Ovarian tissue harvested from lymphoma patients to preserve fertility may be safe for autotransplantation

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BACKGROUND: Ovarian failure is a common sequel to chemo/radiotherapy in patients successfully treated for cancer. Harvesting, cryopreserving and subsequently re-implanting ovarian cortical grafts can be used to re-establish reproductive potential in women with cancer. The safety issue, however, is of great concern because residual disease in autografted ovarian tissues might cause recrudescence of disease. METHODS: A total of 30 non-obese diabetic severe combined immunodeficient (NOD/LtSz-SCID) mice were individually xenografted s.c. with frozen–thawed ovarian tissue from 18 patients with lymphoma [13 Hodgkin’s lymphoma (HL) and 5 non-Hodgkin’s lymphoma (NHL)]. The animals were autopsied at 16 weeks, or earlier if cachectic. The xenograft, liver, spleen, sternum, para-aortic lymph nodes and thymus were prepared for histology, immunohistochemistry and human DNA microsatellite analysis. RESULTS: None of the animals grafted with ovarian tissue from lymphoma patients developed disease. However, all 3 animals grafted with lymph node tissue from an NHL patient developed B-cell lymphomas that were confirmed as human in origin by DNA microsatellite analysis. CONCLUSION: Ovarian tissue harvested before high-dose chemotherapy for HL or NHL may not carry a risk of disease transmission by autotransplantation, although the possibility is difficult to exclude completely.

Key words: cryopreservation/fertility/lymphoma/transplantation/xenografting

Introduction

As a result of the widespread use of intensive chemo/radiotherapy, an increasing number of patients enjoy prolonged disease-free survival following treatment for cancer. Since germ cells are susceptible to cytotoxic treatments (alkylating agents and radiation), infertility and ovarian failure are common complications (Apperley and Reddy, 1995). Sterility has a major impact on the quality of life for young survivors of cancer, and cryostorage of semen is provided routinely for safeguarding the reproductive options of adult males (Hallak et al., 1995). For female patients, on the other hand, the corresponding technology of embryo cryopreservation requires lengthy hormonal stimulation and follicle aspiration. Moreover, cryopreservation of embryos is unsuitable for patients without a male partner, before puberty or for patients requiring immediate treatment. Nevertheless, frozen banking of embryos has occasionally proved helpful for cancer patients (Atkinson et al., 1994).

A new strategy, involving autotransplantation of ovarian cortical slices banked at low temperatures (Gosden et al., 1994), offers the possibility of reinstating fertility in women and children after sterilizing treatment. In addition, this approach may be helpful for women recovering fertility temporarily but who are expected to undergo therapy-related premature menopause. In either circumstance, the endocrine effects of autotransplanted ovarian tissue could be advantageous, and avoid the need for simultaneous hormone replacement therapy. Studies in sheep have demonstrated that frozen–thawed and grafted ovarian tissue can restore ovulatory cycles (Baird et al., 1999), and a single case report has provided proof of principle in humans (Oktay and Karlikaya, 2000).

Before proceeding to clinical applications, it is vital to test whether ovarian tissue harvested from cancer patients harbours residual disease which could be transmitted by autotransplantation. This theoretical risk has been highlighted in a study of AKR strain mice susceptible to lymphoma (Shaw et al., 1996), where the disease was transmitted by grafts to healthy recipients. We have addressed the safety issue in humans by
Tissue Cryopreservation

Ovarian cortical tissue was trimmed to strips of maximum length 8 mm (1–2 mm thick) using a scalpel and equilibrated for 30 min in cryoprotective medium containing 1.5 mol/l dimethyl sulphoxide (DMSO), 0.1 mol/l sucrose and 1% human serum albumin (Sigma, Poole, UK). Each piece was transferred to a 1.5 ml cryovial (Nunc, Roskilde, Denmark), chilled on ice and loaded in an automated freezer (Planer Biomed, London, UK) for cooling at –0.3°C/min to –40°C, and subsequently at –10°C/min to –140°C. The vials were stored in liquid nitrogen (–196°C) for up to 3 years before use. They were thawed rapidly (100°C/min) in water at 30°C followed by washing and the tissue was incubated for 1 h in Leibovitz-L15 medium (Gibco, Paisley, UK) containing 500 IU/ml penicillin G, 50 µg/ml streptomycin and 2.5 µg/ml amphotericin B prior to transplantation.

Preparation of Positive Controls

For NHL controls, a disease-positive lymph node from a patient with recurrent follicular B-cell lymphoma was processed and cryopreserved using the same protocol.

For HL controls, the cell line L1236 derived from Hodgkin’s disease tissue was cultured in RPMI 1640 medium (Sigma) supplemented with 10% heat inactivated fetal calf serum, 50 IU/ml penicillin, 50 µg/ml streptomycin and 4 mmol/l L-glutamine in a 5% CO₂ atmosphere at 37°C. The total cultured tumour cells (45×10⁶) were resuspended in 2500 µl of phosphate-buffered saline (PBS) and prepared into five aliquots (9×10⁶ cells in 500 µl PBS) for inoculation.

Histology and immunohistochemistry

Tissue from the graft sites and host organs was fixed in 4% paraformaldehyde and processed as 4 µm sections stained with haematoxylin and eosin. Selected tissues were stained immunohistochemically using the streptavidin ABC/HRP method (DAKO Ltd, Ely, UK) with the following antibodies: anti-CD3 for T-cell expression; anti-CD79a and anti-CD20/L26 for B-cell expression; anti-CD30/HER2 for Hodgkin’s disease and anti-CD15/Leu M1 (Becton Dickinson, Oxford, UK) for monocye expression (particularly in HL).

Screening for human microsatellite DNA

DNA was prepared from 10 µm sections using procedures previously described (Varley et al., 1997). PCR using primers for human microsatellite DNA sequences were carried out using standard procedures incorporating 32P-dCTP, and the products were visualized by autoradiography after gel electrophoresis. The microsatellites analysed were D2S123, D3S1076, D6S292, D8S255, D9S162, D9S166 and D13S175.

Materials and methods

Sources of ovarian tissue

Human ovarian tissue was collected by laparoscopic ovarian biopsy or unilateral oophorectomy from 18 patients aged 19–39 years (median 25) who had requested fertility conservation by low temperature tissue banking prior to potentially sterilizing first or second line treatment for lymphoma [Hodgkin’s lymphoma (HL), n = 13; non-Hodgkin’s lymphoma (NHL), n = 5]. Patient characteristics, including age, disease status and treatment received after ovarian harvest and survival, are listed in Table I. The individuals gave informed consent for the research use of ovarian tissue under a protocol approved by the Research Ethics Committees of the participating medical centres, namely the Leeds General Infirmary, Leeds (7 patients), the Christie Hospital, Manchester (7), St James’s University Hospital, Leeds (3) and Bourn Hall Clinic, Cambridge (1). The study of ovarian xenografts was carried out in facilities licensed for animal care by the Home Office, UK.

Table I. Characteristics of patients whose ovarian tissue was xenografted into NOD/LtSz-SCID mice

<table>
<thead>
<tr>
<th>Patient</th>
<th>Lymphoma</th>
<th>Age at ovarian harvest</th>
<th>Disease Status</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HL</td>
<td>39</td>
<td>R</td>
<td>Dead</td>
</tr>
<tr>
<td>2</td>
<td>HL</td>
<td>20</td>
<td>P</td>
<td>Alive</td>
</tr>
<tr>
<td>3</td>
<td>HL</td>
<td>22</td>
<td>P</td>
<td>Alive</td>
</tr>
<tr>
<td>4</td>
<td>HL</td>
<td>28</td>
<td>R</td>
<td>Dead</td>
</tr>
<tr>
<td>5</td>
<td>HL</td>
<td>19</td>
<td>P</td>
<td>Alive</td>
</tr>
<tr>
<td>6</td>
<td>HL</td>
<td>26</td>
<td>R</td>
<td>Dead</td>
</tr>
<tr>
<td>7</td>
<td>HL</td>
<td>25</td>
<td>R</td>
<td>Dead</td>
</tr>
<tr>
<td>8</td>
<td>HL</td>
<td>20</td>
<td>R</td>
<td>Alive</td>
</tr>
<tr>
<td>9</td>
<td>HL</td>
<td>23</td>
<td>R</td>
<td>Dead</td>
</tr>
<tr>
<td>10</td>
<td>HL</td>
<td>24</td>
<td>R</td>
<td>Alive</td>
</tr>
<tr>
<td>11</td>
<td>HL</td>
<td>21</td>
<td>R</td>
<td>Alive</td>
</tr>
<tr>
<td>12</td>
<td>HL</td>
<td>23</td>
<td>R</td>
<td>Dead</td>
</tr>
<tr>
<td>13</td>
<td>HL</td>
<td>36</td>
<td>R</td>
<td>Alive</td>
</tr>
<tr>
<td>14</td>
<td>NHL</td>
<td>28</td>
<td>P</td>
<td>Alive</td>
</tr>
<tr>
<td>15</td>
<td>NHL</td>
<td>24</td>
<td>P</td>
<td>Dead</td>
</tr>
<tr>
<td>16</td>
<td>NHL</td>
<td>29</td>
<td>R</td>
<td>Dead</td>
</tr>
<tr>
<td>17</td>
<td>NHL</td>
<td>29</td>
<td>R</td>
<td>Dead</td>
</tr>
<tr>
<td>18</td>
<td>NHL</td>
<td>25</td>
<td>R</td>
<td>Alive</td>
</tr>
<tr>
<td>Totals</td>
<td></td>
<td>HL = 13; Median 25</td>
<td>P = 5;</td>
<td>Alive = 9;</td>
</tr>
<tr>
<td></td>
<td>NHL = 5</td>
<td>(range 19–39)</td>
<td>R = 13</td>
<td>Dead = 9;</td>
</tr>
</tbody>
</table>

aHL = Hodgkin’s lymphoma; NHL = non-Hodgkin’s lymphoma.
bP = primary disease; R = recurrent disease.

Ovarian tissue autotransplants may be safe xenografting ovarian tissue from patients with lymphoma to immunodeficient mice.
Results

Macroscopic examination

The majority of animals remained in good health throughout the study, and only four animals showed macroscopically abnormal lesions. Three animals died or were euthanized post-grafting because of cachexia. One animal from the NHL control group died at 15 weeks of age with a large tumour (20 mm in diameter) at the graft site and enlarged aortic lymph nodes, spleen and liver.

Microscopic observations

Light microscopy showed no evidence of malignant cells in ovarian tissues prior to transplantation. All animals grafted with human ovarian tissues had evidence of spontaneous (murine) lymphoblastic lymphoma in their thymuses (Figure 1a,b), and in eight animals it had spread to one or more of the following: sternal marrow, spleen, liver and para-aortic lymph nodes. Immunohistochemistry of representative samples of these lesions showed that the lymphoma was T-cell derived (CD3+, CD79a–, CD20–, CD15–, CD30–). No human HL was detected in mice injected with cells from cell line L1236. However, human B-cell lymphoma (CD20+, CD79a+, CD3–, CD15–, CD30–) was present at the graft site in 2 of 3 NHL control animals and additionally in the lymph nodes and spleen of animals (including the mouse where B-cell lymphoma could not be identified at the graft site). Histologically and immunohistochemically these tumours were distinct from T-cell lymphoma of murine origin (Figure 2).

Microsatellite analysis

To confirm the human origin of B-cell lymphoma, DNA was analysed from 10 independent human controls and the xenograft, spleen and para-aortic lymph node from three NHL control animals. Additionally, normal tissue and a spontaneous T-cell lymphoma from NOD/SCID mice grafted with human ovarian tissue were studied. The tissues with tumour infiltration in all three NHL control animals were confirmed to contain human DNAs by specific amplification of the human microsatellite markers (Figure 3, lanes 4 and 7). In contrast neither normal tissues (Figure 3, lanes 5 and 6) nor the T-cell lymphoma in animals receiving human ovarian tissue (Figure 3, lane 8) were positive for human sequences, although all contained mouse DNA (data not shown). These findings support the assumption that the T-cell lymphoma in the experimental animals was of murine origin.

Discussion

Low temperature banking of ovarian tissue for women and prepubertal children undergoing sterilizing chemo/radiotherapy is an attractive strategy for preserving fertility. Whilst cryopreservation of mature oocytes has been problematic because of low survival rates, numerous experimental studies using animal and human tissue have indicated that primordial follicles are relatively robust to freezing and thawing (Candy et al., 1997; Gook et al., 1999). Ovarian tissue from cancer patients has been banked at many centres worldwide, and we can expect to see published reports of autotransplantation soon. This step requires prior assurance that the tissue will not transmit disease after grafting, whether the procedure is orthotopic to restore natural fertility or heterotopic to attempt pregnancy after follicular aspiration and IVF (Aubard et al., 1999). Many malignant diseases in young women are now being treated successfully with high-dose chemotherapy and irradiation, and the risks of transmission in autografts are likely to be diseasespecific. In the case of the commonest haematological malignancy affecting young women, HL, it is reassuring that there was no histological evidence of ovarian involvement of disease in either this study or an earlier one (Meirow et al., 1998). Yet, this evidence on its own falls short of providing full reassurance for autotransplants, and there is even less information about other malignancies, such as NHL.

This is the first reported in-vivo investigation of residual disease in human ovarian tissue using a xenograft. Grafting human cells into immunodeficient mice is a well-established method for studying haematological cancer (Greiner et al., 1995), and this model may help to inform clinical decisions.
Ovarian tissue autotransplants may be safe

Figure 2. (a) Lymph node replaced by diffuse lymphoma in NOD/LtSz-SCID mouse which was engrafted with human follicular lymphoma. Haematoxylin and eosin (× 550). (b) The same specimen showing strong immunostaining for CD20 demonstrating that the lymphoma was of B-lineage. Streptavidin/ABC method (original magnification ×550).

Figure 3. A representative autoradiograph showing microsatellite analysis at locus D13S175. Lanes 1–3 are of normal human control samples, lanes 4–7 are from one of the control mice xenografted with human B-cell lymphoma. Lane 4 is the xenograft, lanes 5 and 6 are from histologically normal tissues and lane 7 is from tissue showing evidence of metastatic tumour (spleen). Lane 8 shows the analysis of the spontaneous T-cell lymphoma from a control NOD/SCID mouse.

was no sign of human lymphoma in the animals xenografted with ovarian tissue from 5 NHL patients. In contrast, all 3 control mice grafted with human lymph node tissue from a patient with follicular lymphoma developed B-cell lymphoma of human origin, which was confirmed unequivocally by microsatellite DNA analysis. Our results give hope that ovaries from HL or NHL patients can be safely autotransplanted at the completion of treatment.

One of the most difficult parts of this study was selection of the specimen for the HL control, because the characteristic Hodgkin/Reed-Sternberg (H-RS) cells represent only a minor population in the tumour (<1%) (Kanzler et al., 1996). Therefore, propagation of malignant cells by engraftment or in-vitro culture is very difficult. Among 14 Hodgkin’s disease derived cell lines, only a cell line L1236 has been proven by PCR for Ig gene rearrangement to derive from primary H-RS cells (Wolf et al., 1996). Although it has been reported that SCID mice support growth of Hodgkin’s disease derived cell lines, the frequency of transmission is only ~40% after 4 months observation (Kalle et al., 1992). In our study, none of the 5 animals injected with L1236 cells developed the human disease. This may be due to a limited observation period (16 weeks) or a lower number of injected cells (9×10⁶) compared with other studies (Kalle et al., 1992; Kapp et al., 1994).

This study introduces the NOD/LtSz-SCID mouse as a model for investigating the safety of ovarian transplantation, serving as a preclinical screen for disease. Whilst it has focused on lymphoma, the model could serve for other diseases where it is established that human tumour tissue survives and proliferates in vivo. The maximum period of study is, however, constrained by the short life span of the mice. It has been reported that over 80% of female NOD/LtSz-SCID mice have developed lethal thymic lymphomas by 20 weeks of age (Serreze et al., 1995), and this was consistent with our findings. Xenografting provides a functional test which is complementary to evidence from histopathology or PCR.

Our findings are of immediate relevance to women with lymphoma who are considering banking ovarian tissue for fertility conservation, as well as to their physicians who need to provide them with appropriate advice. We can now be more
optimistic about the application of ovarian cryopreservation and transplantation in cancer patients, but these reassuring findings should not be interpreted as an absolute indication of safety. Hence, efforts to develop new techniques to detect residual disease need to be continued. In the long-term, it is hoped that oocytes in primordial follicles can be safely matured in vitro, since the zona pellucida provides a barrier to somatic cells. This technology is still at an early stage, even in animals (Eppig and O’Brien, 1996), yet it may be available for young females who will not require their stored tissue for a decade or more.

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References


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