Xenotransplantation of testicular tissue into nude mice can be used for detecting leukemic cell contamination

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BACKGROUND: Xeno-grafting of testicular tissue may allow viable gamete maturation. This would be beneficial for prepubertal cancer patients in that it may allow restoration of fertility without the risk of a cancer relapse. However it is unknown whether cancer cells in the testicular graft can transmit the malignancy into the host animal and also if gametes can be retrieved from testicular grafts that are contaminated with malignant cells. METHODS: Rat T-cell leukemia was employed as the source of leukemic lymphoblasts and testicular tissue. This was injected i.p. (lymphoblasts) or grafted s.c. (fresh or cryopreserved testicular tissue) into the back skin of intact nude mice. To simulate clinical autografting, testicular tissue was also transplanted into healthy piebald variegated (PVG) rats. RESULTS: 50–70% of the mice, receiving 200 or 6000 leukemic lymphoblasts, developed terminal leukemia. All mice, grafted with either fresh or cryopreserved testicular tissue from leukemic donor, developed generalized leukemia and/or local tumors. All syngenic PVG rats, treated in the same manner, died of generalized leukemia. In all of the retrieved leukemic grafts, rat spermatogenesis was destroyed and only leukemic infiltration was detected. CONCLUSIONS: Grafting testicular tissue contaminated with leukemic cells led to tumor growth at the injection site without potential to differentiate germline stem cells into gametes. Xenografting could provide a novel functional strategy for simultaneous detection of malignant cell contamination and spermatogonial potential in testicular xenografts collected for fertility preservation.

Keywords: Lymphoblastic leukemia; nude mouse; rat; testis; xenotransplantation

Introduction

The survival rate among children with cancer has increased markedly over the past 20 years and, thus, the growing number of long-term survivors has led to a greater awareness of the long-term adverse consequences of cancer treatment. One such severe side-effect is male infertility due to damage of the germinal stem cells that results in oligospermia or azoospermia (Brougham et al., 2003). The fertility preservation in young boys who have survived childhood cancer has therefore become an important clinical issue.

In the recent years, novel approaches for fertility preservation techniques have developed. Autologous transplantation might be achieved by grafting testicular tissue into an ectopic site, where, once revascularized, it can generate sperm (referred to as testicular tissue transplantation) (Honaramooz et al., 2002). Alternatively, spermatogonial stem cells isolated from testicular biopsy can be retransplanted into their own niche in the testis after cancer therapy, where they may recolonize the seminiferous tubules and ultimately generate mature germ cells (autologous germ cell transplantation) (Brinster and Avarbock, 1994).

The major risk associated with both reimplantation of gonadal tissue and transplantation of autologous germ cells is the possibility of re-introducing tumor cells into the cured cancer patients (Jahnukainen et al., 2001). Promising findings in mice with experimental leukemia suggest that leukemic cells contaminating testicular tissue donor sample may be eliminated by fluorescence-activated cell sorting (FACS) with antibodies against major histocompatibility complex class I and CD45 prior to germ cell transplantation (Fujita et al., 2005, 2006). When this isolation method was applied to eight human leukemia and lymphoma cell lines no malignant cells were detected in the germ cell fraction in seven of the tested cell lines, suggesting the possibility of applying this procedure also to human leukemias (Fujita et al., 2006), although established cell lines are not fully comparable with natural leukemic cells. Contradicting findings suggesting that FACS purification may not sufficiently ensure a total depletion
of malignant cells from murine and human testicular suspensions have been recently reported (Geens et al., 2006).

Xenografting single testicular cells or testicular fragments onto immunodeficient hosts, such as nude mice, might provide a safe means of differentiating germ line stem cells into gametes without re-introducing cancer cells into a cured patient. The limited survival of adult human testicular tissue as ectopic xenograft has been reported (Schlatt et al., 2006), whereas xenografting of the testis of juvenile rhesus monkeys has allowed successful maturation and completion of spermatogenesis (Honaramooz et al., 2004). The close similarities that exist between the testis of the rhesus monkey and human suggest that it would be possible to develop xenografting conditions that allow maturation of the prepuberal human testis as well.

To date, all investigations concerning this type of xenografting have been performed with testicular tissue from healthy or, at least, non-leukemic donors. Consequently, there is a need for a preclinical study designed to explore (i) whether xenografting allows efficient generation of gametes from testicular tissue that is contaminated with cancer cells and (ii) the possible effects of the malignant graft on the immunodeficient, xeno-genic host (Shaw et al., 1996). These questions are also of general clinical importance, since xenotransplantation may even be employed to detect contamination of gonadal tissue by malignant cells. The availability of an animal model which could be used both to generate gametes from a given donor as well as to detect such contamination would be of considerable value in connection with clinical efforts to preserve fertility of childhood cancer patients.

In the present study, rats with Roser’s T-cell leukemia served as a source of leukemic cells and testicular tissue. This radiation-induced leukemia, which closely resembles human acute lymphoblastic leukemia, is maintained with serial i.p. passage in piebald variegated (PVG) rats (Roser and Ford, 1972). Nude mice were the immunodeficient recipients. In order to simulate clinical autografting, testicular tissue was also transplanted into healthy PVG rats. The aim of these experiments was to explore whether donor gametes could be retrieved from leukemic testicular grafts and whether such transplantation to immunodeficient hosts can be employed to detect cancer cell contamination in the fresh or cryopreserved testicular grafts.

Materials and Methods

Experimental animals

Twenty-five-day-old male PVG rats (Scanbur B & K, Stockholm, Sweden) and 6-week-old immunodeficient male Nu-Foxn1nu mice (Nu/Nu mice, Charles River Laboratories, USA) were housed in the animal facilities at the Karolinska University Hospital in Stockholm at 25°C under a 12-h alternating light/dark cycle. The animal experiments performed here were pre-approved by the Northern Stockholm Animal Ethics Committee (N 74/05).

Preparation of leukemic cells and testicular tissue from PVG rats carrying Roser’s T-cell leukemia

Induction of Roser’s T-cell leukemia in PVG rats was performed as described earlier (Jahnukainen et al., 2001). In brief, the PVG rats were injected i.p. with leukemic cells (6000 cells/g body weight) obtained from the lymph nodes of PVG rats with Roser’s T-cell leukemia. When the terminal phase of leukemia had been reached, three of these injected animals were killed by CO₂ inhalation and their enlarged cervical lymph nodes removed and minced with scissors in cold Dulbecco’s modified Eagle’s medium (DMEM)-F12 medium containing 10% fetal calf serum (FCS) and 50 U penicillin and 50 µg streptomycin per milliliter (Gibco, Scotland, UK). This same medium was utilized for all procedures for injection of leukemic cells, preparation of testicular tissue and tissue grafting. The cells were then collected, diluted with this same medium and injected immediately into nude mice for investigation of transmission of leukemia (see below).

For preparation of testicular tissue, the testes of two PVG rats with terminal leukemia were removed, decapsulated, placed into a Petri dish on ice containing 5 ml DMEM-F12 medium and thereafter each cut into 150 pieces (1 mm³) with iris scissors (TG Instrument AB, Helsingborg, Sweden). Half of tissue pieces were used freshly for xenografting, fixed in Bouin’s solution or rapidly frozen in dry ice for morphological examination. Remaining grafts were cryopreserved with 10% FCS and 10% (1.4 M) dimethyl sulphoxide (Sigma, St Louis, MO, USA) (Nestvold et al., 2004) in the DMEM-F12 medium by subsequently cooling to −70°C at a rate of −1°C per minute in NALGÈNE™ freezing containers (Nalgene Co., NY, USA) and thawed 1 week later by placing these vials in a water bath at 37°C.

Transmission of rat leukemia to nude mice

Homozgous, intact outbred Nu/Nu mice were divided randomly into three groups of four animals each, and these groups injected i.p. with either 600, 200 or 20 leukemic cells/mouse. All of these recipients were monitored daily for signs of leukemia (Jahnukainen et al., 2001).

Transplantation of testicular tissue to nude mice and to healthy syngenic PVG rats

The first of three groups of nude mice, each containing four animals, were injected s.c. with four fresh testicular grafts using a 18G needle and metal wire. The mice in the second group received four cryopreserved testicular grafts, whereas those in the third group were injected with eight cryopreserved testicular grafts. As autotransplanted controls, three groups of healthy syngenic PVG rats, each containing two animals, were grafted at the same time with the same numbers of fresh or cryopreserved testicular tissue. As additional controls, three healthy nude mice were each xenotransplanted s.c. with four testicular grafts (fresh) originating from an intact 40-day-old PVG rat and killed 60 days later.

Evaluation of the transmission of leukemia to nude mice following injection of leukemic cells

The body weights, size of the lymph nodes and general condition of each animal were monitored daily, and the mice killed when typical signs of generalized leukemia (i.e. weight loss, enlargement of lymph nodes and dysphonia) had developed. The body, spleen, liver, kidneys and testes were then weighed (Table 1) and the tissues fixed in Bouin’s solution for histological examination.

The extent of enlargement of the thymus and lymph nodes was recorded. Immediately prior to sacrifice a sample of peripheral blood was drawn from each leukemic cell injected mouse by cardia puncture and the white blood cells (WBCs) counted. Blasts were counted by examination of blood smears stained with hematoxylin-eosin under a light microscope (Nikon Eclipse 800; Tokyo, Japan). In addition, the expression of surface markers of Roser’s rat leukemic lymphoblasts (CD4 and CD90), (Nestvold et al., 2004) on WBCs was analyzed by flow cytometry (see below).
Evaluation of the recipients of testicular grafts
The general spread of leukemia and formation of local tumors in recipients with testicular grafts was monitored in the same fashion as for the nude mice injected i.p. with leukemic cells (see above). These animals were sacrificed either when the general phase of leukemia had developed or when a local tumor larger than 10% of the total body weight was present (Table 2). The numbers and diameters of the grafts on each animal were recorded and these grafts and the spleen, liver, testes, brain and kidneys removed and fixed in Bouin’s solution for histological examination (see below).

Histological examination of tissues
Following fixation in Bouin’s solution, tissue samples were embedded in paraffin, cut into 5 μm thin sections and stained with 1% periodic acid (Fluka Buchs SG, Switzerland) and Schiff’s reagent (Merck, NJ, USA) (PAS staining). After rinsing with distilled water, these slides were also stained with Mayer’s hematoxylin solution (Merck). Infiltration by leukemic cells into testes and other tissues (liver, spleen, kidneys and brain), the integrity of the seminiferous tubules and the potential for spermatogenesis in the grafts was finally evaluated by light microscopy (Nikon Eclipse 800).

Labeling of blood cells with antibodies and flow cytometric analysis
The samples of peripheral blood were collected in EDTA-coated tubes and 100 μl of each sample labeled directly with mouse anti-rat CD4^−/PE (5 μl) and anti-rat CD90^−/PerCp (10 μl) fluorescent-conjugated antibodies (BD Bioscience, San Jose, USA) by incubation for 10 min at room temperature with protection from the light. Thereafter, 2 ml of 1× lysis solution (BD Bioscience) was added to each tube in order to lyse the erythrocytes and 5 min later the cells were collected by centrifugation at 394g for 6 min, washed once with 2 ml ice-cold phosphate-buffered saline, and subsequently analyzed by flow cytometry. For negative controls, the same procedure was carried out using irrelevant mouse immunoglobulin (Ig) GI-PE (Chemicon, Boronia, Victoria, Australia) and IgGl^−/PerCp (BD Biosciences) antibodies. Following exclusion of dead cells and granulocytes, the remaining cells were gated and analyzed employing a FACS Calibur flow cytometer equipped with Cell quest Pro acquisition software (BD Bioscience).

Quantitation of data
Altogether, 500 leukocytes in each of three smears from each blood sample were counted in order to determine the percentage proportion of leukemic blast cells. In the case of FACS analysis, the percentages of CD4/CD90 double-positive rat cells among at least 10–20 × 10^3 WBCs from nude mice or leukemic PVG rats were determined on the basis of the gated cell populations and CD4/CD90 expression. Quantitative data in the tables were presented as mean ± SEM.

Results
Roser’s T-cell leukemia can be transmitted from PVG rats to nude mice
Two and three of the four mice injected i.p. with 6000 and 200 leukemic cells, respectively, developed typical Roser’s T-cell leukemia within a period of 31 (± 6.0) and 32 (± 3) days; whereas the remaining animals, including all of those that received 20 leukemic cells, survived the entire 3-month experimental period without developing signs of this disease (Table 1, Fig. 1). No leukemic CD4^+/CD90^+ lymphocytes could be detected among the WBCs of these latter animals (Table 1). Axillary lymph nodes were present in all of the leukemic mice 20–25 days after cell injection and the time-course to development of generalized leukemia was similar in all leukemic animal groups (Table 1), i.e. transmission of leukemia to nude mice appeared to be independent of whether 6000 or 200 leukemic cells were injected. The terminal phase of leukemia was observed 13 days later in nude mice than in syngenic PVG rats injected with the same number of leukemic cells (Jahnukainen et al., 2001).

The spleens of the leukemic mice were larger than those of the animals that did not develop the generalized leukemia (Table 1), whereas the liver and body weights were similar in all the animal groups. No local tumors were detected in the intraperitoneal cavity of i.p. injected mice. The blood of the leukemic mice contained a 26–38-fold greater number of WBCs than that of the non-leukemic animals (Table 1). The general spread of leukemia and formation of local tumors in recipients with testicular grafts was finally evaluated by light microscopy (Fig. 2G) and immunophenotype (CD4^+ CDs90^+) (Fig. 2F) of the leukemic lymphoblasts in nude mice were comparable to Roser’s rat T-lymphoblasts (Fig. 2B and C).

The effects of xenografting fresh or cryopreserved leukemic testicular tissue from rats into nude mice
Local subcutaneous swelling at the site of transplantation, which was the earliest sign of local tumor growth, could be seen 11–13 days post-transplantation with leukemic testicular grafts in all of the mice. Development of subcutaneous swelling

### Table 1: Transmission of leukemia into nude mice and syngenic PVG rats by i.p. injection of T-cell lymphoblasts from leukemic PVG rats

<table>
<thead>
<tr>
<th>Number of leukemic cells injected/animal</th>
<th>Number of animals with general signs of leukemia</th>
<th>Time to overt leukemia (days)</th>
<th>Body weight (g)</th>
<th>Spleen weight (g)</th>
<th>Liver weight (g)</th>
<th>WBC (×10^3/l)</th>
<th>Proportion of blasts cells (%)</th>
<th>Proportion of CD4^+ /CD90^+ cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nude mice</td>
<td>12</td>
<td>5</td>
<td>0.1 ± 0</td>
<td>1.7 ± 0.1</td>
<td>4 ± 1</td>
<td>63 ± 11</td>
<td>65 ± 7</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>4</td>
<td>0</td>
<td>0.6 ± 0</td>
<td>1.8 ± 0.1</td>
<td>92 ± 13</td>
<td>62 ± 1</td>
<td>61 ± 18</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>4</td>
<td>3</td>
<td>31 ± 6</td>
<td>2.2 ± 0.1</td>
<td>138 ± 71</td>
<td>331 ± 99</td>
<td>75 ± 2</td>
<td>94 ± 2</td>
</tr>
<tr>
<td>6000</td>
<td>2</td>
<td>2</td>
<td>15 ± 0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>PVG rats</td>
<td>2</td>
<td>2</td>
<td>15 ± 0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>6000</td>
<td>2</td>
<td>2</td>
<td>15 ± 0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Animals were killed when signs of generalized leukemia had developed. The organ weights, WBC counts and proportion of blast cells and CD4^+/CD90^+ cells among the circulating WBCs are shown. ND, not determined.

×Mean ± SEM.
Table 2: Transmission of leukemia into nude mice and syngenic PVG rats by ectopic transplantation of testicular tissue from leukemic PVG rats

<table>
<thead>
<tr>
<th>Animals</th>
<th>Type of leukemia that developed (N)</th>
<th>Number of refrains recovered /animal</th>
<th>Number of grafts (g)</th>
<th>Survival time in days</th>
<th>Body weight (g)</th>
<th>Liver weight (g)</th>
<th>Spleen weight (g)</th>
<th>WBC (x10^9/l)</th>
<th>Prop. of blast cells (%)</th>
<th>Prop. of CD4^+/CD90^+ cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nude mice</td>
<td>General (6)</td>
<td>0</td>
<td>60^a</td>
<td>37 ± 1</td>
<td>2.1 ± 0.2</td>
<td>0.2 ± 0.0</td>
<td>2 ± 0</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Local tumor (4)</td>
<td>5 ± 1</td>
<td>24 ± 1</td>
<td>37 ± 2</td>
<td>1.9 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>64 ± 27</td>
<td>3 ± 3</td>
<td>14 ± 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5 ± 0.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8 ± 6</td>
<td>2 ± 2</td>
<td>5 ± 2</td>
</tr>
<tr>
<td></td>
<td>Fresh healthy control</td>
<td>3</td>
<td>0.6 ± 0.1</td>
<td>21 ± 0</td>
<td>35 ± 2</td>
<td>2.5 ± 0.2</td>
<td>157 ± 34</td>
<td>71 ± 7</td>
<td>74 ± 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fresh leukemic</td>
<td>4</td>
<td>0.9 ± 0.1</td>
<td>29 ± 2</td>
<td>32 ± 2</td>
<td>1.2 ± 1.3</td>
<td>26 ± 15</td>
<td>17 ± 15</td>
<td>17 ± 8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Frozen leukemic</td>
<td>4</td>
<td>1.9 ± 0.9</td>
<td>29 ± 2</td>
<td>32 ± 2</td>
<td>1.2 ± 1.3</td>
<td>26 ± 15</td>
<td>17 ± 15</td>
<td>17 ± 8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Local tumor (2)</td>
<td>4 ± 0</td>
<td>21 ± 0</td>
<td>33 ± 2</td>
<td>2.3 ± 0.8</td>
<td>1.3 ± 0.2</td>
<td>13 ± 10</td>
<td>7 ± 3</td>
<td>42 ± 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>General (1)</td>
<td>4 ± 0</td>
<td>21 ± 0</td>
<td>33 ± 2</td>
<td>2.3 ± 0.8</td>
<td>1.3 ± 0.2</td>
<td>13 ± 10</td>
<td>7 ± 3</td>
<td>42 ± 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Frozen leukemic</td>
<td>2</td>
<td>3 ± 0.3</td>
<td>13 ± 0</td>
<td>126 ± 4</td>
<td>6.8 ± 0.1</td>
<td>405 ± 25</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>PVG rats</td>
<td>General (2)</td>
<td>2 ± 0</td>
<td>3 ± 1.0</td>
<td>13 ± 0</td>
<td>126 ± 4</td>
<td>6.8 ± 0.1</td>
<td>405 ± 25</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Frozen leukemic</td>
<td>4</td>
<td>4.7 ± 0.0</td>
<td>14 ± 0</td>
<td>130 ± 2</td>
<td>65 ± 0.0</td>
<td>18 ± 0</td>
<td>ND</td>
<td>93</td>
<td></td>
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<tr>
<td></td>
<td>General (2)</td>
<td>5 ± 0</td>
<td>4.7 ± 0.0</td>
<td>14 ± 0</td>
<td>130 ± 2</td>
<td>65 ± 0.0</td>
<td>18 ± 0</td>
<td>ND</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Frozen leukemic</td>
<td>2</td>
<td>4.7 ± 0.0</td>
<td>14 ± 0</td>
<td>130 ± 2</td>
<td>65 ± 0.0</td>
<td>18 ± 0</td>
<td>ND</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>General (2)</td>
<td>11 ± 0</td>
<td>3.4 ± 0.4</td>
<td>13 ± 0</td>
<td>126 ± 4</td>
<td>6.8 ± 0.1</td>
<td>405 ± 25</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

A summary of the grafting procedure, organ weights, WBC counts and proportions of blast cells and CD4^+/CD90^+ cells among the circulating WBCs is shown. ND, not detected; N, number of animals. ^aMean ± SEM. ^bElectively stopped.
tumors 13 or 14 days after grafting with leukemic testicular tissue. This is 2 days earlier than the generalized phase of leukemia in PVG rat after i.p. injection of 6000 leukemic cells/g body weight (Table 1). The proportion of peripheral lymphocytes expressing surface markers CD4/CD90 increased from 14% in control animals (Fig. 2A) to 80% (Fig. 2B) in PVG rats with generalized leukemia. The time required for the development of generalized leukemia was similar after transplantation with four fresh, or four or eight cryopreserved testicular grafts (Table 2). Local subcutaneous swelling, as a primary sign of leukemic tumor, was present in all of the PVG rats 9–15 days after tissue implantation. The biggest graft was recovered after injecting frozen testicular tissue (4.7 g).

Microscopic and histological morphology of the leukemic testicular grafts
Histological examination of the testicular grafts prior to transplantation revealed local infiltration of leukemic cells into the testicular interstitium, as well as blood vessels filled with leukemic blasts (Fig. 3B). Cryopreservation of testicular tissue resulted in minor changes in morphology of interstitial tissue and disturbed morphology of the seminiferous tubules (Fig. 3C).

The median volume of grafts ranged from 0.2 to 5.9 cm³ in PVG and nude hosts (Fig. 2D and H), which is 2–59-fold larger than the original testicular graft (1 mm³) (Fig. 2D insert). Macroscopic examination of the subcutaneous grafts in the nude mice and PVG rats revealed the presence of large hemorrhagic tumors that infiltrated into the skin and subcutaneous muscle layers in PVG rat (D, arrowhead and arrows) and nude mouse hosts (H, arrowhead and arrows).

Figure 2: The expression of rat CD4 and CD90 surface markers on WBCs (A) In control and (B) leukemic PVG rat and in control (E) and leukemic (F) nude mouse after ectopic transplantation with control or leukemic testicular tissue. Altogether, 14% of lymphocytes of control PVG rat and 0% of control nude mouse express rat CD4/CD90 whereas this figure increased to 80% and 63% when PVG rat and nude mouse, respectively, developed generalized stage of leukemia. A morphological characteristic of leukemic blasts on blood smears is similar for leukemic PVG rat (C) and nude mouse (G). Ectopic s.c. transplantation with 1 mm³ of testicular tissue (D, insert) from leukemic PVG rat results in large hemorrhagic tumor that infiltrates into the skin and subcutaneous muscle layers in PVG rat (D, arrowhead and arrows) and nude mouse hosts (H, arrowhead and arrows).

Infiltration of leukemic cells into various tissues of the recipient nude mice
Leukemic testicular infiltration was detected in nude mice and PVG hosts with generalized leukemia. Moreover, such infiltration but lesser in degree was detected also in the testes of the nude mice with local tumors only (Fig. 3E). Infiltration of leukemic cells into the spleen, liver, kidney and brain of the nude mouse hosts was detected only in the animals with generalized leukemia.

Xenografting of fresh testicular tissue from a healthy PVG rat into nude mice
As expected, none of the three healthy mice transplanted with normal testicular tissue (Fig. 3A) from a 40-day-old control PVG rat developed leukemia or local tumors. At the time of the elective sacrifice, 60 days after tissue transplantation, no surviving testicular tissue was observed in the retrieved grafts. No peripheral blood lymphocytes expressing rat CD4/CD90 surface markers were detected in these animals (Fig. 2E, Table 2).
The present study reveals that xenografting of testicular tissue from leukemic rats into immunodeficient mice did not lead to differentiation and restoration of donor spermatogenesis. Leukemic cells that contaminated testicular grafts proliferated and formed tumors at the injection site. This observation suggests that malignant cell contamination in testicular grafts may compromise the testicular tissue transplantation as a means to differentiate germline stem cells from leukemic donors. These findings are in agreement with previous observations that spermatogonial germ cell transplantation by using testicular cells from leukemic donors holds a substantial risk of causing a cancer relapse in the recipient host (Jahnukainen et al., 2001).

No spermatogenesis was detected in any of the xenotransplanted leukemic testicular grafts. Histological morphology of retrieved grafts showed a total disintegration of the testicular architecture and an overt infiltration with proliferating leukemic cells, while seminiferous tubules turned into hyalinized casts. Leukemic tumors in immunodeficient hosts appeared within 11–13 days after testicular tissue transplantation and the size of tumors reached 10% of host body weight in 4–5 weeks. This aggressive proliferation of leukemic cells and destruction of spermatogenesis were similar to that previously observed in the Roser’s rat leukemia model after intratesticular injection of leukemic lymphoblasts (Jahnukainen et al., 1993, 2001) or in cryptorchid testes (Jahnukainen et al., 1994). It has been speculated that the changes in the paracrine testicular cytokine network and function of testicular vasculature can regulate the testicular infiltration and the proliferation capacity of the leukemic cells (Jackson et al., 1984). The present observation suggests that transplantation related changes in testicular xenografts favor the proliferation of contaminating cancer cells.

The present observation that spermatogenesis was not preserved in control testicular grafts 60 days after transplantation either, confirmed the previous observation that spermatogenesis cannot be maintained in testicular xenografts from mature rodents (Schlatt et al., 2002). It has been shown earlier that recovery of spermatogenesis in adult mouse and hamster tissue is limited while complete spermatogenesis can be observed 50 days after transplantation of grafts from immature rodents (Honaramooz et al., 2002, 2004; Schlatt et al., 2002, 2003). Unfortunately, the presently used rodent model does not allow the use of immature testicular xenografts from donors with overt leukemia. The appearance of Roser’s rat leukemia takes 16–17 days (Roser and Ford, 1972). This is later than the postnatal age at which rat spermatogenesis starts (van Haaster and de Rooij, 1993). To gain a better insight into the maturation of the grafted tissue from leukemic animals, immature testicular tissue from leukemic donor animals needs to be employed in future studies. Immature testicular tissue from PVG rats could possibly be obtained shortly after inoculation of Roser’s rat T-cell leukemia. The fast tumor formation at the injection site in the present study suggests that leukemic cell proliferation may destroy the immature testicular grafts well before the spermatogenesis shows maturation.

In this study, we unequivocally show that leukemic cells can survive cryopreservation in testicular grafts. Xenotransplantation...
with cryopreserved grafts was associated with an increased incidence of generalized leukemia in the nude mouse hosts. Three of the eight nude hosts receiving cryopreserved testicular grafts showed signs of generalized leukemia whereas none of the nude mice grafted with fresh testicular tissue showed such signs. It can be speculated that the minor morphological disintegration of the testicular tissue that was observed after cryopreservation may have an effect on the invasive capacity of the contaminating leukemic cells. Disturbances of the vessel barriers of the cryopreserved grafts may have enhanced the hematogenous invasion of surviving leukemic cells. This suggests that the cryopreservation per se does not abolish leukemic cell contamination. On the contrary, autotransplantation with cryopreserved testicular grafts may carry an even higher risk of disseminating leukemia.

Although the current conditions resulted in a complete regression of most of the leukemic testicular tissue, it appeared that the present method successfully detected leukemic cell contamination in testicular tissue. The nude hosts developed a localized leukemic tumor in the area of each site of transplantation and the first subcutaneous swelling, at the injection site, appeared concurrently in the nude and syngenic hosts. These observations suggest that tumor formation at the injection site could be used as a sign of leukemic cell contamination in the grafts.

It would be of general importance to know how sensitive the presently used xenografting method is to detect cancer cell contamination. Leukemic testicular tissue was collected from overtly leukemic donors. According to our previous studies, ~15% of the testicular interstitium of these testes were infiltrated by leukemic cells (Jahnukainen et al., 1994). This is well in accordance with the clinical observation that 20% of leukemic biopsies at the time of leukemia diagnosis show leukemic infiltration when judged by light microscopy (Kim et al., 1981). Thus, the extent of the leukemic infiltration in rat testes represents well the clinical situation. One leukemic rat testis could be cut into 150 grafts and each testis contains about 30 × 10⁶ cells (unpublished observations). One leukemic testicular graft (1 mm³) was therefore estimated to contain about 0.2 × 10⁶ cells. This figure is comparable to earlier published estimations; one adult rat testis (1910 ± 74 mg) contains on average 400 × 10⁶ cells (Orwig et al., 2002). Assuming that the testicular leukemic cell infiltration at the time of appearance of overt Roser’s rat leukemia is ~2–4% of the total testicular volume (Jahnukainen et al., 1994), one testicular graft is expected to be infiltrated by 4000–8000 leukemic cells. The sensitivity of nude mice to the estimated minimum of 4000 leukemic cells in the testicular grafts was confirmed by injecting a known number of leukemic cells into the nude hosts. Altogether, 70% of the nude mice recipients developed signs of generalized rat T-cell leukemia when more than 200 leukemic lymphoblasts were injected i.p. These observations suggest that the present nude mouse model would have formed leukemic tumors even if 20–40 times less leukemic cells were contaminating the testicular grafts.

Xenografting of small pieces of human testicular tissue into immunodeficient mouse hosts may provide an accessible approach to evaluate spermatogonial stem cell potential in testicular biopsies collected and cryopreserved for fertility preservation before cancer therapy (Jahnukainen et al., 2007). The present observations suggest that this approach may provide a unique possibility to evaluate also the cancer contamination in the xenografts. The risk of cancer re-introduction through testicular tissue cells (Jahnukainen et al., 2001) highlights the necessity of devising reliable strategies to screen the testicular tissue prior to cryopreservation and transplantation (Fujita et al., 2005; Wallace et al., 2005; Geens et al., 2007). To date, the most sensitive methods for detection of minimal residual leukemic cells in hematological samples are FACS or PCR. At their best, these methods may detect one leukemic cell among 10³ and 10⁴ bone marrow cells (Mandrell and Pritchard, 2006). However, analysis using these methods requires dispersion of solid tissue samples which is not possible prior to testicular tissue transplantation. A successful testicular tissue transplantation is possible only when the donor germ cells are co-transplanted with their surroundings and remain in undisturbed contact with their original niches (Honaramooz et al., 2002, 2004; Schlatt et al., 2002, 2003).

The xenografting method presented here provides a first and promising step toward developing a functional malignancy detection assay in the live testicular grafts. It will be interesting to explore whether malignant cell contamination in human testicular samples can be detected when xenografted into nude hosts. In addition, it remains to be determined whether malignant cells in human testicular biopsies may affect the success of the testicular xenografting, possibly preventing the maturation of fully functional sperm.

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