Isolation of Germ Cells from Leukemia and Lymphoma Cells in a Human In vitro Model: Potential Clinical Application for Restoring Human Fertility after Anticancer Therapy

Kazutoshi Fujita, Akira Tsujimura, Yasushi Miyagawa, Hiroshi Kiuchi, Yasuhiro Matsuoka, Tetsuya Takao, Shingo Takada, Norio Nonomura, and Akihiko Okuyama

Department of Urology, Osaka University Graduate School of Medicine, Suita Osaka, Japan

Abstract

More than 70% of patients survive childhood cancer, but chemotherapy and radiation therapy may cause irreversible impairment of spermatogenesis. To treat infertility secondary to anticancer treatment for childhood cancer, we have developed a procedure to isolate germ cells from leukemic mice by fluorescence-activated cell sorting with two surface markers, and transplantation of isolated germ cells successfully restored fertility without inducing leukemia. In the present study, we analyzed human germ cells and human malignant cells, including five leukemia cell lines and three lymphoma cell lines, by fluorescence-activated cell sorting with antibodies against MHC class I and CD45. Testicular specimens were obtained from a patient who underwent surgery for testicular rupture. In the high forward scatter and low side scatter region, no malignant cells were found in the MHC class I-negative and CD45-negative fraction (the germ cell fraction), with the exception of K562 cells. A total of 39.2% of the germ cells were found in the germ cell fraction. A total of 1.43% of K562 cells were found in the germ cell fraction. Treatment with IFNγ induced the expression of MHC class I on K562 cells but not on germ cells and made it possible to isolate germ cells from K562 cells. In conclusion, we isolated human germ cells from malignant cells with two surface markers after treatment with IFNγ. Immunophenotyping for each patient will be necessary before isolation and induction of surface marker will be clinically applicable.

Introduction

The incidence of childhood cancer is 141 per million annually in the United States. The development of chemotherapy and radiation therapy has improved survival from childhood cancer. At present, >70% of patients survive (1). The prevalence of cancer survivors among young adults will increase to one in 250 persons in the year 2010 (2). Anticancer therapies can cause late side effects in long-term survivors. For example, growth abnormalities occur in 51%, neurocognitive abnormalities occur in 30%, and infertility occurs in 30% of long-time survivors of childhood acute myelogenous leukemia (AML; ref. 3). Infertility is a common late side effect and is a common source of stress in survivors (4). Thirty percent of male survivors of childhood cancer suffer from azospermia (5) and cannot father children even with assisted reproductive techniques. The only established option is the cryopreservation of sperm before treatment. However, prepubertal boys have no sperm or spermatids and therefore do not benefit from cryopreservation or assisted reproductive techniques. Recently, the preservation of prepubertal fertility potential has been developed in animals. One method involves transplantation of spermatogonial stem cells into the testis (6). Donor germ cells injected into seminiferous tubules undergo spermatogenesis. This technique has been successful in primates and presents new possibilities for preservation of fertility potential in prepubertal patients. Before cancer therapy, testicular tissues may be harvested and cryopreserved. After recovery, germ cells may be autotransplanted. However, autotransplantation of germ cells from cancer patients poses the risk of transmission of malignant cells. Jahnukainen et al. (7) reported that transplantation of testicular cells from leukemic rats induced transmission of leukemia. Therefore, germ cells should be completely isolated from malignant cells. We previously developed a procedure for the isolation of germ cells from leukemic mice by fluorescence-activated cell sorting (FACS) with antibodies against two surface markers, MHC class I and common leukocyte antigen (CD45), and successfully transplanted germ cells into recipient testes without transmission of leukemia (8). We used a murine AML cell line; however, humans can develop many kinds of cancer. Whether all malignant cells can be excluded from human germ cells to be transplanted remains to be determined. In addition, the validity of MHC class I and CD45 as markers for isolation should be evaluated in human cell lines. The most common childhood cancer is leukemia followed by central nervous system neoplasm and lymphoma. The main treatment strategy for leukemia and lymphoma is the combination of chemotherapy, radiotherapy, and bone marrow transplantation, whereas that for central nervous system neoplasm is surgical resection. Leukemia and lymphoma account for approximately 30% and 10%, respectively, of cancers diagnosed in children <15 years of age. In the present study, we examined human leukemia cell lines and lymphoma cell lines by FACS with antibodies against MHC class I and CD45 to completely isolate human germ cells from malignant cells.

Materials and Methods

Preparation of cells. Testicular specimens were obtained from a patient undergoing surgery for testicular rupture due to blunt trauma. The patient provided written informed consent to participate in the study. Testicular tissues were placed in PBS containing collagenase type IV (1 mg/mL; Sigma-Aldrich, St. Louis, MO), and the tubules were incubated for 15 minutes at 37°C, including manual agitation for 5 minutes. The tubules were then incubated in PBS containing 0.25% trypsin and DNase I (100 μg/mL; Sigma-Aldrich) for 5 minutes at 37°C with manual agitation.
isolation of human germ cells from malignant cells

Table 1. Human cell lines used in flow cytometric analysis and results

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jurkat</td>
<td>T-cell leukemia</td>
<td>Success</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>T-cell leukemia</td>
<td>Success</td>
</tr>
<tr>
<td>HL60</td>
<td>Promyelocytic leukemia</td>
<td>Success</td>
</tr>
<tr>
<td>KU812</td>
<td>CML</td>
<td>Success</td>
</tr>
<tr>
<td>K562</td>
<td>CML</td>
<td>Failure</td>
</tr>
<tr>
<td>U-937</td>
<td>Histiocyte lymphoma</td>
<td>Success</td>
</tr>
<tr>
<td>RAJI</td>
<td>Burkitt lymphoma</td>
<td>Success</td>
</tr>
<tr>
<td>DHL8</td>
<td>B-cell diffuse large non-Hodgkin’s lymphoma</td>
<td>Success</td>
</tr>
</tbody>
</table>

After the addition of a half volume of α-MEM containing 10% fetal bovine serum (FBS), the cell suspension was filtered through 30-µm pore size nylon mesh to remove large clumps of cells.

Human cell lines used in the present study are listed in the Table 1. K562, a human chronic myelogenous leukemia (CML) cell line, was supplied by Yoshihiro Oka (Osaka University, Osaka, Japan). DHL8, a human lymphoma cell line, was supplied by Masaki Yasukawa (Ehime University, Ehime, Japan). HL60, a human promyelocytic leukemia cell line; MOLT-4, a human T-cell leukemia cell line; Jurkat, a human T-cell leukemia cell line; KU812, a human CML cell line, U-937, a human histiocytic lymphoma cell line; and RAJI, a human Burkitt lymphoma cell line were purchased from Riken Cell Banks (Saitama, Japan). The leukemia and lymphoma cell lines were maintained in RPMI 1640 containing 10% FBS, kanamycin sulfate (80 ng/mL), and 2-mercaptoethanol at 37°C.

Endogenous peroxidase activity was blocked by incubation in H2O2. Testicular tissues were embedded in OCT (Tissue-Tek OCT embedding medium, Miles) and 6-µm-thick sections were cut with a cryostat. The specimens were fixed in 4°C acetone. Endogenous peroxidase activity was blocked by incubation in H2O2.

The primary antibodies were mouse monoclonal antibodies generated against the human leukocyte antigen (HLA)-A, B, C antigen (W6/32; DakoCytomation, Glostrup, Denmark) or CD45 (2B11+PD7/26; DakoCytomation, Glostrup, Denmark) or CD45 (2B11+PD7/26; DakoCytomation, Glostrup, Denmark) and 45 minutes on ice and washed with PBS/FBS. Finally, cells were resuspended in PBS/FBS (1 × 106 cells/mL) containing 1 µg/mL propidium iodide (PI; Sigma-Aldrich) and kept in the dark on ice until FACS analysis. In the analysis of the expression of α6 integrin, cells were incubated with FITC-conjugated anti-human HLA-A, B, C monoclonal antibody, R-PE (PE-Cy5)-conjugated anti-human CD45, B, C monoclonal antibody (G46-2.6), and R-PE-conjugated anti-human α6 integrin monoclonal antibody (GoH3; BD Biosciences-PharMingen) for 45 minutes on ice and washed with PBS/FBS. Finally, cells were resuspended in PBS/FBS (1 × 106 cells/mL) without PL.

FACS analysis. FACS analysis was done with a FACSvantage (BD Biosciences) equipped with a 488-nm argon laser (250 mW). The laser was used to excite FITC, phycoerythrin, and PI, and fluorescence emissions were collected with a 530-nm filter for FITC and a 575-nm filter for phycoerythrin and PI. The gate (G1) was set on forward scatter (FSC) versus side scatter (SSC) variables. Analysis of both HLA-A, B, C, and CD45 staining in the G1 population was done with quadrant statistics. Analysis of α6-integrin staining in cells in HLA-A, B, C–negative and CD45-negative fraction (germ cell fraction) was done with histogram statistics independently for four times. Isotype-matched rat R-PE-conjugated antibody (BD Biosciences-PharMingen) was used as controls.

FACS sorting. Cell sorting was done with a FACSVantage (BD Biosciences) equipped with a 488-nm argon laser (250 mW). The laser was used to excite FITC, phycoerythrin, and PI, and fluorescence emissions were collected with a 530-nm filter for FITC and a 575-nm filter for phycoerythrin and PI. The gate (G1) was set on forward versus SSC variables. Cells in the germ cell fraction were sorted by negative labeling for HLA-A, B, C, phycoerythrin and CD45 FITC. Cells were sorted into 5-mL tubes containing 2 mL DMEM supplemented with 10% FBS. The FSC threshold was set to exclude cell debris. The sorted cells were collected, centrifuged, and resuspended in RNA Later (Ambion, Austin, TX) at 4°C for reverse transcription-PCR (RT-PCR).

RT-PCR. Germ cells before isolation were used as positive control. Total RNA was extracted from isolated germ cell fraction with the Micro-to-Midi Total Purification System (Invitrogen Corp., Carlsbad, CA). Total RNA (1 µg) was treated with DNase I (Invitrogen). First-strand cDNA was produced with random hexamers as per the manufacturer’s recommendations (GeneAmp RNA PCR Core kit/Applied Biosystems, Inc., Foster City, CA). Reverse transcription was done at 48°C for 15 minutes, 95°C for 5 minutes, and 4°C for 5 minutes. PCR amplification of β-actin, DAZZL, HIWI, VASA, NANOG, STELLAR, and OCT4 was done with pure Taq Ready-To-Go PCR beads (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Primers used were as follows: HIWI, 5′-AGCATTTGTATTCCACGGCTTCCTTCT-3′ (forward) and 5′-CCACCTATGGTTGTAGTGACCATC-3′ (reverse); DAZZL, 5′-ATGGTAGGATGGAGTGAAACCTGAACTG-3′ (forward) and 5′-CCATGGAAAATTTATCTGTGTTCTACT-3′ (reverse); VASA, 5′-AGAAGATGTTGATCTCAAGGACC-AAC-3′ (forward) and 5′-TGCAGAGTTAGGTTTCTCTCAG-3′ (reverse); NANOG, 5′-CAGCTGGTGTGACTCATAAGGATTT-3′ (forward) and 5′-ACACACTGTTGATTTCTCAGGGATGG-3′ (reverse); STELLAR, 5′-GTTACGCGCCGGATGGCTCTA-3′ (forward) and 5′-TGAAAGTGGCTTACG-3′ (reverse); and OCT4, 5′-ACATGACGCTACTCCAGAAGACT-3′ (forward) and 5′-TACGGTTGCTTGTCTTC-3′ (reverse).

Figure 1. Immunohistochemical staining for HLA-A, B, C (A) and CD45 (B) in the human testis. Antibody binding was visualized with diaminobenzidine as the chromogen to generate a brown color. Sections were counterstained with hematoxylin, which stained nuclei blue. No immunostaining for HLA-A, B, C and CD45 was observed in germ cells. Bar, 100 µm.
(forward) and 5'-CTGAATACCTTCCCAAATAGAACCC-3' (reverse); and β-actin, 5'-CAAGAGATGGCCACGGCTGCT-3' (forward) and 5'-TCCTTCTGCGATCCTGTCGGCA-3' (reverse). Amplification conditions were as follows: 5 minutes at 95°C (one cycle) and 30 seconds at 94°C; 30 seconds at the annealing temperature (60°C for HIWI, DAZL, VASA, NANOG, and STELLAR and 55°C for OCT4); and 60 seconds at 72°C (35 cycles for HIWI, DAZL, VASA, NANOG, STELLAR, and OCT4 and 30 cycles for β-actin) and 72°C for 5 minutes (one cycle). After electrophoretic separation on 2.0% agarose gels, PCR products were visualized with Gelstar (Cambrex, East Rutherford, NJ).

Results

Human germ cells are negative for MHC class 1 and CD45 staining. First, the testicular tissues were analyzed immunohistochemically to confirm that human germ cells did not stain for HLA-A, B, C and CD45. Extratubular cells stained for HLA-A, B, C. However, intratubular cells, including germ cells and Sertoli cells, did not stain for HLA-A, B, C (Fig. 1). Cells positive for CD45 staining, which are leukocytes, were present in the extratubular region, and germ cells did not stain for CD45. These results indicated that germ cells, including spermatogonia, would be included in the HLA-A, B, C-negative and CD45-negative fraction.

Isolation of germ cells from leukemia and lymphoma cells. For autotransplantation of germ cells in the clinical setting, the germ cell fraction must not contain any malignant cells that may cause the recurrence of cancer. Dissociated human germ cells and leukemia or lymphoma cells clustered in a similar area of the bivariate histogram of FSC and SSC (Fig. 2). In the FSC<sup>high</sup> and SSC<sub>low</sub> area (G<sub>1</sub>), in which spermatogonial stem cells are included (8, 9), all of the leukemia cells and lymphoma cells stained for both the anti-HLA-A, B, C and anti-CD45 antibodies. A total of 1.45% of the K562 cells were found in the HLA-A, B, C-negative and CD45-negative fraction. No other cell lines were found in the HLA-A, B, C-negative and CD45-negative fraction. A total of 39.2% of the germ cells were found in the HLA-A, B, C-negative and CD45-negative fraction (germ cell fraction), 35.1% were found in the HLA-A, B, C-positive and CD45-negative fraction, and 24.9% were found in the HLA-A, B, C-positive and CD45-positive fraction. Without gating of the FSC<sup>high</sup> and SSC<sub>low</sub> cell population, a few cells from other cell lines were found in the germ cell fraction.
The results are summarized in the Table 1. The combination of two surface markers with the gating of FSC<sup>high</sup> and SSC<sup>low</sup> cell population made it possible to exclude all malignant cells, except K562 cells from the germ cell fraction.

The germ cell fraction contained germ cells. To confirm that the isolated germ cell fraction contained germ cells, we examined the expressions of HIWI, DAZL, VASA, NANOG, STELLAR, and OCT4 by RT-PCR (Fig. 3A and B). HIWI, DAZL, VASA, and NANOG were markers of testicular germ cells, and STELLAR and OCT4 were markers of stem cells. The results of RT-PCR showed that cells in the isolated germ cell fraction expressed HIWI, DAZL, VASA, and NANOG, which are the germ cell marker, as well as unsorted germ cells (T). Cells in the isolated germ cell fraction also expressed STELLAR and OCT4, which are the markers of stem cells. These results indicate that the germ cell fraction was germ cells, including spermatogonia. Next, we analyzed the expression of α6-integrin in the germ cell fraction by flow cytometry (Fig. 3C). α6-Integrin is a surface marker of spermatogonial stem cells in mice. Cells positive for α6-integrin staining represented 76.7 ± 4.1% (mean ± SD) of the germ cell fraction; cells positive for α6-integrin staining in the germ cell fraction represented 18.3 ± 6.3% of the total germ cells. Isolation of the germ cell fraction produced 4.2-fold enrichment of the cells positive for α6-integrin staining.

**Isolation of germ cells from K562 cells by treating with IFNγ.** To prevent contamination of the germ cell fraction by K562 cells, we induced MHC class I expression on K562 cells by treatment with IFNγ. α-MEM is suitable for maintenance of donor germ cells before transplantation (10). Whereas IFNγ did not alter the proportion of germ cells in the germ cell fraction, it induced expression of MHC class I on K562 cells and purged K562 cells from the germ cell fraction (Fig. 4).

**Discussion**

Cytotoxic agents, including alkylating agents (cyclophosphamide, ifosfamide, and chlorambucil), procarbazine, cisplatin, and vinblastine can produce long-lasting or permanent damage to germ cells, resulting in oligospermia or azoospermia. Azoospermia is permanent in 90% of men treated with cyclophosphamide doses >7.5
Germ cells are also susceptible to fractionation by radiotherapy, with doses >1.2 Gy resulting in permanent azoospermia (12). Following treatment with total body irradiation (9.9 or 13.2 Gy) and cyclophosphamide for bone marrow transplantation, azoospermia was found in 85% of adult male patients (13). Whereas postpubertal males can cryopreserved their own sperm, to date, there are no options for preventing infertility in prepubertal boys undergoing chemotherapy and/or radiotherapy. Hormonal protection of germ cells by gonadotropin-releasing hormone agonist has been successful in rodents (14) but not in humans (15). Thus, the only available option is to harvest testicular tissues before treatment and immature germ cells could somehow be matured either by in vitro maturation, xenografting, or autotransplantation.

In vitro maturation of human germ cells to spermatid-like cells has been successful, but the spermatid-like cells do not develop normally (16). Xenografting of testis tissues from pig or goat under the skin of immunodeficient mice has been reported to result in complete spermatogenesis (17). Xenografting of human testis tissues has been attempted, but only spermatogonia were obtained (18, 19). Xenogeneic spermatogenesis also raises ethical issues and presents the risk of transmission of animal pathogens. Autotransplantation of germ cells had been successful in primates (20). Autotransplantation would be advantageous in that spontaneous conception could occur.

We have developed a procedure for isolating germ cells from the testes of leukemic mice and have successfully transplanted isolated germ cells without transmission of leukemia. However, there are some issues to be resolved before autotransplantation can be applied clinically. The first is that humans can develop many types of malignant disease and disease subtypes. Germ cells from patients with cancer have been transplanted without isolation from cancer cells; however, results with respect to propagation of cancer have not been reported (21). Because the risk of contamination of donor cells by malignant cells exists (7), germ cells must be isolated before transplantation. We selected MHC class I as surface marker for spermatogonial stem cells and CD45 as a surface marker for leukemic cells to aid in the isolation of spermatogonial stem cells from testis infiltrated by leukemic cells. It should be confirmed that FACS with two surface markers can isolate germ cells without contamination by malignant cells in humans. Our results that no malignant cells from seven of eight cell lines were found in the germ cell fraction indicate the possibility of applying this procedure to treat infertility in long-term survivors of childhood cancer. CML K562 cells were not completely excluded from the germ cell fraction by the two surface markers because of the low expression of MHC class I on the surface of K562 cells. Other human leukemias also show decreased surface expression of MHC class I (22). Treatment with IFNγ induced MHC class I expression on K562 cells and made it possible to exclude K562 cells from the germ cell fraction. IFNγ induces expression of MHC class I on the cell surface by up-regulating proteasomes (23). Approximately 10% of childhood acute lymphoblastic leukemias are CD45 negative (24). Therefore, it is necessary to immunophenotype malignant cells of each patient before isolation and to either induce the expression of surface markers or add appropriate antibodies against specific surface markers of malignant cells that are not expressed on spermatogonial stem cells.

A second issue is that the sorted germ cell fraction must contain spermatogonia, including spermatogonial stem cells, to undergo spermatogenesis. Transplantation of cells into the testis is necessary to identify cells as spermatogonial stem cells. Spermatogenesis of transplanted cells shows that spermatogonial stem cells are present.

Transplantation experiments in animals do not present the ethical difficulties of experiments in human. The MHC class I-negative fraction in mice reportedly includes spermatogonial stem cells (25), and our previous study also showed that the MHC class I-negative and CD45−negative fraction includes spermatogonial stem cells (8) because the transplanted fraction underwent spermatogenesis. In humans, germ cells have also been shown to be MHC class I negative (26, 27) and CD45 negative. Somatic cells, such as interstitial cells, are MHC class I positive and CD45 negative. Testis also contains leukocytes, which are MHC class I positive and CD45 positive. RT-PCR experiments showed that cells in the germ cell fraction expressed mRNA of DAZL, HIWI, VASA, NANOG, STELLAR, and OCT4, which are markers specific for germ cells or stem cells in human. HIWI is expressed in CD34-positive hematopoietic stem cells, and germ cells, especially spermatocytes, not in Sertoli cells (28). DAZL is a germ-cell specific marker, expressed only by gonocytes, spermatogonia, spermatocytes, and spermatids in humans (29). VASA is expressed by fetal and adult gonadal germ cells in both males and females, which includes gonocytes, spermatogonia, spermatocytes, and spermatids (30). NANOG, STELLAR, and OCT4 are expressed by human embryonal stem cells, primordial germ cells, gonocyte, and spermatogonia (31, 32). NANOG is also expressed by spermatocytes and spermatids in humans (31). OCT4 is expressed in adult human stem cells, such as breast, liver, pancreas, kidney, mesenchyme, and gastric stem cells (33). It is reasonable to believe that the germ cell fraction in humans contains spermatogonia and spermatogonial stem cells. Our results that no malignant cells were found in the MHC class I-negative and CD45−negative fraction in this in vitro model indicate that FACS

**Figure 4.** Flow cytometric analysis of human germ cells and K562 cells cultured with IFNγ. K562 cells and germ cells were cultured in medium containing no IFNγ (control; left) or 1,000 IU/mL IFNγ (right) for 2 days. IFNγ did not alter the proportion of human germ cells in the germ cell fraction (bottom) but induced MHC class I on K562 cells and purged K562 cells from the germ cell fraction (top).
with antibodies against MHC class I and CD45 can isolate spermatogonial stem cells without contamination by malignant cells. We also examined a surface marker of human germ cells. There is no knowledge about surface markers of human spermatogonial stem cells, whereas surface markers of mouse spermatogonial stem cells were well investigated by the spermatogonial transplantation technique. Selection of mouse testis with anti-CD6-integrin antibody resulted in 8- to 10-fold enrichment of spermatogonial stem cells from intact testis cells, and cells positive for CD6-integrin staining represented ~15% of cryptorchid germ cells (9, 34). CD6-Integrin is also expressed by malignant cells and cannot be used for the isolation of germ cells from malignant cells (35, 36). In the present study, we showed that cells positive for CD6-integrin staining represented 76.7% of the germ cell fraction and 18.6% of the total germ cells, although it remains to be examined whether the selection by anti-CD6-integrin antibody enriches the human spermatogonial stem cells.

The remaining issue is the feasibility of the transplantation technique. Attempts have been made to infer the human testis with dye via the rete testis (37). Harvested testis tissues may not contain enough spermatogonial germ cells to undergo spermatogenesis, and in vitro culture of human spermatogonial stem cells may be required to increase the rate of successful transplantation (38). The normality of meiosis after cryopreserving and transplanting human spermatogonial stem cells should be also assessed (39).

In conclusion, our procedure to isolate germ cells from malignant cells by two surface markers was successfully applied to human leukemia and lymphoma. Results with other malignant diseases should be obtained. In addition, immunophenotyping should be done for each patient before isolation and induction of surface markers will be clinically applicable.

Acknowledgments
Received 6/26/2006; revised 9/1/2006; accepted 9/20/2006.

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

We thank Yoshihiro Oka, Hiroko Nakajima, and Masaki Yasukawa for providing cell lines and Satoru Otsubu for the technical advice.

References
Isolation of Germ Cells from Leukemia and Lymphoma Cells in a Human In vitro Model: Potential Clinical Application for Restoring Human Fertility after Anticancer Therapy

Kazutoshi Fujita, Akira Tsujimura, Yasushi Miyagawa, et al.