 30 mg/m^2 on days four and five, and etoposide (VP16) 100 mg/m^2 on days 1–5, according to the JPLT protocol (6), and with additional two courses of irinotecan (CPT-11) 20 mg/m^2 daily for five days. Even after those therapies, however, the size of the tumor did not change, and the AFP level remained high at 170 000 ng/mL.

At the age of four yr and eight months, the patient was transferred to our hospital for treatment of unresectable hepatoblastoma. He underwent LRLT from his mother and was treated with three courses of CPT-11 at 20 mg/m^2 daily for five days post-operatively. The AFP level normalized, and abdominal and chest CT scans showed no evidence of disease. He received tacrolimus orally and there was no sign of graft rejection.

Nine months after LRLT, the AFP levels increased to 68 ng/mL, and chest CT demonstrated a solitary tumor measuring 6.5 mm in the upper lobe of left lung. He underwent a wedge resection of left lung and histological examination of the tumor confirmed relapse of the disease. As the tumor is thought to be at least partially resistant to the standard chemotherapy used prior to LT, we planned to use L-PAM and TEPA with stem cell rescue, both of which had not been used and were expected to retain anticancer effect. Thereafter, PBSC containing 5.1×10^6 cells/kg CD34$^+$ cells were harvested after mobilization with nogitecan at 1 mg/m^2 daily for five days and G-CSF.

The clinical course of auto-PBSCT is shown in Fig. 1. For fear of a higher risk of severe regimen-related toxicity, the patient received a modified double-conditioning regimen (two cycles of drug combinations with a one-wk interval) that was originally reported by Hara et al. (7). The regimen consisted of 50 mg/m^2 L-PAM on days 11, 10, four, and three; TEPA of 150 mg/m^2 on days 11 and 10 and 200 mg/m^2 on days four and three. He also received ursodeoxycolic acid, low-molecular-weight heparin, antithrombin III for prophylaxis for VOD of the liver. Tacrolimus was discontinued just before the start of conditioning for fear of severe renal toxicity because of concomitant administration of L-PAM. PBSC containing 3.3×10^6/kg CD34$^+$ cells were infused, and G-CSF was started from day six until engraftment.

Hematological engraftment was prompt; absolute neutrophil counts reached more than 0.5×10^9/L on day 10; reticulocytes were more than 10‰ on day 14; platelet counts were more than 5.0×10^9/L on day 10. There were no severe regimen-related toxicities, such as mucositis, renal toxicity, or VOD, and no severe infections.

Pyrexia occurred on day four, and was diagnosed as clinical engraftment syndrome. The patient was treated with mPSL intravenously for four days starting on day five, which improved the symptom. Although tacrolimus was resumed on day 11, marked elevation of serum AST (477 IU/L), ALT (452 IU/L), LDH (654 IU/L), ALP (831 IU/L), and GGT (187 IU/L) levels occurred on the same day. Abdominal CT scan showed normal findings and there was no reactivation of CMV or EBV. He was diagnosed as having mild acute cellular rejection based on histological examination of a liver biopsy specimen (Fig. 2). mPSL was given intravenously for 10 days starting on day 11, and liver dysfunction rapidly improved. The patient was alive and free from disease two yr after the auto-PBSCT with no signs of graft rejection.

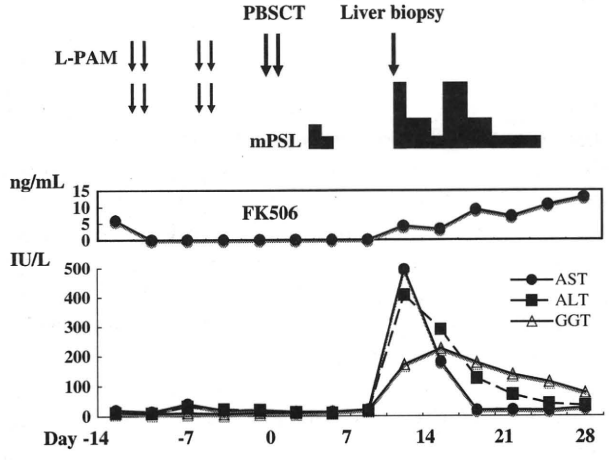

Fig. 1. The clinical course of auto-PBSCT after LT.

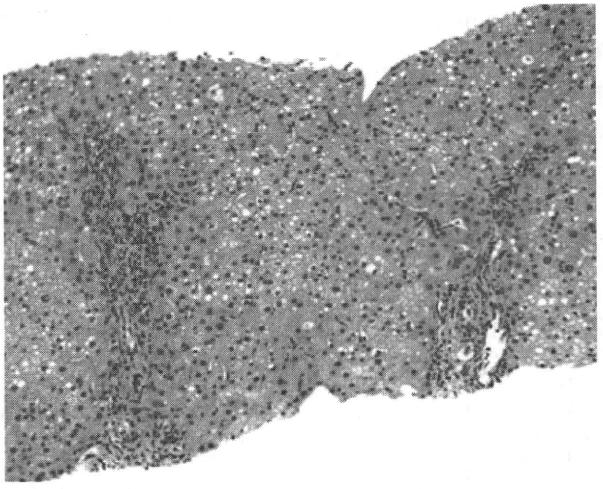

Fig. 2. Histology of liver biopsy on day 11 showing a predominantly lymphocyte infiltration in portal tracts, which was diagnosed as acute cellular graft rejection.

Discussion

Chemotherapy has improved the prognosis of hepatoblastoma (1, 2). However, the outcome of chemoresistant cases remains extremely poor, and effective treatment for such cases has not been established. Although the role of high-dose chemotherapy in the treatment of hepatoblastoma remains controversial, there are some reports describing auto-PBSCT for hepatoblastoma (7–9). Our case underwent LRLT but a solitary lung metastasis developed. Although the metastatic lesion could be removed completely, the prognosis was thought to be poor, since previous reports indicated that most patients with relapse after LT died their disease (4). Moreover, these cases were thought to be resistant to CDDP, THP-ADR, IFO, CBDCA, and VP16 at standard doses, which are the key drugs to treat hepatoblastoma. Therefore, we used high-dose chemotherapy with stem cell rescue using two alkylating agents, L-PAM and TEPA, which had not been used in this case and are reported to have potent anti-tumor activity (7).

There has not been any report of successful auto-PBSCT after LT. Compared with conventional auto-PBSCT, it remains unknown whether the preconditioning regimen could be tolerated by patients with a transplanted liver. Double-conditioning regimen (two cycles of drug administration with a one-wk interval), a modification of the treatment reported by Hara et al. (7), was selected to reduce regimen-related toxicities to a minimum while retaining the anti-cancer effect. Indeed, there was no serious regimen-related toxicity in this case. There was no severe infection either, despite using immunosuppressive agents to prevent graft rejection after LT. Although the follow-up period remains relatively short, high-dose chemotherapy with auto-PBSCT might be effective as a consolidation therapy after resection of a metastatic lesion. Thus, high-dose chemotherapy with stem cell rescue could be considered for cases with metastatic or relapsed tumors that were resistant to the standard chemotherapy.

Even in conventional auto-PBSCT, the combination of L-PAM and TEPA has been reported to cause severe renal toxicity (7). To reduce the risk of severe renal toxicity, the total dose of L-PAM was reduced from 280 mg/m^2, that was originally reported (7), to 200 mg/m^2. Furthermore, to gain sufficient time for the elimination of L-PAM, tacrolimus was discontinued from the beginning of the conditioning regimen until hematological engraftment. As a result, graft rejection did occur on day 11 although it had not previously been observed before auto-PBSCT. In cases undergoing allogeneic BMT from HLA-matched sibling donor after LT, graft rejection was not reported when immunosuppressive agents were transiently discontinued during administration of preconditioning drugs, then restarted from one day before transplantation (10, 11). Therefore, it is suggested that at the early stage of auto-PBSCT, only a few engrafted cells can cause graft rejection in the absence of immunosuppressive agents. Low dose of tacrolimus or steroids after conditioning might reduce the risk of graft rejection.

In conclusion, we successfully performed auto-PBSCT after the double-conditioning regimen with L-PAM and TEPA for recurrent hepatoblastoma after LT. If the patient is in good condition, the conditioning regimen with L-PAM and TEPA can be performed safely after LT, and possibly prevent relapse of hepatoblastoma that is refractory against standard chemotherapies. More sophisticated immunosuppressive therapy after conditioning will be required to prevent graft rejection.

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Generation of transplantable, functional satellite-like cells from mouse embryonic stem cells

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ABSTRACT **Satellite cells are myogenic stem cells responsible for the postnatal regeneration of skeletal muscle. Here we report the successful *in vitro* induction of Pax7-positive satellite-like cells from mouse embryonic stem (mES) cells. Embryoid bodies were generated from mES cells and cultured on Matrigel-coated dishes with Dulbecco's modified Eagle medium containing fetal bovine serum and horse serum. Pax7-positive satellite-like cells were enriched by fluorescence-activated cell sorting using a novel anti-satellite cell antibody, SM/C-2.6. SM/C-2.6-positive cells efficiently differentiate into skeletal muscle fibers both *in vitro* and *in vivo*. Furthermore, the cells demonstrate satellite cell characteristics such as extensive self-renewal capacity in subsequent muscle injury model, long-term engraftment up to 24 wk, and the ability to be secondarily transplanted with remarkably high engraftment efficiency compared to myoblast transplantation. This is the first report of transplantable, functional satellite-like cells derived from mES cells and will provide a foundation for new therapies for degenerative muscle disorders.—Chang, H., Yoshimoto, M., Umeda, K., Iwasa, T., Mizuno, Y., Fukada, S., Yamamoto, H., Motohashi, N., Yuko-Miyagoe-Suzuki, Takeda, S., Heike, T., Nakahata, T. Generation of transplantable, functional satellite-like cells from mouse embryonic stem cells. *FASEB J.* 23, 1907–1919 (2009)**

Key Words: long-term engraftment · secondary transplantation · high engraftment efficiency · self-renewal

DUCHENNE MUSCULAR DYSTROPHY (DMD; ref. 1) is a progressive, lethal muscular disorder (2) with no effective cure despite extensive research efforts. DMD results from mutations in the X-linked *dystrophin* gene (3). Dystrophin and its associated proteins function to link the intracellular actin cytoskeleton of muscle fibers to laminin in the extracellular matrix (4), thereby protecting myofibers from contraction-induced damage (5). Skeletal muscle fibers are continuously regenerated following exercise and injuries when satellite cells (6) are induced to differentiate into myoblasts that form myotubes and replace the damaged myofibers (7, 8). This muscular regeneration is observed at a much higher frequency in DMD patients (9). Continuous damage to myofibers and constant activation of resident satellite cells due to loss of dystrophin leads to the exhaustion of the satellite cells (10, 11), and the eventual depletion of satellite cells is primarily responsible for the onset of DMD symptoms.

Successful transplantation of normal satellite cells into the skeletal muscle of DMD patients may enable *in situ* production of normal muscle tissue and create a treatment option for this otherwise fatal disease. A recent report has shown that the transplantation of satellite cells collected from mouse muscle tissues can produce muscle fibers with normal dystrophin expression in mdx mice (12-14), a model mouse for DMD (15). This study suggests that stem cell transplantation may be a viable therapeutic approach for the treatment of DMD (16).

Satellite cells are monopotent stem cells that have the ability to self-renew and to differentiate into myoblasts and myotubes to maintain the integrity of skeletal muscle (17). Satellite cells lie dormant beneath the basal lamina and express transcription factors such as Pax3 (13, 18) and Pax7 (19). Pax7, a paired box transcription factor, is particularly important for satellite cell function. A recent study of *Pax7-null* mice revealed that Pax7 is essential for satellite cell formation (19) and that the *Pax7-null* mice exhibit a severe deficiency in muscle fibers at birth and premature mortality with complete depletion of the satellite cells. Surface markers such as M-cadherin and c-met (20) are also expressed by satellite cells. However, these markers are not specific to satellite cells because they are also expressed in the cerebellum (21) and by hepatocytes (22). To specifically identify quiescent satellite cells, a

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novel monoclonal antibody, SM/C-2.6, has recently been established (23). Satellite cells purified with this antibody regenerate muscle fibers on implantation into mdx mice (15).

The use of satellite cells for clinical therapies would require the establishment of a reliable source of these cells. Embryonic stem (ES) cells are totipotent stem cells that are able to differentiate into various types of somatic cells *in vitro*. While mouse embryonic stem (mES) cells can be readily induced to differentiate into muscle fibers (24, 25) and the myogenicity of human ES cells was recently validated (26), the induction of mES cells into functional satellite cells has not been reported. Here we have successfully induced mES cells to generate cells expressing Pax7 *in vitro* by forming embryoid bodies (EBs). These ES cell-derived (ES-derived) Pax7-positive cells can be enriched using the SM/C-2.6 antibody (23) and possess a great potential for generating mature skeletal muscle fibers both *in vitro* and *in vivo*. The Pax7-positive cells display a self-renewal ability that can repopulate Pax7-positive cells *in vivo* in the recipient muscles following an injury. Furthermore, these ES-derived Pax7-positive cells could engraft in the recipient muscle for long periods, up to 24 wk, and could also be serially transplanted. These results indicate that ES-derived Pax7-positive cells possess satellite cell characteristics. This is the first report of effective induction of functional satellite cells from mES cells, and these novel findings may provide a new therapeutic approach for treatment of DMD.

MATERIALS AND METHODS

Cell culture

D3 cells, mES cells (27) that ubiquitously express the *EGFP* gene under the *CAG* promoter (28) (a gift from Dr. Masaru Okabe, Osaka University, Osaka, Japan), were used in this study. ES cells were maintained on tissue culture dishes (Falcon) coated with 0.1% gelatin (Sigma, Oakville, CA, USA), in DMEM (Sigma) supplemented with 15% fetal bovine serum (FBS; Thermo Trace, Melbourne, Australia), 0.1 mM 2-mercaptoethanol (Nakalai Tesque, Japan), 0.1 mM nonessential amino acids (Invitrogen, Burlington, CA, USA), 1 mM sodium pyruvate (Sigma), penicillin/streptomycin (50 μg/mL), and 5000 U/ml leukemia inhibitory factor (Dainippon Pharmaceutical Co., Japan).

In vitro differentiation of ES cells into a muscle lineage

To induce EB formation, undifferentiated ES cells were cultured in hanging drops for 3 d at a density of 800 cells/20 μl of differentiation medium, which consisted of DMEM supplemented with penicillin/streptomycin, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptonethanol, 5% horse serum (HS), and 10% FBS. EBs were transferred to suspension cultures for an additional 3 d (d 3+3). Finally, the EBs were plated in differentiation medium in 48-well plates (Falcon) coated with Matrigel (BD Bioscience, Bedford, MA, USA). The medium was changed every 5 d.

Immunofluorescence and immunocytochemical analysis

Immunostaining of cultured cells and recipient mouse tissues were carried out as described previously (29). Briefly, the left tibialis anterior (LTA) muscle of the recipient mouse was fixed with 4% paraformaldehyde and cut into 6 μm cross sections using a cryostat, and samples were fixed for 5 min in 4% paraformaldehyde (PFA) in PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min. After incubation in 5% skim milk for 10 min at room temperature to block nonspecific antibody binding, cells were incubated for 12 h at 4°C with anti-mouse monoclonal antibodies. Antibodies used in this study were mouse anti-Pax7, which was biotinylated using a DSB-X Biotin Protein Labeling Kit (D20655; Molecular Probes, Eugene, OR, USA), mouse anti-Pax3 (MAB1675, MAB2457; R&D Systems, Minneapolis, MN, USA), rabbit anti-mouse Myf5 (sc-302; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-mouse M-cadherin (205610; Calbiochem, San Diego, CA, USA), mouse anti-myosin heavy chain (MHC; 18-0105; Zymed Laboratories, San Francisco, CA, USA; reacts with human, rabbit, rat, mouse, bovine, and pig skeletal MHC), mouse anti-mouse myogenin and mouse anti-mouse Myo-D1 (M3559, M3512; Dako, Carpinteria, CA, USA), monoclonal rabbit anti-mouse laminin (LB-1013; LSL, Tokyo, Japan), and mouse anti-mouse dystrophin (NCL-DYS2; Novocastra Laboratories, Newcastle-upon-Tyne, UK). Cy3-labeled antibodies to mouse or rabbit IgG, fluorescein isothiocyanate-labeled antibodies to mouse or rabbit IgG (715-005-150, 711-165-152; Jackson ImmunoResearch Laboratory, Bar Harbor, ME, USA), or Alexa 633-labeled goat anti-rabbit IgG (A21070; Invitrogen, Molecular Probes) were applied as secondary antibodies. Hoechst 33324 (H3570; Molecular Probes) was used for nuclear staining. The samples were examined with a fluorescence microscope (Olympus, Tokyo, Japan) or an AS-MDW system (Leica Microsystems, Wetzlar, Germany). Micrographs were obtained using an AxioCam (Carl Zeiss Vision, Hallbergmoos, Germany) or the AS-MDW system (Leica Microsystems). In sections of muscles transplanted with ES-derived satellite cells, the number of GFP-positive muscle fascicles and GFP/Pax7-double-positive cells were counted, per field, at ×100. More than 10 fields in each tissue sample were observed. To prevent nonspecific secondary antibody binding to Fc receptors, all immunostaining of frozen sections used the Vector® M.O.M™ Immunodetection Kit (BMK-2202; Vector Laboratories, Burlingame, CA, USA).

PCR analysis

Total RNA was isolated from cultured cells in 48-well plates, using TRIzol reagent (Invitrogen). The following specific primers were used for PCR:

Pax3, sense, 5′-AACACTGGCCCTCAGTGAGTTCTAT-3′, and antisense, 5′-ACTCAGGATGCCATCGATGCTGTG-3′; Pax7, sense, 5′-CATCCAGTGCTGGTACCCCACAG-3′, and antisense, 5′-CTGTGGATGTCACCTGCTTGAA-3′; Myf5, sense, 5′-GAGCTGCTGAGGGAACAGGTGG-3′, and antisense, 5′-GTTCTTTCGGGACCAGACAGGG-3′; MyoD, sense, 5′-AGGCTCTGCTGCGCGACCAG-3′, and antisense, 5′-TGCAGTCGATCTCTCAAAGC-3′; myogenin, sense, 5′-TGAGGGAGAAGCGCAGGCTCAAG-3′, and antisense, 5′-ATGCTGTCCACGATGGACGTAAGG-3′; M-cadherin, sense, 5′-CCACAAACGCCTCCCCTACCC-3′, and antisense, 5′-GTCGATGCTGAAGAACTCAGGGC-3′; C-met, sense, 5′-GAATGTCGTCCTACACGGCCAT-3′, and antisense, 5′-CACTACACAGTCAGGACACTGC-3′; GAPDH, sense, 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′, and antisense, 5′-TGTTGGGGGCCGAGTTGGGATA-3′. AmpliTaqGold (Applied

Biosystems, Foster City, CA, USA) was used for PCR amplification. The amplification program used was 35 cycles of 30 s at 94°C, 30 s at 64°C, and 40 s at 72°C, with a final incubation of 7 min at 72°C.

Flow cytometry and cell sorting

Cultured cells were incubated with enzyme-free Hank's-based Cell Dissociation Buffer (Invitrogen) for 30 min at 37°C and gently dissociated into single cells. The cells were then washed with PBS twice, probed with biotinylated-SM/C-2.6 (23) antibody for 15 min at room temperature, and stained with phycoerythrin-conjugated strepavidin (12-4312; eBioscience, San Diego, CA, USA) for 15 min at room temperature. Dead cells were excluded from the plots based on propidium iodide staining (Sigma), and SM/C-2.6-positive cells were collected using a FACS Vantage instrument (Becton Dickinson, San Jose, CA, USA). Sorted cells were plated (1×10^4 cells/well) with differentiation medium in 96-well plates (Falcon) coated with Matrigel (008504; BD Bioscience). The medium was changed every 5 d, and 7 d after plating the cultured cells were analyzed.

Intramuscular cell transplantation (primary transplantation)

Recipient mice were injected with 50 μl of 10 μM cardiotoxin (CTX; Latoxan, Valence, France) (30) in the LTA muscle 24 h before transplantation (31). CTX is a myotoxin that destroys myofibers, but not satellite cells, and leaves the basal lamina and microcirculation intact. Since proliferation of host myogenic cells may prevent the incorporation of transplanted cells, recipient mdx mice (15) received 8 cGy of systemic irradiation (32) 12 h before transplantation to block muscle repair by endogenous cells. An average of 4.53×10^4 ES-derived SM/C-2.6-positive or -negative cells were washed twice with 500 μl of PBS, resuspended in 20 μl of DMEM, and injected into the LTA muscle of recipient mdx mice using an allergy syringe (Becton Dickinson). Mdx mice, which are derived from the CL/B16 strain, were used as the recipient mice in all experiments. Similarly, D3 ES cells, which are derived from the 129X1/SvJ ES cells, were used in all experiments. The major histocompatibility complex (MHC) of mdx mouse and D3 cells are very similar, both possessing type *b* MHC H2 haplotypes. All animal-handling procedures followed the Guild for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the Guidelines of the Animal Research Committee of the Graduate School of Medicine, Kyoto University.

Secondary transplantation

The LTA muscles of recipient mice were collected 8 wk after the primary transplantation. The muscles were minced and digested into single cells with 0.5% collagenase type I (lot S4D7301; Worthington Biochemical Corp., Lakewood, NJ, USA). After washing with PBS and filtration through a 100 μm filter, Pax7-positive cells were sorted by FACS using the SM/C-2.6 antibody. SM/C-2.6-positive cells (200 cells/mouse) were injected into preinjured LTA muscles of secondary recipient mice. The LTA muscles were analyzed 8 wk after transplantation.

Isolation and immunostaining of single fibers

To detect muscle satellite cells attaching to single fibers with Pax7, muscle fibers from the LTA muscle of recipient mice were prepared essentially according to the method of Bischoff in Rosenblatt *et al.* (33). Briefly, dissected muscles were incubated in DMEM containing 0.5% type I collagenase (Worthington) at 37°C for 90 min. The tissue was then transferred to prewarmed DMEM containing 10% FBS. The tissue was gently dissociated into single fibers by trituration with a fire-polished wide-mouth Pasteur pipette. Fibers were transferred to a Matrigel-coated 60 mm culture dish (Falcon) and fixed in 4% PFA for 5 min at room temperature. Fibers were permeabilized with 0.1% Triton X-100 in PBS for 10 min, and nonspecific binding was blocked by incubation in 5% skim milk for 10 min at room temperature. Primary mouse monoclonal antibodies against mouse Pax7 were applied for 12 h at 4°C. Antibodies were detected using the secondary antibodies described above.

Statistics

Data are presented as means ± SD. For comparison of the numbers of MHC and Pax7-positive cells in the sorted SM/C-2.6-positive and -negative fractions and the numbers of GFP-positive muscle fascicles and GFP/Pax7-double-positive cells in reinjured and noninjured groups, the unpaired Student's t test was used, and a value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Myogenic lineage cells are effectively induced from mES cells *in vitro*

EBs were formed in hanging drop cultures for 3 d followed by an additional 3 d in suspension cultures (**Fig. 1*A***). These EBs were then plated onto Matrigel-coated 48-well plates in differentiation medium, which contained 5% HS. This culture method is a modified version of the classical ES cell differentiation method (25) and the skeletal muscle single fiber culture method (33). After plating, EBs quickly attached to the bottom of the coated dishes, and spindle-shaped fibers appeared surrounding the EBs by the seventh day of plating (d 3+3+7; Fig. 1*B*). As these spindle fibers grew, they began to fuse with each other, forming thick multinucleated fibers resembling skeletal myofibers (Fig. 1*C, D*). At the same time we observed spontaneous contractions by the fibers (Supplemental Videos 1 and 2), a trait commonly seen in cultured skeletal muscle fibers. Immunostaining showed that these fused fibers were positive for skeletal-muscle-specific MHC (Fig. 1*E*). Furthermore, cells expressing muscle regulatory factor (MRF) proteins, including Pax7 (Fig. 1*F*), Myf5 (Fig. 1*G*), MyoD (Fig. 1*H*), and myogenin (Fig. 1*I*) were observed. On d 3 + 3 + 14, the average number of MHC-positive wells was 73.6 ± 5.8% (n=144). In all the MHC-positive wells, cells expressing Pax7, an essential transcription factor in satellite cells, were also observed. Double staining for Pax7 and MyoD confirmed the existence of cells staining for Pax7 alone, indicating the presence of quiescent-state satellite cells (34) within the culture (Supplemental Fig. 1). Next, the time course of MRF expression was examined by RT-PCR (Fig. 1*J*). Expression of Pax3 and Pax7 both peaked on d 3 + 3 +

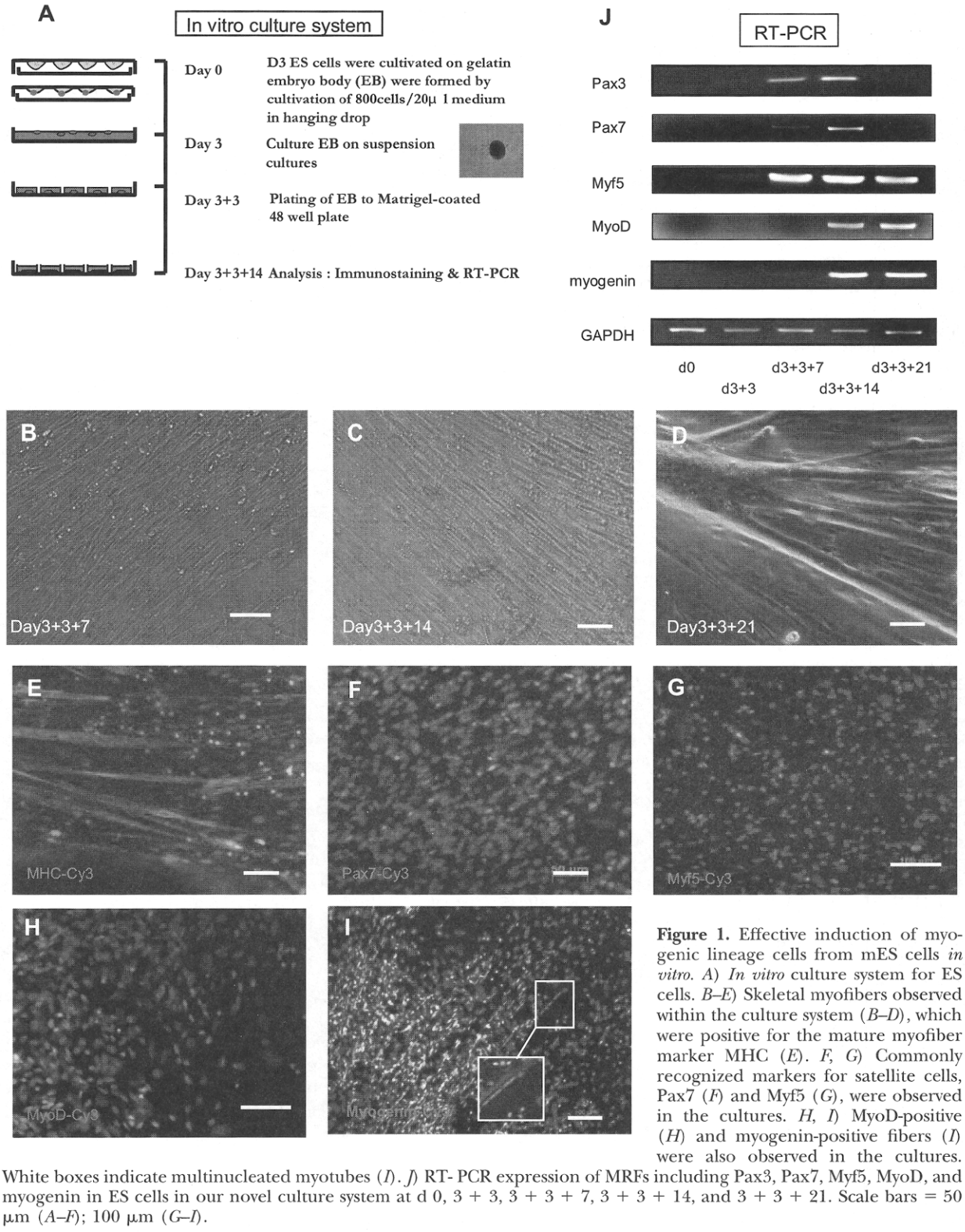

Figure 1. Effective induction of myogenic lineage cells from mES cells *in vitro*. *A*) *In vitro* culture system for ES cells. *B–E*) Skeletal myofibers observed within the culture system (*B–D*), which were positive for the mature myofiber marker MHC (*E*). *F, G*) Commonly recognized markers for satellite cells, Pax7 (*F*) and Myf5 (*G*), were observed in the cultures. *H, I*) MyoD-positive (*H*) and myogenin-positive fibers (*I*) were also observed in the cultures. White boxes indicate multinucleated myotubes (*I*). *J*) RT- PCR expression of MRFs including Pax3, Pax7, Myf5, MyoD, and myogenin in ES cells in our novel culture system at d 0, 3 + 3, 3 + 3 + 7, 3 + 3 + 14, and 3 + 3 + 21. Scale bars = 50 μm (*A–F*); 100 μm (*G–I*).

14, but Myf5, MyoD, and myogenin continued to be expressed after d 3 + 3 + 14.

Thus, using Matrigel plates and differentiation medium containing HS, myogenic lineages including Pax7-positive satellite-like cells were successfully induced from mES cells.

A novel antibody, SM/C-2.6, can enrich for Pax7-positive satellite-like cells derived from ES cells

To examine the characteristics of ES-derived Pax7-positive satellite-like cells, we needed to isolate these cells from the culture. Since Pax7 is a nuclear protein rather than a

surface marker, anti-Pax7 antibodies cannot be used for living cell separation by FACS. Therefore, a novel antibody, SM/C-2.6 (23), was used to detect satellite cells. SM/C-2.6 detects quiescent adult mouse satellite cells, as well as satellite cells in neonatal muscle tissue, as determined by immunostaining (Supplemental Fig. 2). RT-PCR confirmed that sorted SM/C-2.6-positive cells expressed Pax3, Pax7, Myf5, and c-met, whereas sorted SM/C-2.6-negative cells did not (Supplemental Fig. 3). Thus, the SM/C-2.6 antibody was shown to be useful for isolating living satellite cells by FACS.

We collected all the differentiated ES cells (1×10^6 cells) from cultures on d 3 + 3 + 14. FACS analysis using the SM/C-2.6 antibody showed that 15.7% of the cells were SM/C-2.6 positive (**Fig. 2*A***). RT-PCR analysis revealed that sorted SM/C-2.6-positive cells strongly expressed Pax3, Pax7, Myf5, c-met, and M-cadherin (Fig. 2*B*). Using a cytospin preparation of sorted SM/C-2.6-positive cells, we also confirmed the expression of M-cadherin (Fig. 2*C*) and Pax7 (Fig. 2*D;* 70.7±16.5% and 59.9±1.1% positive, respectively); only 2.3 ± 0.49% of the sorted SM/C-2.6-negative cells expressed M-cadherin, and 2.7 ± 0.1% expressed Pax7. Thus, the SM/C-2.6 antibody could enrich for satellite-like cells derived from mES cells *in vitro.*

ES-derived satellite-like cells have strong myogenic potential *in vitro*

To evaluate the myogenic potential of ES-derived SM/C-2.6-positive satellite-like cells *in vitro,* both SM/C-2.6-positive and -negative cells were sorted by FACS and plated in 96-well Matrigel-coated plates (see Fig. 4A). One week after cultivation, the number of muscle fibers in the wells was assessed. Although there were fibroblast-like and endothelium-like cells, MHC-positive fibers (787.3±123.7/well, 10.7±0.8% of the total cells per well, n=3) and Pax7-positive cells (222±81.4/well, 2.9±1.1% of the total cells per well, n=9) were observed in the SM/C-2.6-positive wells. In contrast, very few MHC-positive fibers (8.75±32.6/well, n=15; 0.12±0.46%) or Pax7-positive cells (2.6±2.0/well, n=8; 0.03±0.01%) were seen in the SM/C-2.6-negative wells

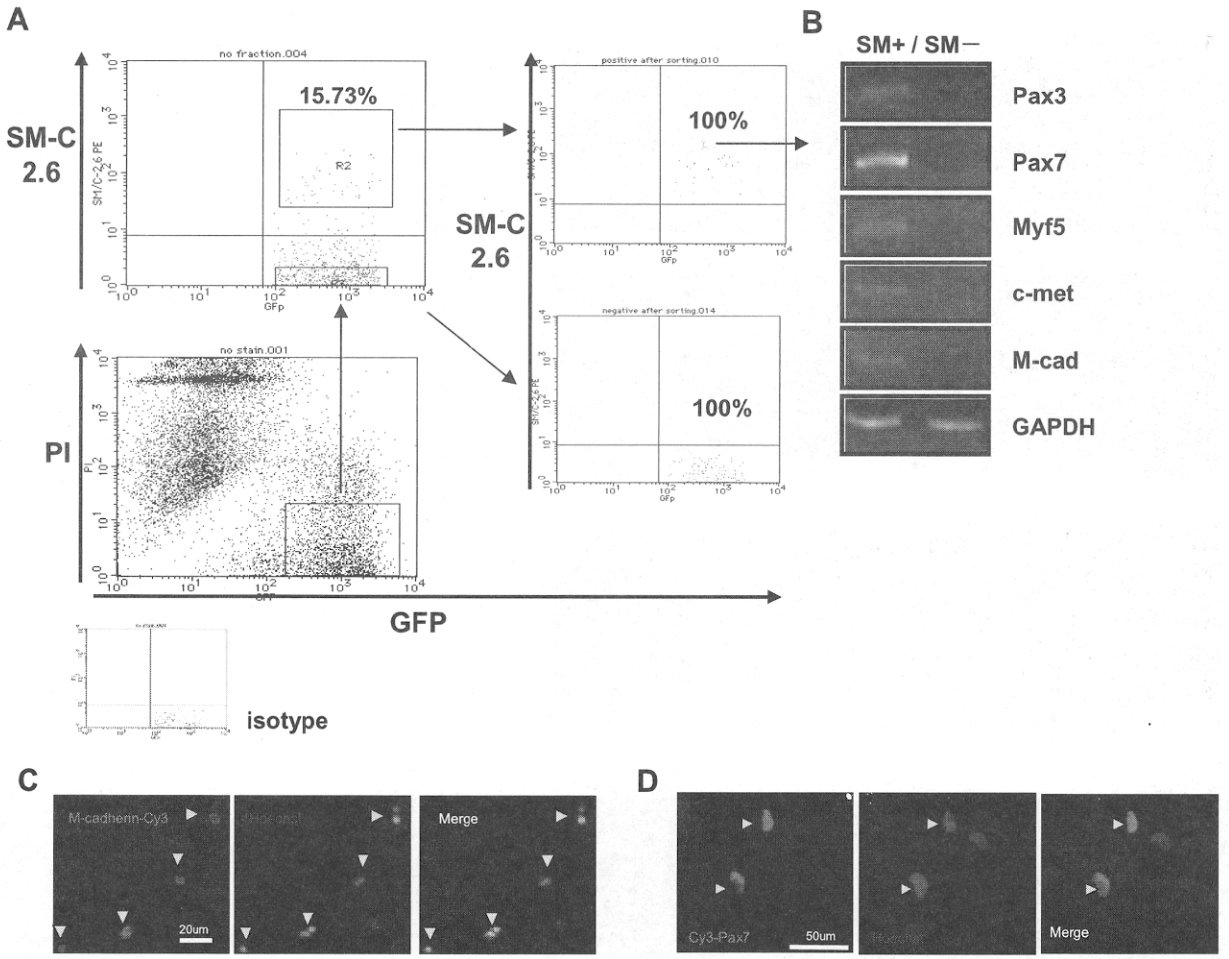

Figure 2. A novel antibody, SM/C-2.6, can enrich Pax7-positive satellite-like cells derived from ES cells. *A*) FACS data of cultured ES cells at d 3 + 3 + 14 indicate that 15.7% of total cultured cells are SM/C-2.6-positive cells. *B*) RT-PCR of the SM/C-2.6-positive fraction showed strong expression of Pax3, Pax7, Myf5, c-met, and M-cadherin. Immunostaining of a cytospin preparation of the sorted SM/C-2.6-positive cells showed that these cells were positive for M-cadherin (*C*), and Pax7 (*D*) (white arrowheads). Scale bars = 20 μm (*C*); 50 μm (*D*).

(both $P<0.05$; **Fig. 3**). Thus, ES-derived satellite-like cells isolated using the SM/C-2.6 antibody possess strong myogenic potential *in vitro.*

Damaged muscle can be repaired by transplantation of ES-derived satellite-like cells

To examine the myogenic potential of ES-derived satellite-like cells *in vivo,* SM/C-2.6-positive and -negative cells were transplanted into conditioned mdx mice (15). The LTA muscles of recipient mdx mice were preinjured with CTX (primary injury; ref. 30) 24 h prior to transplantation, and mice were exposed to 8 cGy of γ-irradiation (whole body) 12 h prior to transplantation (**Fig. 4*A***). GFP-positive ES cells were used as donor cells in this experiment. GFP^+ES-derived SM/C-2.6-positive and -negative cells were directly injected into the predamaged LTA muscles. The recipient mice were analyzed 3 wk post-transplantation. By fluorescence stereomicroscopy, GFP-positive tissues were clearly observed within the LTA muscles injected with SM/C-2.6-positive cells (Fig. 4*B* and **Table 1**). In contrast, no GFP-positive tissue was observed in muscles injected with SM/C-2.6-negative cells (Fig. 4*C*). These GFP-positive tissues were further confirmed by diaminobenzidine staining using anti-GFP and a peroxidase-conjugated secondary antibody (Supplemental Fig. 4) to exclude the possibility of autofluorescence of the muscle tissues. Immunostaining with anti-MHC confirmed that these GFP-positive tissues were mature skeletal myofibers (Fig. 4*D*). In addition, GFP/Pax7 double-positive cells were observed within the LTA muscles of the recipient mice (Fig. 4*E* and Supplemental Fig. 5) and in isolated single fibers (Fig. 4*F* and Table 1). The GFP-positive cells were also confirmed to be positive for other satellite cell markers such as Myf5 and M-cadherin (Supplemental Figs. 6 and 7). These GFP/Pax7-double-positive cells were located along the periphery of the muscle fascicle. With laminin immunostaining we verified that the location of the GFP-positive mononuclear cells was between the basal lamina and the muscle cell plasma membrane, a location consistent with the anatomical definition of satellite cells

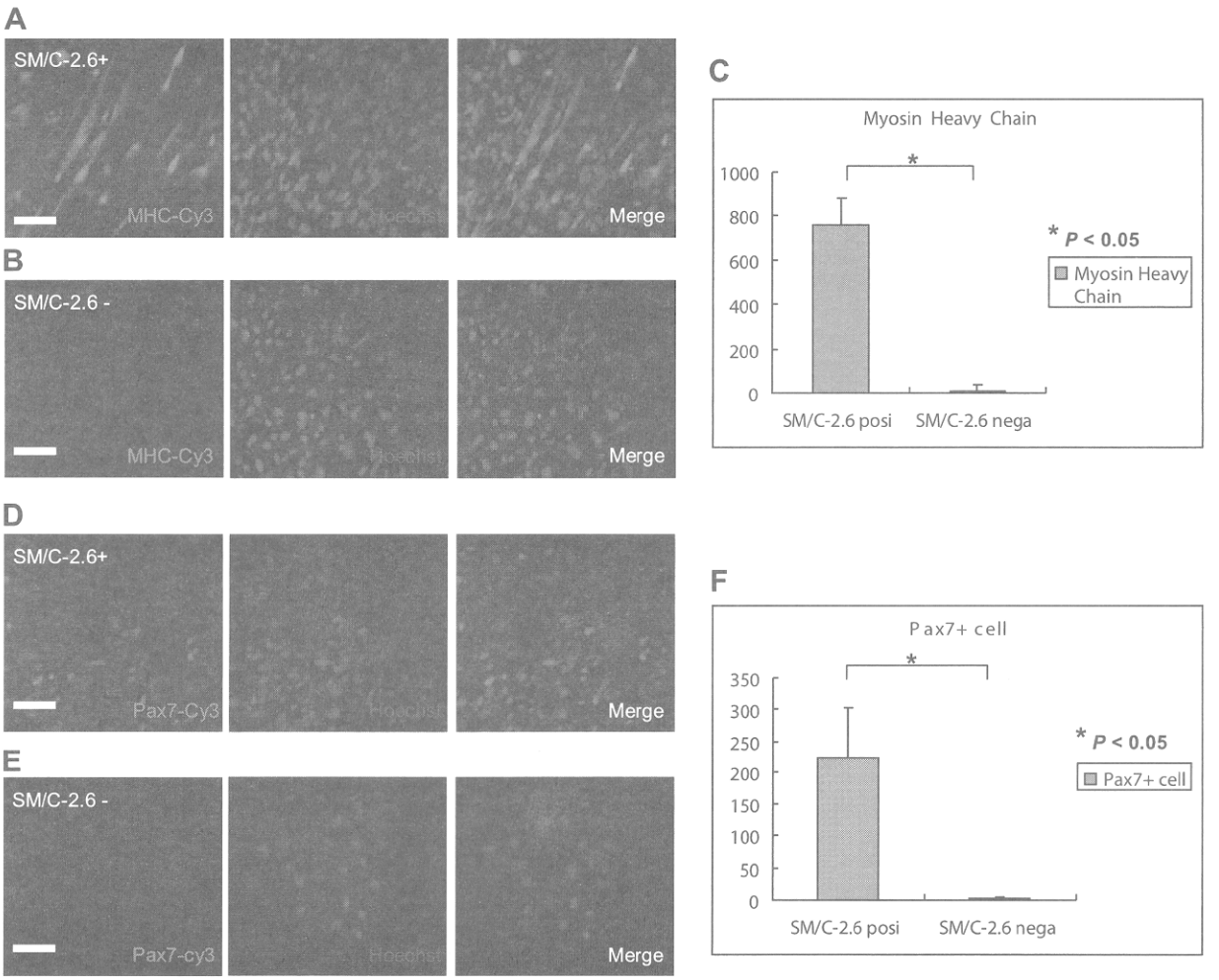

Figure 3. ES-derived satellite-like cells have strong myogenic potential *in vitro.* Immunostaining detected an abundant number of MHC-positive fibers and Pax7-positive cells in SM/C-2.6-positive cell culture (*A, D*) but not SM/C-2.6-negative cells (*B, E*) after 1 wk in culture. Scale bars = 50 μm. Significant differences were observed in the number of MHC-positive fibers and Pax7-positive cells per well between sorted SM/C-2.6-positive and -negative cell cultures (*C, F*).