Testicular Xenografts: A Novel Approach to Study Cytotoxic Damage in Juvenile Primate Testis

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Abstract
The underlying primary damage to the testis caused by chemotherapeutic regimens during childhood is largely unknown. Xenografting of monkey testes was successfully applied in maturation of juvenile testis to the point of complete spermatogenesis. This allows us to manipulate developing primate testis without direct treatment of patients. This new model is validated establishing the effects of cytotoxic treatment in the immature primate testis. Male castrated nude mice received eight s.c. grafts of juvenile monkey testicular tissue and, 28 weeks later, were injected with busulfan (38 mg/kg, i.p.) or vehicle. Graft numbers, size, and histology were examined. Grafts showed pubertal induction of spermatogenesis to the level of pachytenocyte's at point of busulfan treatment and further progressed to the level of round spermatids in control samples at 4 weeks. Busulfan treatment caused a statistically significant decrease in the number of seminiferous tubules containing germ cells. Type B spermatogonia and more advanced stages of spermatogenesis were depleted. A statistically significant decrease to pretreatment level was observed in the number of type A pale and centrally located spermatogonia. Busulfan did not affect type A dark spermatogonia. Occasionally, elongating spermatids were detected in busulfan-treated grafts. Observations show that busulfan selectively destroys differentiating spermatogonia whereas some of the spermatocytes present at the moment of cytotoxic insult are able to continue differentiation. Data indicate that xenografting of testicular monkey tissue is a valid approach to detect the busulfan-induced germ cell damage and serves as a powerful experimental tool to study cytotoxic effects in developing primate testis. (Cancer Res 2006; 66(7): 3813-8)

Introduction
Patients undergoing marrow ablation therapy before bone marrow transplantation or peripheral stem cell rescue are at high risk of testicular dysfunction. Majority of patients who receive combined treatment with busulfan and cyclophosphamide as marrow ablation therapy show late severe impairment of reproductive function (1, 2). The underlining causes of cytotoxicity, including damage from busulfan, to spermatogenesis in the prepubertal testis are not studied in humans.

Busulfan (1,4-butanediol dimethanesulfonate, Myleran) is an alkylating agent which does not affect DNA synthesis but causes inhibition of subsequent mitosis by alkylation of nucleotides leading to DNA intrastrand cross-links (3). The damaging effect of busulfan on the spermatogenic epithelium is well studied in rodent models (4, 5). High-dose administration of busulfan (45 mg/kg) in mice has been shown to eliminate spermatogenic stem cells and render animals permanently sterile (6). Administration of a low dose (15-30 mg/kg) reduces the number of stem cells. Duration of sterility is related to the extent of stem cell depletion (5, 6). During the first week after cytotoxic treatment, surviving stem cells selectively undergo self-renewal. This first period of stem cell colonization is only later followed by differentiating divisions of premeiotic germ cells starting a few weeks after the busulfan exposure (6). The spermatogonial population is selectively affected by busulfan. The majority of post spermatogonial development stages continue differentiation, gradually maturing into spermatids and disappearing after maturation with normal spermatogenic kinetics (4, 6). It is not known if this is true of primate testis. Significant differences in spermatogonial morphology and physiology, as well as in the regenerative processes of spermatogenesis, after irradiation have been described between rodents and primates. In primates, irradiation causes azoospermia by depleting the spermatogonial pool, whereas in rodents azoospermia is caused by an arrest of spermatogonial expansion (7, 8).

Testicular development is similar in rhesus monkeys and men, including the regulation of the neonatal hypothalamic-pituitary-testis axis, the existence of two marked periods of Sertoli cell proliferation, and the prepubertal increase in the number of spermatogonia unassociated with the initiation of spermatogenesis at puberty (9, 10). A specific feature during initiation of spermatogenesis in primate testis is the appearance of spermatogonia in the central region of the seminiferous tubules (11, 12). These spermatogonia do not have contact to the basement membrane and often show a morphologic appearance of type A spermatogonia (12). Some of them degenerate (11). In the testes of young boys, increased incidence of these adluminal spermato-gonia is seen at 3 and at 8 to 9 years of age (11). These periods are followed by periods of marked spermatogonial proliferation. Centrally located spermatogonia have also been observed in juvenile Rhesus monkey testis (12, 13). Testosterone treatment is shown to significantly increase their number in these immature testes (12). The rhesus monkey is an excellent established animal model for exploring the cytotoxicity of cancer treatments in the immature human testis (14). On the downside, high costs associated with the generation and maintenance of macaque colonies and special demands on experimental studies with nonhuman primates have prevented intense use of rhesus monkeys to explore the testicular toxicity of oncological therapies.
Grafting immature rhesus monkey tissue into immunodeficient mice was recently introduced as a novel strategy to mature the immature testicular tissue (15). Grafting of testis tissue from immature rhesus monkeys (13-month-old) into host mice resulted in the acceleration of testicular maturation, producing fertilization-competent sperm 7 months after grafting (16). This maturation process was confirmed by other studies using rhesus (15) and marmoset monkeys (17). It is therefore intriguing to explore whether ectopic xenografting of immature testis tissue from monkeys and men is a novel model to determine gonadotoxic actions of drugs and other agents on testicular differentiation and spermatogenesis.

The aim of the present study was to describe the depletion and subsequent recovery of spermatogonial stem cells and differentiating germ cells in rhesus monkey xenografts when the mouse host was exposed to a single i.p. injection of busulfan (38 mg/kg) or vehicle. We are presenting evidence that the xenograft model is a powerful and novel experimental approach to explore cytotoxic effects in the immature primate testis.

**Materials and Methods**

**Animals and treatments.** Testicular tissue was obtained from two juvenile male rhesus monkeys (16 and 19 months of age). In this species, the onset of puberty, as reflected by the initiation of nocturnal testosterone secretion, occurs at ~30 months of age (18). Monkey testes were decapsulated and cut into 50 to 80 fragments. The fragments of tissue (~0.5-1.0 mm³) were dissected and maintained in ice-cold sterile Leibovitz-L15 medium (Life Technologies, Inc., Paisley, United Kingdom) until grafting, which occurred within 3 hours.

Five- to seven-week-old immunodeficient male nude mice (CrlNu/Nu; Charles River Laboratories, Wilmington, MA) were used as recipients (n = 10). Animals were castrated through scrotal incisions and eight testicular fragments of monkey tissue were placed under the dorsal skin on either side of dorsal midline by using cancer implant G13 needle (Popper Precision Instruments, Lincoln, RI). Mice were maintained in groups of five to six per cage with food and water available ad libitum.

Recipient mice were randomly distributed among three experimental groups. The pretreatment group contained two mice, which were analyzed 28 weeks after grafting without other treatments. At the same time, four mice (mean body weight ± SE, 25 ± 2 g) were treated with a single i.p. injection of busulfan (Sigma-Aldrich, Inc., St. Louis, MO) dissolved in vehicle (saline/DMSO, 1:1) at 38 mg/kg body weight corresponding to body surface area–related doses of 100 ± 3 mg/m² (ref. 19; busulfan group). The control mice only received i.p. injection of vehicle (control group). Four weeks after treatment, mice were weighed and blood was collected by cardiac puncture. The seminal vesicles were dissected and weighed, and the number of visible grafts was recorded. The testicular tissue was dissected from the skin, weighed, and fixed in Bouin solution. The fixed grafts were weighed before embedding. All animal experiments were approved by and done under the guidance of the Animal Care and Use Committee at the University of Pittsburgh School of Medicine.

**Histology and statistical analysis.** The tissue was fixed for 18 to 24 hours in Bouin solution, transferred for storage into 70% ethanol, and embedded in Technovit (Kulzer, Germany) for sectioning at 2 µm. Tissue sections were stained with periodic acid–Schiff’s reagent/Gill’s hematoxylin and examined with oil immersion under the light microscope. The degree of tissue necrosis was approximated in the sections representing the most expanded 20 to 23 cross-sectional areas of individual grafts. At least 20 cross sections of seminiferous tubules were analyzed from each of the 13 grafts in the pretreatment group, the 23 grafts in the control group, and the 20 grafts in the busulfan group. The necrotic area in the graft was expressed as a percentage of total area after computing the data using MetaMorph imaging software (Universal Imaging, West Chester, PA). The same cross sections were also scored to determine the relative number of tubules showing defined germ cell types (spermatogonia, preleptotene spermatocytes, pachytene spermatocytes, round, and/or elongating spermatids) or a complete absence of germ cells (Sertoli cell only). The identification of type A dark and type A pale spermatogonia followed the scheme of Clermont and Leblond (20). Tubules with spermatogonia, which were located in the center of seminiferous tubule and were detached from the basement membrane, were scored as a separate category named adluminal spermatogonia. The light microscopic determinations were conducted by one observer (K.J.).

The data are presented as mean ± SE. The Mann-Whitney U test was employed for single statistical comparison of independent groups of samples and the Kruskall-Wallis analysis with Dunn's posthoc test for multiple comparisons of independent groups of samples. *P < 0.05* was considered to be statistically significant.

**Results**

**Effects of a single i.p. dose of busulfan on graft size and survival.** Four weeks after a single i.p. injection of busulfan, a median number of 5 ± 1 testicular grafts from the eight originally placed grafts were recovered per recipient (62% survival). This was not significantly different from the graft numbers that were recovered from the control group (6 ± 1; 75% survival) or from the two mice sacrificed at the time of busulfan/vehicle injection (7 ± 1; 88% survival). There was also no difference in postfixation graft weights between the three groups (Table 1). No significant increase in graft necrosis was detected (Table 1). Recipient body weight or weight of their seminal vesicles was not affected by busulfan treatment (Table 1).

**Effects of a single i.p. dose of busulfan on testicular differentiation.** At the time of grafting, the testicular tissue from both donor monkeys consisted of seminiferous cords with spermatogonia as the most advanced germ cell type (not shown).

**Table 1.** Host and graft descriptive and number of surviving grafts per host when studied before and after treating host with busulfan (38 mg/kg) or vehicle as control

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Pretreatment (mean ± SE)</th>
<th>After vehicle treatment (mean ± SE)</th>
<th>After busulfan treatment (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>2</td>
<td>27.5 ± 4.5</td>
<td>24.5 ± 0.3</td>
<td>25.5 ± 1.6</td>
</tr>
<tr>
<td><strong>Seminal vesicle weight (mg)</strong></td>
<td>2</td>
<td>8.0 ± 1.0</td>
<td>6.0 ± 0.7</td>
<td>6.0 ± 1.3</td>
</tr>
<tr>
<td><strong>No. grafts per host</strong></td>
<td>13</td>
<td>7 ± 1</td>
<td>6 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td><strong>Graft weight (mg)</strong></td>
<td>14</td>
<td>6.6 ± 1.3</td>
<td>5.4 ± 1.3</td>
<td>7.2 ± 1.4</td>
</tr>
<tr>
<td><strong>Proportion of necrotic area on section (%)</strong></td>
<td>13</td>
<td>22 ± 8</td>
<td>23 ± 6</td>
<td>16 ± 5</td>
</tr>
</tbody>
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Twenty-eight weeks after grafting (Fig. 1A and B), spermatogenesis was initiated. Altogether, 21% of the seminiferous tubules contained type B spermatogonia and 11% of them spermatocytes (Figs. 1A and 2). Spermatids were not seen. Four weeks later, significantly higher number of seminiferous tubules (37%) contained type B spermatogonia (Fig. 2) and spermatids appeared in the seminiferous tubules (Fig. 1C). Tendency towards a lower incidence of tubules with type A pale and central spermatogonia (Fig. 2) was detected 32 weeks after xenografting when compared with that at 28 weeks. This decrease did not reach statistical significance. At both time points, 33% to 39% of seminiferous tubules showed Sertoli cell only pattern (Fig. 2, SCO).

When the most advanced germ cell type was studied in the grafts from pretreatment and vehicle-treated controls, 85% (11 of 13) of pretreatment grafts and 74% (17 of 23) of vehicle-treated grafts showed spermatogenesis that progressed well beyond type B spermatogonia (spermatocytes and spermatids; Fig. 3). The most advanced germ cell types were preleptotene spermatocyte in 31% (4 of 13) and pachytene spermatoocyte in 54% (7 of 13) of the pretreatment grafts. The most advanced germ cell types were preleptotene spermatocyte in 35% (8 of 23), pachytene spermatoocyte in 30% (7 of 23), and round spermatids in 9% (2 of 23) of the vehicle-treated grafts. Thirty-two weeks after grafting, monkey spermatogenesis had reached the postmeiotic stage.

Four weeks after busulfan treatment (32 weeks after grafting), incidence of seminiferous tubules depleted from germ cells (Sertoli cell only pattern) was significantly increased (Figs. 1D and 2). Significantly fewer tubules contained type B spermatogonia or more advanced stages of spermatogenesis when compared with vehicle-treated controls (Fig. 2). Significantly fewer tubules contained type A pale spermatogonia and adluminal spermatogonia when compared with pretreatment controls (Fig. 2). No significant change was detected in the number of tubules containing type A dark spermatogonia (Fig. 2).

When most advanced germ cell types were studied in the busulfan-treated grafts, 40% (8 of 20) of grafts showed no initiation of spermatogenesis. Only type A spermatogonia or adluminal spermatogonia were detected (Figs. 1D and 3). Spermatogenesis was shown to differentiate to the level of type B spermatogonia in 25% (5 of 20) of the busulfan-treated grafts and beyond this level in 35% (7 of 20) of grafts (Fig. 3). In 30% (6 of 20) of the grafts, the spermatogenesis reached the level of preleptotene spermatocytes, and in 1 of 20 grafts (5%), elongated sperm without other maturing germ cells were detected (Fig. 1D).

This observation highlights that 32 weeks were sufficient to complete the first round of spermatogenesis in the grafted tissue. In all grafts, a morphologically normal interstitium with blood vessels, macrophages, peritubular, and Leydig cells was observed. Busulfan did not affect the light microscopic morphology of Sertoli cells.

Discussion

Our results show that xenografted testicular tissue from juvenile nonhuman primates is sensitive to the cytotoxic drug administered to the host animal. Detailed histologic analysis revealed that busulfan treatment eradicated the differentiating germ cells from the primate testis. A significant decrease in the number of seminiferous tubules containing type B spermatogonia was detected along with an increase in Sertoli cell only pattern. Taking this into consideration, we also observed a significant decrease in the number of seminiferous tubules with spermatocytes and spermatids. A rational summary of this significant observation would be that the lack of visible progression of spermatogenesis to the point of spermatocytes and spermatids 4 weeks after busulfan treatment indicates the depletion of type B spermatogonia at the time of this treatment. These data are in concordance with previous reports on the depletion and repopulation of monkey spermatogonia.

References

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spermatogonia following testicular irradiation (21, 22), suggesting that the type B spermatogonia in the primate testis is highly sensitive to DNA damage and that, exclusively, these cells are depleted (21). During the 11-day postirradiation period, the pool of differentiating type A pale spermatogonia is also depleted whereas the number of type A dark does not change significantly (21). In the present study, analyzing xenografts 4 weeks after busulfan exposure, we detect spermatogenic damage comparable to the histologic pattern at 1.5 weeks after irradiation. The delayed response may be related to the rather low dose of busulfan used in our study or to a potentially different busulfan response in type A dark spermatogonia when compared with irradiation.

During the initiation of human spermatogenesis, the number of seminiferous tubules with spermatogonia and the number of type A pale spermatogonia are known to decline concurrently with the increase in number of type B spermatogonia (11). This developmental effect is seen in the testis of young boys (11). A similar effect was also detected in monkey xenografts 32 weeks after transplantation (Fig. 2). This developmental decrease in number of type A pale spermatogonia must be separated from the toxic effects of busulfan in pubertally developing xenografts. Busulfan was shown to significantly decrease the number of tubules with type A pale and adluminal spermatogonia from the pretreatment level but it did not affect the number of tubules with type A dark spermatogonia. These observations further suggest that busulfan toxicity in testicular xenografts closely resembles the previously reported irradiation toxicity in the primate testis (21).

Our data show that busulfan was acting selectively on spermatogonia. Elongating sperm were observed in one xenograft in the absence of other differentiating germ cells (Fig. 1D). The 4-week postexposure period used in our study represents 2.5 times the length of the seminiferous epithelial cycle in the rhesus monkey (23). The presence of elongated spermatids indicates that the preleptotene or leptotene spermatocytes, which were present in the xenografts at the moment of the cytotoxic insult, continued their differentiation. The absence of any other germ cell reveals that type B spermatogonia were the primary target of busulfan and that these cells were efficiently depleted. We assume that during the 4-week period, any meiotic germ cell present at the time of busulfan treatment matured and was released from the epithelium via spermiation. Quite commonly, incomplete cellular associations due to the absence of one or two generations of differentiating germ cells are noted following testicular irradiation in men and monkeys (22, 24) whereas in rodents, such incomplete epithelial germ cell associations are rarely observed (25). In 30% of busulfan-treated grafts, seminiferous tubules with spermatogenesis up to level of preleptotene were detected. In accordance to the length of epithelial cycle in rhesus monkeys (23), these cells must have been type A spermatogonia at the time of busulfan treatment. This observation indicates that type A spermatogonial proliferation

![Figure 2. Relative number of seminiferous tubules fully depleted of germ cells (Sertoli cell only), containing type A dark spermatogonia (Ad), type A pale spermatogonia, adluminal spermatogonia, type B spermatogonia and more advanced (spermatocytes and spermatids) stages of spermatogenesis (mean ± SE) in the testis of xenografted juvenile monkey testicular tissue before and 4 weeks after injection of vehicle or busulfan into the host.](image-url)
leads to an immediate differentiation into type B spermatogonia after busulfan treatment, which is strikingly similar to the pattern of recovery in men and monkeys after irradiation (22). However, this pattern of recovery is different from that in rodent where the initial period after cytotoxic treatment or irradiation is characterized by self-renewing divisions of type A spermatogonia in the absence of germ cell differentiation (6).

In the xenografts, spermatogenesis was initiated with no morphologic distinction from normal pubertal development. The process proceeded with time after grafting and reached the level of pachytene spermatocytes at 28 weeks and the postmeiotic level at 32 weeks. These findings are in accordance with earlier observations on the acceleration of spermatogenesis in testicular xenografts from immature (13-month-old) rhesus monkeys (16). A high frequency of seminiferous tubules with adluminal spermatogonia was detected at both control time points (Figs. 1A and B and 2), confirming the earlier reports both in monkeys and men (11, 12). Adluminal spermatogonia were depleted after busulfan treatment, indicating that they share the high sensitivity to busulfan with type A pale and type B spermatogonia. Observations suggest that xenotransplantation can be successfully applied to generate models for the study of the physiologic changes at the beginning of primate puberty and also any toxic interference at different developmental phases by using time period modifications to variables such as xenografting and toxic treatments.

An important issue to validate testicular grafting as a model for cytotoxic exposures in cancer patients will be the drug penetration into the xenografted tissue. Does the drug reach the xenografted tissue? What are the equivalent doses to human treatments? In the present study, a busulfan dose of 38 mg/kg body weight was used. In previous mouse experiments, this dose was effective to severely reduce spermatogonial stem cell numbers but recovery was possible (5, 6). In the mouse, 70 days after injecting a busulfan dose of 30 mg/kg body weight, 47% of tubular cross sections show recovery of spermatogenesis whereas this figure changes to only 3.4% after injection of 45 mg/kg (6). In the present study, 28 days after 38 mg/kg of busulfan per host body weight, 21% of monkey seminiferous tubules showed type B spermatogonia and must therefore be considered regenerating seminiferous tubules. Because similar proportions of stem cells were killed in mouse testis and xenografts after comparable busulfan doses had been delivered, we assume that drug penetration and drug actions are identical in the mouse testis and the monkey xenograft.

It is more difficult to assess if the presently used dose serves as a model for the doses and testicular effects observed after clinical use of busulfan. The presently used 38 mg/kg of host mouse body weight corresponds to the body surface area–related dose of 100 mg/m². Busulfan doses of 16 mg/kg (corresponding to 600 mg/m²) are clinically used for marrow ablation before bone marrow transplantation or peripheral stem cell rescue (26). This busulfan dose, when combined to cyclophosphamide (120 mg/kg), is known to cause azoospermia in adult male patients (27). In a majority of these patients, recovery of fertility starts within the second year posttransplantation and spermatogenesis will continue recovery up the first 5 years posttreatment (27). Our observation that a busulfan dose of 100 mg/m² did not affect the number of type A dark spermatogonia in xenografts indicates that a fast recovery of spermatogenesis would occur. The effect in monkey xenografts would therefore correspond well to the testicular effect expected after a six times lower busulfan dose than that clinically used as marrow ablation (600 mg/m²). Further studies are needed to obtain an accurate dose-response of busulfan in xenografted monkey testes.

We conclude that ectopic xenografting is a simple and powerful approach for the study of cytotoxic damage in juvenile and pubertal primate testes. Grafting allows timed initiation of primate spermatogenesis, which can then be manipulated using cytotoxic treatment of the host mice. Limited numbers of immature monkey testis can be subjected to a large number of conditions, exploring the response of testicular tissue to drugs and toxic agents. Treatment of groups of nude mice seems to be more ethical and is certainly more cost-efficient than the treatment of large groups of immature monkeys. The small amount of tissue needed in these experiments could, in the near future, also enable similar studies using human tissue. This approach opens new avenues to study the poorly characterized testicular toxicity caused by cancer treatment at childhood.

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