Brief Report

Leydig cell injury as a consequence of an acute graft-versus-host reaction

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Abstract

Hematopoietic stem cell transplantation (HSCT) is associated with significant posttransplant gonadotoxicity. This deficit has been mainly attributed to pretransplant conditioning but lower sperm counts in humans also appear to be associated with graft-versus-host disease (GVHD) following allogeneic HSCT. However, the mechanisms leading to diminished spermatocyte levels during GVHD remain unknown. Here we demonstrate that injury to intratesticular cells occurs in unconditioned F1 mice following the infiltration of donor alloreactive T-cells during an acute graft-versus-host reaction (GVHR). Using computer-aided quantitative microscopic morphology we demonstrate that the nadir of Leydig cell volume density coincides with the peak of intratesticular infiltration by donor T-cells. Injury to Leydig cells correlates with an intratesticular inflammatory response characterized by interferon-γ and tumor necrosis factor-α production. These results demonstrate impairment of testosterone-producing Leydig cells during a local alloresponse, thus representing a mechanism that contributes to gonadal insufficiency following allogeneic HSCT.
Introduction

Hematopoietic stem cell transplantation (HSCT) is the preferred therapy for malignant and non-malignant disorders \textsuperscript{1,2}. To maintain quality of life following HSCT, an appropriate reproductive status is required. A retrospective study conducted by the European Group for Blood and Marrow Transplantation (EBMT) determined, however, that the overall posttransplant pregnancy rate is currently as low as 0.6\% \textsuperscript{3}. These result suggested that female HSCT recipients become transiently or permanently infertile. Likewise, most male HSCT recipients are reversibly or irreversibly infertile due to germ cell injury and/or Leydig cell (LC) insufficiency \textsuperscript{4-6}.

Posttransplant gonadotoxicity has been attributed to the type and intensity of the pre-HSCT conditioning used. Here, myeloablative and nonmyeloablative reduced-intensity conditioning regimens can both induce spermatocyte injury and impaired LC function \textsuperscript{5,7,8}. Gonadal dysfunction has, however, also been associated with the development of graft-versus-host disease (GVHD) since sperm counts in long-term survivors of allogeneic HSCT are much lower in GVHD patients \textsuperscript{9}. GVHD remains a major transplant-related toxicity that is initiated by the recognition of host alloantigens by donor-derived mature T-cells, leading to injury in a restricted set of target tissues (i.e. skin, liver, gastrointestinal tract) \textsuperscript{10,11}. The mechanisms leading to diminished spermatocyte levels secondary to GVHD remain unknown. Here we tested the hypothesis that injury to intratesticular cells occurs following the infiltration of alloreactive donor T-cells during an acute graft-versus-host reaction (GVHR).

Materials and Methods

Male B6.SJL-PtprcaPep3b/BoyJ mice (B6.CD45.1; H-2\textsuperscript{b}, CD45.1\textsuperscript{+}) were purchased from the Jackson Laboratories (Bar Harbor, ME) and male [C57BL/6 x DBA/2]F\textsubscript{1} (B6D2F\textsubscript{1}, H-2\textsuperscript{bd}) mice were obtained from Charles River, France. Animals between 6 and 10 weeks of age were kept according to federal regulations. GVHD was induced in non-irradiated B6D2F\textsubscript{1} mice by infusion of 60x10\textsuperscript{6} allogeneic splenocytes from B6.CD45.1 mice containing \textasciitilde15x10\textsuperscript{6} T-cells \textsuperscript{12,13}. This transplantation model allowed us to examine in a longitudinal study the acute GVHR-mediated testicular pathology independent of tissue injury effected by chemo/radiotherapy. Control mice received the same number of cells from syngeneic B6D2F\textsubscript{1} donors.

Testosterone in blood serum was measured by radioimmunoassay validated for murine samples \textsuperscript{14}. For analysis of the LC compartment, we used computer-aided quantitative microscopic morphometry. Transversal paraffin sections of paraformaldehyde-fixed testicular tissue (6 \textmu m) were treated with 0.1\% protease/0.5 M Tris and labeled with an antibody directed against Cytochrome p450scc (Cy p450scc; Ab1244; VWR International Life Science, Lucerne, Switzerland). Cy p450scc is a key enzyme for steroid biosynthesis and, in testis, it is exclusively
localized to mitochondria in LC\textsuperscript{15}. Bound antibody was revealed with biotin-conjugated goat anti-rabbit polyclonal Ab, ExtrAvidin (Sigma, Buchs, Switzerland) and aminoethylcarbazole. The aggregate area of the specific immunostaining within a random high-powered microscope field (758.6 x 508.4 \mu m\textsuperscript{2}) was determined by detection of gray-scale in digitized microscopic 2-D images (Leica DMRE microscope, Nidau, Switzerland, and Soft Imaging System, Lakewood, CO). The LC volume density (\%) was then calculated by standard methods as described\textsuperscript{16,17}.

For analysis of cytokine mRNA expression, quantitative PCR was performed using SYBR Green\textsuperscript{TM} (PE Biosystems, Rotkreuz, Switzerland), with GAPDH as a reference\textsuperscript{13}. Primers used were: IFN-\gamma sense: GCTGATGGGAGGAGATGTCTACAC, antisense: GACACATTGAGTGCTGTCTGG; TNF-\alpha sense: AGGTCTACTTTGGAGTGTCATTGC, antisense: ACATTGAGGCTGCCAGTGATCGG; GAPDH sense: ACCATGATGGAAGTCCAATGAAG, antisense: GGTGAAGGTCGGTGTAAGC. PCR data from the two transplant groups were compared to each other by calculating \Delta\DeltaCt values.

For immunohistochemical analyses of CD3\textsuperscript{\epsilon} and CD11b, frozen testicular sections (6 \mu m) were incubated with specific biotin-conjugated moAbs (clones 145-2C11 and M1/70, respectively; Pharmingen, San Diego, CA), ExtrAvidin- and aminoethylcarbazole. For confocal microscopy, frozen sections were simultaneously stained with biotin and fluorescein-conjugated moAbs to CD3\textsuperscript{\epsilon} and CD45.1 (clone A20), respectively. Strepatavidin-Cy3 was used as secondary reagent (Zymed Laboratories, Switzerland). Two-color immunofluorescent sections were analyzed using a confocal microscope (Carl-Zeiss AG, Feldbach. Switzerland).

**Results and Discussion**

To evaluate gonadal insufficiency during acute GVHR, we assessed serum testosterone. Two to three weeks following the infusion of alloreactive T-cells into unconditioned, haploidentical F\textsubscript{1} mice, median serum testosterone levels were diminished relative to syngeneically transplanted mice (1.7, range 0.1-4.3 vs. 3.4, range 1.2-63.5 nmol/l; Fig. 1a). As lower systemic testosterone did not \textit{per se} prove injury to intratesticular cells, however, we then analyzed testosterone-producing cells using expression of Cy p450scc as marker for Leydig cells\textsuperscript{15}. In recipients of alloreactive T-cells, expression levels of Cy p450scc were lower than in syngeneically transplanted mice (Fig. 1b-e). To quantify this reduction, computer-aided microscopic morphometry was employed to determine the LC volume density (Fig. 1f). We found that three weeks posttransplant, the LC volume density significantly decreased from a normal level of 4.70±1.06 \% to a value of 1.78±1.28 \% in mice with acute GVHD. Since transplant recipients had not received pretransplant conditioning, this result indicated injury to LC (decreased LC number or, alternatively, LC dysfunction as a result of mitochondrial damage) due to an acute GVHR. This testicular injury was temporally related to GVHD-associated impairment of other organs, (i.e. gastrointestinal tract and liver) as evidenced by histopathology at 3 weeks after T-cell transfer (data not shown). Similar to the other target organs in mice surviving GVHD (data
not shown), the LC compartment had recovered from the insult at 7 weeks posttransplant (Fig. 1f).

Following activation by host professional antigen presenting cells (APC) in secondary lymphoid organs, local APCs recruit primed alloreactive T-cells where they elicit cutaneous and hepatic GHVD during the disease effector phase. To establish whether donor T-cells infiltrated the gonads, we examined testes from transplant recipients at two weeks after transplantation (Fig. 2a-b). We found that CD3+ cells were very rare in normal testes but were abundant in the LC area of mice with GVHD, i.e. the interstitial space separating the seminiferous tubules. Confocal microscopy confirmed that these T-cells were CD45.1-positive and were thus derived from the donor inoculum (Fig. 2c). The infiltration of donor T-cells peaked at three weeks after transplantation (data not shown), coinciding with the nadir in LC volume density. To further detail their function, testicular tissue was analyzed for cytokine expression since the activation of alloreactive T-cells has been linked to secretion of the Th1 signature cytokine Interferon-\(\gamma\). Quantitative RT-PCR analysis revealed that intratesticular transcription of IFN-\(\gamma\) mRNA was enhanced by more than 100-fold as a result of the GVHR (Fig. 2d, upper panel). Expression levels were upregulated for the entire observation period although they decreased with time.

Our results support the premise that LC are lost or dysfunctional as a consequence of a local alloresponse, representing a mechanism that contributes to gonadal insufficiency following HSCT. Similar to other target organs, tissue-resident professional or semiprofessional APC may recruit activated T-cells to the testis which initiate a local inflammatory immune response. Evidence for such intratesticular inflammation was provided by enhanced numbers of intratesticular macrophages (Fig. 2e-f) and increased transcripts for TNF-\(\alpha\) (Fig. 2d, lower panel) in mice with GVHD. Due to the spatial proximity of donor T-cells to MHC-bearing host LC, it is conceivable that direct T-cell-mediated cytotoxicity is responsible for target cell injury. Alternatively, inflammatory cytokines such as IFN-\(\gamma\) and TNF-\(\alpha\), which impair LC function, may affect LC in an antigen-nonspecific mechanism similar to other target organs. In our experimental system, we did not find evidence for T-cell infiltration into the seminiferous tubules, a fact that correlated with the absence of overt abnormalities in this compartment (data not shown). Thus, it remains to be investigated whether spermatogenesis is indirectly altered during acute GVHD as a consequence of loss and/or dysfunction of Leydig cells.

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**Literature**


Legends to Figures

**Figure 1. Injury to Leydig cells during an acute GVHR.** Experimental acute GVHD was induced by transfer of 60 x 10⁶ parental B6.CD45.1 splenocytes to unirradiated B6D2F₁ mice whereas syngeneically transplanted B6D2F₁ mice served as controls. **Panel a:** Determinations of serum testosterone by radioimmunoassay were done at the Institute for Reproductive Medicine (University of Muenster, Germany). The graph represents pooled data from eight (non-GVHD) and seven (GVHD) mice, respectively, that were analyzed between two and three weeks after transplantation in one independent experiment. Three experiments were performed, with similar results. Medians are represented by horizontal bars within the quartile boxes, the ranges are given by vertical bars. Mann-Whitney U-test p=0.05 syngeneic vs. allogeneic transplantation. **Panels b-e:** Paraffin sections (6 µm) of testicular tissue were analyzed for LC-specific Cy p450scc expression (in red color) at three weeks after transplantation in mice without (b,d) and with GVHD (c,e). The nuclear counterstain (hematoxylin) is displayed in panels b and c for both groups in blue color. The horizontal bars represent 100 µm. **Panel f:** Sections from mice without and with GVHD were stained with antibody to Cy p450scc and were subjected to computer-aided quantitative morphometric analysis of the Leydig cell compartment. The Leydig cell volume density (%) was calculated from 5 random high powered microscope fields per tissue section, with 5 sections per testis (serial sections separated by 30 µm; total of 25 fields per mouse). The figure depicts representative data (mean ± SD) from one (out of three) independent experiments; with 5 mice analyzed for each group. B6D2F₁ mice infused with syngeneic (□) or allogeneic (■) donor T-cells. Unpaired t-test p<0.005 syngeneic vs. allogeneic transplantation. Untransplanted, naïve B6D2F₁ mice served as additional controls (●).

**Figure 2. Alloreactive donor T-cells infiltrate the testis during an acute GVHR.** Acute GVHD was induced as in Fig. 1. **Panels a,b:** Frozen sections from testicular tissues were analyzed at two weeks posttransplant for the presence of CD3⁺-positive cells in mice without (a) and with (b) GVHD. A cluster of CD3⁺ T-cells present in the interstitial space between the seminiferous tubules is highlighted in panel b. Magnification x 100. **Panel c:** Donor T-cells are present in testis. Co-localization of CD3⁺ and CD45.1 was detected by confocal microscopy in allogeneically transplanted mice. The panel shows cells that stain simultaneously for red (CD3/Cy3) and green (CD45.1/FITC) color. Magnification x 630. **Panel d:** Analysis of cytokine mRNA expression by quantitative PCR. Transcripts for IFN-γ and TNF-α were measured in frozen testicular tissue isolated from mice at 2-4 weeks after T-cell infusion. The y-axis represents the x-fold increase of cytokine mRNA in mice with acute GVHD when compared to mice without GVHD (normal transcriptional activity is set as 1). **Panels e,f:** Frozen sections from testicular tissues were analyzed at two weeks posttransplant for the presence of CD11b-
positive cells (shown in red) in mice without (e) and with (f) GVHD. Magnification x 200.
B6D2F1 mice infused with syngeneic (□) or allogeneic (■) donor T-cells.

Fig. 1
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B6D2F1 → B6D2F1 (2 wk)  B6.CD45.1 → B6D2F1 (2 wk)

CD8
CD3
CD45.1 x CD3

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