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The influence of retinoic acid-induced differentiation on the radiation response of male germline stem cells

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ABSTRACT

Lifelong mammalian male fertility is maintained through an intricate balance between spermatogonial proliferation and differentiation. DNA damage in spermatogonia, for instance caused by chemo- or radiotherapy, can induce cell cycle arrest or germ cell apoptosis, possibly resulting in male infertility. Spermatogonia are generally more radiosensitive and prone to undergo apoptosis than somatic cells. Among spermatogonial subtypes the response to DNA damage is differentially modulated; undifferentiated spermatogonia, including the spermatogonial stem cells (SSCs), are relatively radio-resistant, whereas differentiating spermatogonia are very radiosensitive. To investigate the molecular mechanisms underlying this difference, we used an *in vitro* system consisting of mouse male germline stem (GS) cells that can be induced to differentiate. Using RNA-sequencing analysis, we analyzed the response of undifferentiated and differentiating GS cells to ionizing radiation (IR). At the RNA expression level, both undifferentiated and differentiating GS cells showed a very similar response to IR. Protein localization of several genes found to be involved in either spermatogonial differentiation or radiation response was investigated using mouse testis sections. For instance, we found that the transcription factor PDX1 was specifically expressed in undifferentiated spermatogonia and thus may be a novel marker for these cells. Interestingly, also at the protein level, undifferentiated GS cells showed a more pronounced upregulation of p53 in response to IR than differentiating GS cells. The higher p53 protein level in undifferentiated spermatogonia may preferentially induce cell cycle arrest, thereby giving these cells more time to repair inflicted DNA damage and increase their radio-resistance.

1. Introduction

Spermatogenesis is an intricate process that takes place in the seminiferous tubules within the testis. In mammals, the entire process of spermatogenesis is comprised of three consecutive phases: a mitotic phase (spermatogonial proliferation and differentiation), a meiotic phase (spermatocyte meiotic divisions to generate haploid spermatids) and spermiogenesis (elongation and maturation of spermatids) [1]. For continuous spermatogenesis spermatogonial stem cells (SSCs) are essential. SSCs can be defined as a subpopulation of undifferentiated spermatogonia able to generate and maintain donor-derived spermatogenesis when transplanted into infertile recipient testes [2,3]. Continuous spermatogenesis requires a constant balance between SSC self-renewal, proliferation and differentiation [1]. Within the seminiferous tubules, spermatogenesis occurs in an orchestrated spatio-temporal fashion in which specific germ cell types are grouped in specific stages of the seminiferous epithelium. The undifferentiated spermatogonia may divide freely during all of these epithelial stages. In contrast, differentiating spermatogonia are irreversibly committed towards meiosis and their subsequent divisions are strictly dictated by the epithelial stage in which they are present [4].

Because DNA damage, for instance caused by gonadotoxic chemicals or ionizing radiation (IR), can result in gene mutations or chromosomal aberrations, DNA damage often causes spermatogonial

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apoptosis or activates a male-specific meiotic arrest checkpoint [5–9]. These forms of spermatogenic arrest then prevent genomic aberrations being transmitted *via* the sperm. Indeed, in the human, treatment with chemo- or radiotherapy in adult males often results in impaired fertility [10]. Spermatogenic cells generally exhibit a slow rate of DNA repair together with a high incidence of unrepaired DNA damage, which renders them more radiosensitive than somatic cells [11,12].

Because genetic aberrations in SSCs have the potential to result in lifelong generation of mutated sperm, one might expect that SSCs, when compared to all other spermatogonia, are most prone to undergo apoptosis in response to IR. However, this appears not to be the case. It turns out that differentiating spermatogonia are actually much more radiosensitive and show a stronger apoptotic response [13–15]. Among undifferentiated spermatogonia, the self-renewing SSCs are most resistant to DNA damage induced by either the alkylating agent busulfan or IR [16–18]. Evidently, while the damaged differentiating spermatogonia are more easily sacrificed, preservation of SSCs, and thus the long-term fertility, seem to outweigh a certain risk of mutated offspring.

What determines the differences in radio-sensitivity among spermatogonial subtypes is currently unknown. Several DNA damage response proteins have been reported to be differentially regulated during spermatogonial differentiation. For instance, phosphorylated histone H2 AX (ɣ-H2 AX), usually marking DSBs, has been described to increase with spermatogonial differentiation [12,19] and is highly expressed in intermediate and B spermatogonia [20]. The DNA damage response protein p53 has been found to be induced in all spermatogonia by irradiation, but knockout of p53 seems to predominantly affect the apoptotic response of undifferentiated spermatogonia [5,21,22]. Nevertheless, transplantation assays of mutated SSCs revealed that deficiency in a specific p53 pathway (Trp53-Trp53inp1-Tnfrsf10b) actually increased survival of SSCs after irradiation [23]. The same study also reported that the apoptosis-inducing protein BBC3 was specifically active in differentiating spermatogonia after irradiation [23].

To investigate the relation between the IR-induced DNA damage response and spermatogonial differentiation, we used an established culture system for undifferentiated mouse spermatogonia [24,25]. In this culture system, primary isolated mouse SSCs, then referred to as male germline stem (GS) cells, can propagate *in vitro* for years without losing SSC properties [25]. GS cells can also be induced to differentiate by adding retinoic acid (RA) to the culture medium [26,27]. Moreover, by way of RNA-sequencing (RNA-seq), the transcriptome of RA-induced differentiating GS cells was reported recently [27]. To gain insights into the differential DNA damage responses of undifferentiated and differentiating spermatogonia, we investigated the transcriptomes of irradiated and non-irradiated GS cells with or without RA treatment.

2. Materials and methods

2.1. Animals

Neonatal (4–5 d.p.p) DBA/2 J male mice were used for GS cell isolation, and adult (~8 weeks) C57BL/6 J male mice were used for irradiation and immunohistochemical analysis. For histological analysis on neonatal testis sections, 8 d.p.p old C57BL/6 J male mice were used. All animal procedures were in accordance with and approved by the animal ethical committee of the Academic Medical Center, University of Amsterdam or in accordance with the National Institutes of Health and US Department of Agriculture criteria approved by the Institutional Animal Care and Use Committees of Johns Hopkins University.

2.2. GS cell culture

A mouse GS cell line was established as previously reported [24,28]. Briefly, testes were harvested from neonatal DBA/2 J male mice, and after removing the tunica albuginea, testicular tissues were mechanically dissociated and subjected to a collagenase-trypsin dissociation to obtain a single-cell suspension. Germ cells were enriched by an overnight differential plating and cultured in a medium mainly composed of StemPro-34 SFM medium (Thermo Fisher Scientific), StemPro-34 Supplement (Thermo Fisher Scientific), 1% fetal bovine serum (FBS), recombinant human GDNF (10 ng/ml, Peprotech), recombinant human bFGF (10 ng/ml, Peprotech), as well as other components as previously reported [24]. The cells were cultured on mitotically inactivated mouse embryonic fibroblasts (MEFs) since the third passage and were refreshed every 2–3 days and passaged every 5–7 days at a ratio of 1:4-6. The cells were maintained at 37 °C in an atmosphere of 5% CO_2 in air.

2.3. RA treatment

Before RA treatment, GS cells cultured on MEFs were transferred to laminin (20 μg/ml, Sigma-Aldrich)-coated wells. On the next day, GS cells were treated with 2μM all-trans-RA (Sigma-Aldrich) in culture medium for 48–72 hours. In control groups, vehicle (0.1% ethanol in medium) was applied to the cells.

2.4. Ionizing irradiation (IR)

Before IR treatment, GS cells cultured on MEFs were transferred to laminin (20 μg/ml, Sigma-Aldrich)-coated wells. On the next day, GS cells were subjected to 1 Gy of IR emitted by a ^{137}Cs source, a dose that causes substantial DNA damage but does not necessarily kill spermatogonia *in vivo* [15]. Because spermatogonial p53 is significantly induced in response to IR after 3 h [5], cells were used 3 h after IR or sham irradiation (same treatment but leaving out the actual exposure to IR). To prepare irradiated mice, adult C57BL/6 J male mice were exposed to a whole-body IR (1 Gy) and killed 3 h post IR (or sham IR), after which the testes were fixed in 4% paraformaldehyde (PFA).

2.5. Quantitative-real time PCR (Q-PCR)

Total RNA was extracted from GS cells using ISOLATE II RNA Mini Kit (Bioline) and following the protocol provided by the manufacturer. After treatment with DNase (Qiagen) and tests for genomic DNA-free, RNA samples were reversely transcribed, using SensiFAST cDNA Synthesis Kit (Bioline). The synthesized cDNA was then used for Q-PCR reactions, using the Roche LightCycler 480 platform (the 384-well plate format). The Q-PCR reaction was performed in a 10 μl volume system including 2× LightCycler 480 SYBR Green I Master (Roche). *Ppt2* and *Mtg1* were used as reference genes, and the data were analyzed using the $-\Delta\Delta C_t$ method. Data were presented as the mean ± standard error of mean (SEM) of 3 independent experiments (n = 3). Differences between groups were assessed using the Student's *t*-test. $P < 0.05$ was considered statistically significant and $P < 0.01$ was considered extremely significant. The primers for Q-PCR analysis are listed in Table 1.

2.6. Western blot

Proteins were extracted from the cells and quantified with Qubit Protein Assay Kit (Thermo Fisher Scientific). Then Western blot analysis was performed as reported previously [28–30], using the LI-COR Odyssey imaging system (LI-COR Biosciences). The primary antibodies used were mouse anti-PLZF (1:100; D-9, Santa Cruz Biotechnology), mouse anti-OCT4 (1:200; C-10, Santa Cruz Biotechnology), rabbit anti-STRA8 (1:500; ab49602, Abcam), rabbit anti-p53 (1:100; FL-393, Santa Cruz Biotechnology), rabbit anti-GAPDH (1:400; FL-335, Santa Cruz Biotechnology) and mouse anti-β-actin (1:5000; A1978, Sigma-Aldrich). For quantification of the relative p53 protein expression, p53 band intensities were divided by those of GAPDH. Data were presented as the mean ± SEM of 4 independent experiments (n = 4). Differences among groups were assessed using the one-way ANOVA followed by the LSD test. $P < 0.05$ was considered statistically significant and