Spermatogonial survival after grafting human testicular tissue to immunodeficient mice

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BACKGROUND: The xenografting of pre-pubertal human testicular tissue to an immunodeficient mouse is a theoretical strategy for restoring fertility in childhood cancer patients, while circumventing the risk of malignant recurrence. This study aimed at comparing the grafting of pre-pubertal and adult murine testicular tissue, as well as that of human adult testicular tissue, to two immunodeficient recipients, i.e. Swiss Nude mice and SCID-NOD mice.

MATERIALS AND METHODS: In this study, we evaluated the survival of pre-pubertal and adult murine testicular tissues, and that of adult human testicular tissue after subcutaneous grafting to immunodeficient mice.

RESULTS: After allografting pre-pubertal testicular tissue pieces, meiotic cells were observed in 69.1% of the grafts, while complete spermatogenesis was observed in 30.9%. All grafts of adult murine testicular tissue and 59.5% of the adult human testicular grafts showed sclerosis. However, in 21.6% of the adult human testicular grafts, spermatogonia were still observed, with increasing sclerosis in time. No significant differences were observed between the two mouse models under evaluation.

CONCLUSION: After xenografting human adult testicular tissue to a recipient mouse, spermatogonia were maintained over a period of >195 days. However, in order to prove xenografting as a method for external germ line storage, the transplants should have a more immature developmental stage. Moreover, not only the developmental status of the tissue at the time-point of grafting, but also the structural organisation of the seminiferous epithelium, might influence the development of the testicular tissue.

Key words: human/mouse/spermatogonia/testicular tissue/xenograft

Introduction

Approximately 1 in 475 children will develop cancer before the age of 15 years. Thanks to recent advances in chemo- and radiotherapy, cure rates of childhood cancers are as high as 80%. It has been estimated that by 2010, 1 in 250 young adults in the USA may be long-term survivors of childhood cancer (Bleyer, 1990) and cancer incidence is also on the rise in Europe (Steliarova-Foucher et al., 2004). Unfortunately, these treatments not only destroy malignant cells, but may also have a cytotoxic effect on the rapidly dividing spermatogonia (Howell and Shalet, 1998; Müller, 2003). As a result, spermatogenic failure and infertility may occur during adulthood (Wallace et al., 1991; Meistrich, 1993; Aslam et al., 2000).

In order to improve survival, a small proportion of children, e.g. children with high-risk acute leukaemia, will have marrowablative chemo-radiotherapy followed by haematopoietic stem cell transplantation with stem cells from an allogeneic donor (Duerst et al., 2000). In this population, the risk for long-term sterility is extremely high.

Adolescents and adult men have the option of cryobanking their semen before cancer treatment and, by artificial insemination, IVF or ICSI, they can father children who are genetically their own (for review, see Tournaye et al., 2004). In contrast, pre-pubertal boys cannot benefit from this approach since they do not have completed spermatogenesis. Their seminiferous epithelium contains only Sertoli cells and different types of spermatogonia, among which are the stem cells. In recent years, however, alternative strategies for preserving their fertility have been introduced (for review, see Tournaye et al., 2004).

One of the possibilities under research is protection of the testis by hormonal pre-treatment. Hormonal deprivation schemes could decrease the gonadotoxicity by down-regulating the proliferative activity of the stem cells. This therapy is very time-consuming and has not so far proved effective in men (Meistrich, 1999).

Another possibility is fertility restoration by autologous germ cell transplantation. This technique was introduced by Brinster and Zimmerman (1994) and may become a method with potential clinical use for fertility protection. Recovery of testicular tissue before starting cancer treatment, cryopreservation of the tissue and retransplantation of the spermatogonial stem cells into the seminiferous tubules after cure, might result...
in efficient reconstitution of spermatogenesis (Avarbock et al., 1996). Germ cell transplantation has already proved successful in mice and rats, and recently even in primates (Schlatt et al., 1999). Although this technique seems very promising, there are some major points of concern that should be evaluated before stem cell transplantation in humans can be considered. Beside the low number of spermatogonial stem cells (Meistrich et al., 1993), the testicular tissue taken from the cancer patient may, in the case of leukemia or any other cancer with blood-borne metastases, contain malignant cells. Retransplantation of these cells to the patient might cause malignant relapse and should certainly be avoided.

Xenografting may be a strategy to prevent the transfer of malignant cells into the patient. Grafting possibly contaminated tissue to immunodeficient animals may have major benefit for cancer patients because there is no risk of transferring malignant cells back into the patient. Whenever sperm are produced in the graft, offspring can be obtained through assisted fertilization procedures. Complete spermatogenesis has already been observed in grafts from immature rodents and rabbits, and using the sperm obtained from these grafts resulted in fertile offspring (Honaramooz et al., 2002; Schlatt et al., 2003). In tissue from neonatal marmosets, development up to the stage of spermatocytes has been observed (Schlatt et al., 2002; Wistuba et al., 2004), while grafting of tissue from immature rhesus monkeys resulted in the production of fertilization-competent sperm (Honaramooz et al., 2004).

So far, there has been little research on grafting of human tissue. In this study, the aim was to compare the grafting of testicular tissue to immunodeficient mice from pre-pubertal and adult mice on the one hand, and from adult men on the other. We explored two recipient mouse models: a recipient with a deficient T-lymphocyte function and another with both T- and B-lymphocyte deficiency.

**Materials and methods**

**Tissue donors**
For the murine testicular tissue, donor testes were dissected from F1-hybrid males (C57BL/Cba; Iffa Credo, Belgium) bred in our own facilities. We used both pre-pubertal (3 day old) and adult (3 month old) mice. After dissection, the testes were placed in Dulbecco’s modified Eagle’s medium supplemented with F-12 nutrient mix (DMEM/F-12) (Invitrogen, Belgium) for grafting (see below).

Human adult gonadal tissue was obtained, after written informed consent, from patients undergoing vasectomy reversal. All men had normal spermatogenesis, as proven by histology. The tissue was placed in PBS (Sigma, Belgium) and transferred on ice to the laboratory for immediate grafting within 1 h.

All experiments in this study were approved by both the Ethical Review Board of the university hospital and the Animal Care and Use Committee of the Brussels Free University.

**Host mice**
Two immunodeficient mouse models were evaluated: the Swiss Nude and the SCID-NOD mouse. Swiss Nude mice (Iffa Credo, Belgium) are homozygous for the autosomal recessive nude gene (nu/nu) and develop an abnormal thymus leading to a deficient T-lymphocyte function.

The SCID-NOD mice (generously donated by Prof. Plum from the Rijksuniversiteit Gent, Ghent, Belgium) are homozygous for the severe combined immunodeficiency (scid) mutation and show a blockage in both B- and T-lymphocyte development. Moreover, these mice are deficient in natural killer cells, macrophages and complement activity (Shultz et al., 1995). Therefore, they should theoretically be a more efficient model in order to obviate potential rejection problems during long-term xenografting. Nude mice, however, require simpler rearing conditions and cost less.

**Grafting procedure**
The testes of the pre-pubertal murine donor mice were kept intact. All testes measured between 1.5 and 2.5 mm³. The murine adult and human adult testicular tissue was cut into pieces of 4 mm³. Before grafting, the testicular tissue was immersed in saline containing 2% enrofloxacin (Baytril®; Bayer, Belgium) to reduce the risk of infection.

Recipient mice were anaesthetized with a mixture of ketamine 100 mg (75mg/kg) (Ceva Santé Animale, Belgium) and medetomidin hydrochloride 1 mg/ml (1.0 mg/kg) (Pfizer Animal Health, Belgium), dissolved in saline. The recipients were orchidectomized bilaterally. For grafting, the skin was incised on either side of the dorsal midline, and four to eight testicular tissue pieces were brought under the skin without further fixation.

**Origin and destiny of the testicular tissue**
A total of 171 testicular tissue pieces were transplanted into 41 mice. To some mice, only adult murine or human testicular tissue was grafted. To other mice, both adult human and pre-pubertal murine tissue was grafted. Table 1 gives an overview of the transplanted tissue pieces and recipient mice.

**Histological preparation and staining of the grafts**
Thirty to 195 days after transplantation, the recipient males were evaluated. The dorsal skin was opened, and visible testicular tissue pieces were collected and fixed in Bouin solution. The tissue was dehydrated by treatment with Vacuum Infiltration Processor (Bayer, Germany) and embedded in paraffin. Cross-sections of 5 μm thickness were examined by light microscopy after haematoxylin–eosin staining.

| Table 1. Origin and destiny of the grafted testicular tissue pieces |
|---------------------|---------------------|---------------------|
| Donor tissue       | Recipient: SCID-NOD | Recipient: Swiss Nude |
| Co-grafting        | Co-grafting         |
| Pre-pubertal murine| Adult human         | Pre-pubertal murine |
| Adult human        | Adult human         | Adult human         |
| No. of individual donors | 6                  | 22                 |
| No. of tissue grafts | 12                 | 43                 |
| No. of recipients  | 6                   | 16                 |
overall testicular architecture of all the grafts together with the most advanced germ cell type was assessed. When assessing the most advanced germ cell type, at least one tubule had to contain this cell type for categorizing the tissue. The criteria used to define the spermatogenic cell type were those outlined by Russell et al. (1990).

Statistics
The grafting results were analysed using a $\chi^2$-test. $P < 0.05$ was considered significant.

Results

Murine pre-pubertal tissue

Comparison of two immunodeficient recipient models

A total of 55 pieces of murine pre-pubertal tissue were grafted, 12 to SCID-NOD mice, 43 to Swiss Nude mice. Sclerosis and/or atrophy of the tissue was observed in 33.3% of the SCID-NOD grafts and in 14.0% of the Swiss Nude grafts. In respectively 9.3 and 7.0% of the Swiss Nude grafts, we found Sertoli cell-only pattern or some spermatogonia. Meiotic cells were found in 66.6 and 69.8% of SCID-NOD and Swiss Nude grafts respectively. Complete spermatogenesis with the production of sperm was observed in 33.3% of the SCID-NOD grafts and in 30.2% of the tissue pieces grafted to Swiss Nude mice (see Figure 1a). There were no significant differences between the two mouse models (see Table II). Figure 1b shows the distribution of our results found in murine pre-pubertal tissue grafts.

Influence of time on the development of the transplanted tissue

In 22.2% of the tissue pieces that were analysed <120 days after grafting, sclerosis or atrophy of the tissue was observed. In the pieces analysed ≥120 days after grafting, this proportion was 16.2%. In 11.1% of the transplants analysed <120 days after grafting and in 5.4% of those analysed after 120 days, Sertoli cell-only pattern was found. Differentiated spermatogenic cells were observed in 61.1 and 73.3% of the grafts analysed respectively <120 and >120 days after grafting. Complete spermatogenesis, with production of sperm, was observed in only 11.1% of the tissue pieces that were analysed <120 days after grafting, whereas this proportion rose to 43.2% in the tissue pieces that were analysed ≥120 days after grafting ($P = 0.038$). An overview of these results can be found in Figure 1c.

Murine adult tissue

Since we found no difference between SCID-NOD and nude mice in the grafting experiment with murine pre-pubertal tissue, and considering the higher costs of the SCID-NOD mice, pieces of murine adult testicular tissue were only grafted to Swiss Nude mice. All grafts featured total sclerosis or atrophy. In 59.5% of the grafts, occasional sperm were observed in otherwise completely sclerotic tubules and without the presence of other spermatogenic cells (see Figure 2). These sperm were not considered to be derived from active spermatogenesis but rather remnants of earlier arrested spermatogenic activity; these tubules were therefore considered to be sclerotic.

Human adult tissue

Comparison of two immunodeficient recipient models

Thirteen pieces of human adult testicular tissue were grafted to SCID-NOD mice and 61 to Swiss Nude mice. Again, no significant differences were found between the two immunodeficient mouse models (see Table II). There was no significant difference either between the pieces grafted alone to immunodeficient mice or those co-grafted with pre-pubertal murine tissue (see Table II). In no sample was any meiotic spermatogenic cell observed. However, in 23.1% of the SCID-NOD grafts and in 21.3% of the Swiss nude grafts, some rare spermatogonia
<table>
<thead>
<tr>
<th></th>
<th>Pre-pubertal murine</th>
<th>Adult human</th>
<th>Adult human grafts alone</th>
<th>Total results of adult human grafts</th>
<th>Adult murine</th>
<th>Adult murine grafts alone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SCID-NOD Swiss Nude</td>
<td>Total</td>
<td>SCID-NOD Swiss Nude Total</td>
<td>SCID-NOD Swiss Nude Total</td>
<td>SCID-NOD Swiss Nude Total</td>
<td>SCID-NOD Swiss Nude Total</td>
</tr>
<tr>
<td>Atrophy/sclerosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(with sperm)</td>
<td>4 (33.3)</td>
<td>6 (14.0)</td>
<td>10 (18.2)</td>
<td>5 (62.5)</td>
<td>25 (55.6)</td>
<td>30 (56.6)</td>
</tr>
<tr>
<td>Sertoli cell only</td>
<td>0</td>
<td>4 (9.3)</td>
<td>4 (7.3)</td>
<td>1 (12.5)</td>
<td>10 (22.2)</td>
<td>11 (20.8)</td>
</tr>
<tr>
<td>Up to spermatogonia</td>
<td>0</td>
<td>3 (7.0)</td>
<td>3 (5.5)</td>
<td>2 (25.0)</td>
<td>10 (22.2)</td>
<td>12 (22.6)</td>
</tr>
<tr>
<td>Up to spermatocytes</td>
<td>3 (25.0)</td>
<td>14 (32.6)</td>
<td>17 (30.9)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Up to spermatids</td>
<td>1 (8.3)</td>
<td>3 (7.0)</td>
<td>4 (7.3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sperm</td>
<td>4 (33.3)</td>
<td>13 (30.2)</td>
<td>17 (30.9)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total grafts</td>
<td>12</td>
<td>43</td>
<td>55</td>
<td>8</td>
<td>45</td>
<td>53</td>
</tr>
</tbody>
</table>

Values within parentheses are percentages.
were observed on the basal lamina (see Figure 3a). Most grafts presented either severe sclerosis (69.2 and 57.4% for SCID-NOD and Swiss Nude respectively), or the tubules contained only Sertoli cells (7.7 and 21.3%). Figure 3b shows the results of adult human tissue grafting.

**Influence of time on the development of the transplanted tissue**

In the tissue pieces that were analysed <120 days after grafting, digestion or sclerosis was observed in 40.7%. Significantly more sclerotic tissue pieces were found ≥120 days after grafting (70.2%, \( P = 0.025 \)). Sertoli cell-only pattern was found in 25.9 and 14.9% <120 and >120 days after grafting respectively, and spermatogonia were observed in 33.3 and 14.9% of the tissue grafts analysed <120 and >120 days after grafting respectively (Figure 3c).

**Discussion**

Autologous germ cell transplantation is a promising technique for pre-pubertal cancer patients at risk of losing their fertility. One of the major concerns, however, is the risk of transferring malignant cells back into the patient. Jahnukainen et al. (2001) demonstrated that as few as 20 leukaemic cells were able to cause a malignant recurrence in rats. It is therefore mandatory to develop techniques to detect and eventually remove these malignant cells from the cell suspension to prevent them being reintroduced into the patient. Fluorescence-activated cell sorting is one strategy. Although unique surface markers for spermatogonial stem cells have not yet been described (Dobrinski et al., 1999; Shinohara et al., 2000), Fujita et al. (2005) demonstrated that FACS sorting of murine germ cells allows retransplantation without inducing leukemia. Further research will be needed, however, before this technique might find its way to a clinical application.

As a theoretical alternative option for the restoration of fertility without possible malignant relapse, testicular tissue may be frozen before childhood cancer treatment and subsequently thawed and xenografted to immunodeficient animals, or alternatively, fresh tissue may be xenografted, and the spermatids or spermatogonia obtained from the xenografts cryopreserved. The spermatids or sperm produced in the grafts may be used for assisted fertilization.

Xenografting of fresh and cryopreserved human ovarian tissue under the skin or kidney capsules has already led to some interesting results. Several groups found that primordial follicles in xenografts developed up to the antral stage and that...
these antral follicles had the ability to undergo peri-ovulatory changes following a luteinizing stimulus (Weissman, 1999; Aubard, 2003; Gook et al., 2004). Recently, Gook et al. (2005) even observed metaphase II oocytes in frozen–thawed human ovarian follicle grafts, thereby proving that the developmental potential of the follicles was preserved.

The testis looks less promising for grafting because it has a thick tunica, a complex vascular architecture and is very sensitive to ischaemia (Nugent et al., 1997). Our results show that the differentiation of neonatal murine testicular tissue in an immunodeficient recipient is possible. We found meiotic cells in 69.1% and complete spermatogenesis in 30.9% of the pre-pubertal murine tissue grafts, thereby indicating that our grafting procedure was successful. These results corroborate those reported by another group who further showed that the sperm from neonatal murine grafts were functional (Honaramooz et al., 2002; Schlatt et al., 2003). Moreover, time was found to have a positive influence on the development of the transplanted tissue, since there were significantly more tissue pieces with complete spermatogenesis if analysis was done after ≥120 days than there were if analysed <120 days after grafting. This might be explained by the time needed for proper angiogenesis before the initiation of spermatogenesis. There was no difference between the two immunodeficient mouse strains. Ohta and Wakayama (2004) also demonstrated that, for the xenografting of testicular tissue in mice, the recipient mouse strains. Ohta and Wakayama (2004) also demonstrated that, for the xenografting of testicular tissue in mice, the recipient is of less importance.

Our experiment, which involved the use of adult murine tissue, was less successful since none of the grafts showed spermatogenic activity. The human adult testicular tissue grafted to immunodeficient mice showed no spermatogenic activity either. None of the grafts showed any meiotic germ cell and most of the grafts were sclerotic. Moreover, the longer before the tissue was recovered from the host, the more atrophy or sclerosis was found. However, in 21.6% of the grafts, survival of some spermatagonia was observed. We obtained similar results with the murine adult tissue grafts. Schlatt et al. (2002), who also achieved poor results with adult mouse and photoregressed hamster testicular tissue, suggested that immature tissue might have a better ability to survive periods of ischaemia or might be more effective for angiogenesis in the host than the adult tissue. However, after grafting of neonatal marmoset testicular tissue, they found an arrest of spermatogenesis at the spermatogonial level, similar to our results obtained with adult human testicular tissue. These findings were confirmed by Wistuba et al. (2004) who added that the marmoset blockade was independent of donor age. The reasons for this developmental failure have not been solved so far. However, since Honaramooz et al. (2004) demonstrated that the grafting of testis tissue from immature macaque into host mice resulted in the production of fertilization-competent sperm in the xenografts, there might be two possible explanations. Except for the developmental status of the tissue at the time-point of grafting, the structural organisation of the seminiferous epithelium might influence the development of the testicular tissue, since the macaque’s epithelium is organized more similarly to that of rodents than to that of human or marmoset (Luetjens et al., 2005).

We certainly cannot simply extrapolate the results obtained with the murine tissue to the human testes, because of different hormonal requirements and architecture. However, there is still hope that the grafting of human pre-pubertal testicular tissue might result in differentiation of the stem cells and initiation of spermatogenesis, as was seen with pre-pubertal murine tissue.

Xenografting may also be a method of testing the risk of transplanting malignant cells. If over time no malignant cells are observed in the xenografts, tissue of the same testis may be autotransplanted or autografted to a cured patient. This method has already been proposed for ovarian cortex grafting (Shaw et al., 1996).

Currently, the application of xenografting of human tissue should be limited to research. If the xenografting of pre-pubertal human tissue were to prove successful, many ethical, legal and clinical issues would need to be evaluated before the technique could be clinically applied. First, there are the biological risks: the introduction of unknown animal infectious agents into the grafted tissue is a serious reason for concern, especially when the matured germ cells are to be used for fertilization (Aslam et al., 2000). Not only is the risk of zoonosis in the offspring important, but the risk of a possible epidemic in the human population should also be considered (Brown et al., 1998). Also, ethical guidelines need to be outlined that concern the procurement, preservation and use of pre-pubertal testicular tissue. Obtaining proper informed consent from children is a problem and substituted consent from the parents should be limited to the safe-keeping of the tissue (Bahadur and Ralph, 1999; Bahadur et al., 2000). Testicular biopsy is an invasive procedure that may have complications such as haematoma and infection. Therefore, the procedure must be well motivated and can only be justified when it is assumed to lead to an improvement in the quality of future life. Moreover, the surgery should be optimally timed, with regard to cancer treatment and other necessary invasive procedures (Bahadur et al., 2000; Hovatta, 2001).

From our study, we may conclude that mouse hosts can at least maintain human spermatogonial activity for a period of 195 days. However, in order to prove xenografting as a method for external germ line storage, the transplants should have a more immature developmental stage. Moreover, development of the grafted testicular tissue might be influenced, both by the developmental status of the tissue at the time point of grafting, as by the structural organisation of the seminiferous epithelium. Therefore, we believe that research on the xenografting of pre-pubertal human testicular tissue is needed. In the future, the effects of cryopreserving the human tissue before transplantation should be evaluated since, in a clinical approach, cryopreservation of the tissue might be needed. If xenografting of fresh or cryopreserved pre-pubertal testicular tissue results in spermatogenesis, and if this approach proves to be safe, it may become a strategy for omitting malignant relapse.

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