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Mechanism of Protection of Rat Spermatogenesis by Hormonal Pretreatment: Stimulation of Spermatogonial Differentiation After Irradiation

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ABSTRACT: Pretreatment of rats with hormones that suppress testosterone levels and sperm production enhances the recovery of spermatogenesis from stem cells after a cytotoxic insult. It is not known whether the enhanced recovery results from an increase in the numbers of surviving stem cells or whether their ability to differentiate is enhanced. In this study, untreated rats and rats pretreated with testosterone plus estradiol-17β (T + E) were irradiated with 3.5 or 6 Gy, and the recovery of spermatogenesis from surviving stem cells was assessed at 6, 10, and 20 weeks after irradiation. T + E pretreatment did not significantly affect the numbers of A spermatogonia remaining in the tubules at 6 weeks after irradiation. In rats that were given irradiation alone, spermatogenesis steadily declined after 6 weeks because the stem cells lost their ability to differentiate. However, when rats were treated with T + E before irradiation, this decline was prevented, and in fact, at least at the lower dose of radiation, there was a progressive recovery of spermatogenesis. Given the similar spermatogonial counts at 6 weeks after irradiation in the irradiated-only and T + E–treated, irradiated rats, the hormone treatment appears not to protect stem cells from being killed by the cytotoxic agent. Rather, the later enhancement of spermatogenic recovery results from prevention of an injury-induced change in spermatogonia or in their environment, which would have otherwise resulted in failure of spermatogonial differentiation.

Key words: Testosterone, estrogen, procarbazine, cancer therapy, spermatogonia.

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Sterility is a frequent side effect in men who have been treated with cytotoxic agents for cancer (Meistrich et al, 1997a). In particular, radiation and alkylating agents, such as cyclophosphamide and procarbazine, produce testicular damage that results in prolonged azoospermia. It is important to develop methods to prevent this damage if fertility is to be preserved in men who want to father children after treatment for cancer. Although it is generally considered that azoospermia is caused by the killing of stem cells, it has recently been recognized that the failure of surviving stem cells to differentiate into spermatozoa is also a factor (Meistrich, 1998).

In several different strains of rats, methods already exist that involve hormonal pretreatment with analogues of gonadotropin-releasing hormone (GnRH) or steroids to enhance the recovery of spermatogenesis from stem cells after exposure to radiation, procarbazine, or cyclophosphamide (Delic et al, 1986; Kurdoglu et al, 1994; Meistrich et al, 1995). However, attempts to directly translate these observations to reduce the damaging effects of cytotoxic therapies on human spermatogenesis have not been successful (Johnson et al, 1985; Morris and Shalet, 1990). A contributing factor to the failure of the clinical studies is a lack of knowledge of the mechanism by which the hormonal treatment works in the rat. Without this knowledge, there is no rationale for optimizing the protocol for humans.

The mechanism originally proposed for ''protecting'' spermatogenesis with hormonal treatment was interruption of the pituitary-gonadal axis, thereby reducing the rate of spermatogenesis and rendering the resting testis more resistant to the effects of chemotherapy (Glode et al, 1981). However, we have conclusively shown that enhancement of spermatogenic recovery is achieved under conditions that do not modify the kinetics of spermatogonial proliferation before cytotoxic therapy (Meistrich et al, 1997b). Other mechanisms that have been considered involved changes in drug metabolism, delivery, and bioactivation, but these were ruled out by observations that the hormone treatment did not alter the effectiveness of procarbazine in killing the differentiating germ cells (Meistrich et al, 1997b) and by achieving protection of spermatogenesis recovery from stem cells against gamma radiation (Kurdoglu et al, 1994). Possible mechanisms

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that would protect against both radiation and alkylating agents include changes in levels of oxygen, thiols, and DNA repair. However, the similar protective effect of hormone treatment against neutron radiation damage as opposed to gamma radiation indicated that these factors are unlikely to be significant in the hormonal protection mechanism (Wilson et al, 1999). Alternatively, it is possible that hormone pretreatment may increase the number of spermatogonia or alter their proliferation status before irradiation, as has been observed in monkeys after treatment with follicle-stimulating hormone (FSH; van Alphen et al, 1989); however, this is not the case in rats (Meistrich et al, 1997b).

We have proposed that hormonal pretreatment may act by enhancing the recovery of spermatogenesis from surviving stem spermatogonia (Meistrich, 1996). In irradiated rats, surviving stem cells can fail to produce sperm for prolonged periods of time, which opens the possibility that enhancement of recovery could occur (Kangasniemi et al, 1996). To test this proposal in this study, we measured the recovery of spermatogenesis at a sequence of times in rats receiving irradiation alone and in irradiated rats pretreated with testosterone and estradiol-17β (T + E). The comparisons of the time courses of recovery or failure of spermatogenesis support the notion that the mechanism of ''protection'' involves enhancement of recovery of spermatogenesis from surviving stem cells.

Materials and Methods

All of the principal methods have been described in previous publications (Meistrich and van Beek, 1993; Parchuri et al, 1993; Kangasniemi et al, 1996; Meistrich and Kangasniemi, 1997; Wilson et al, 1999) and are briefly summarized here.

Animals and Irradiation

$LBNF_1$ rats were obtained at 10 weeks of age from Harlan Sprague-Dawley, Inc (Indianapolis, Ind). They were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care, in accordance with current regulations and standards.

Rats were irradiated with either 3.5 or 6 Gy using a 60_{Co} gamma ray unit (Meistrich and Kangasniemi, 1997). The single doses of radiation were administered to the lower half-body of rats, under general anesthesia, with 5 mm of tissue-equivalent bolus material placed over the scrotum to provide a build-up layer.

Hormone Pretreatment

Rats were implanted with hormone-containing Silastic tubing capsules (Parchuri et al, 1993). The T + E treated rats were given a 2-cm testosterone implant and a 0.5-cm estradiol-17β implant. Control rats used in this experiment (3.5 Gy, all weeks; 6 Gy, 6 weeks) were given a 3-cm capsule containing cholesterol, which is an inactive steroid precursor. Implants were placed in the rats 6 weeks before irradiation and were removed 1 day after irradiation. The immediate effect of T + E treatment on the testis (Parchuri et al, 1993) was confirmed by evaluating sperm production in 3 rats that were killed at the time the others were irradiated. Testis weight was reduced from a control value of 1.49 ± 0.04 grams to 0.47 ± 0.01 gram; sperm head count per testis was reduced from a control value of 2.9×10^8 ($\pm 0.2 \times 10^8$) to 1.6×10^6 ($\pm 1 \times 10^6$), which is consistent with previously reported values (Parchuri et al, 1993).

Experimental Design

In this study, 35 rats were used. Of these, 27 were implanted with T + E capsules. Three were killed after 6 weeks of hormone treatment, 12 were given 3.5 Gy of irradiation, and 12 were given 6 Gy of irradiation. Four rats from each irradiation group were killed at 6, 10, and 20 weeks after irradiation.

Because of the reproducibility of numerous other experiments performed in this laboratory to analyze the time course of recovery and decline of spermatogenesis in irradiated $LBNF_1$ rats, only 8 concurrent controls that were given irradiation only with no T + E treatment were used. These 8 rats were implanted instead with cholesterol-containing capsules. Six were given 3.5 Gy of irradiation and 2 each were killed 6, 10, and 20 weeks later. Two rats were given 6 Gy of irradiation and were killed 6 weeks after irradiation.

Additional control data values were taken from previously reported studies (Kangasniemi et al, 1996; Meistrich and Kangasniemi, 1997; Meistrich et al, 1999) and unpublished experiments in this laboratory, all of which used the same procedures, with the exception of implantation of the cholesterol capsules, and were done within 2 years (before and after) of the experiment just described. We believe that it is justifiable to include these control data values. To confirm the lack of effect of these small cholesterol capsules, we performed an experiment to show that capsule implantation had no effect on the recovery of spermatogenesis 10 weeks after 3.5 Gy of irradiation (repopulation index—RI = 11 ± 3% with implantation and 7 ± 2% without). However, in the accompanying figure and tables, the data from the concurrently irradiated rats are presented separately from the other ones.

Evaluation of Recovery of Spermatogenesis

Rats were killed at 6, 10, and 20 weeks after irradiation. One testis was fixed in Bouin's fluid and embedded in methacrylate, and sections were stained with Harris hematoxylin. The level of spermatogenesis after treatment was evaluated by determining the RI, which is the percentage of tubules that contained 3 or more differentiated spermatogenic cells (Meistrich and van Beek, 1993) derived from cells that were stem cells at the time of irradiation. When analyzing rats killed at 6 weeks after irradiation, spermatids were excluded from the counts because they could have been derived from cells that were differentiating at the time of irradiation. At least 200 seminiferous tubules were scored in each testicular section.

A spermatogonia and their mitoses were counted in at least 100 nonrepopulating tubules. In every fifth tubule, Sertoli cell nuclei with a visible nucleolus were counted. Only nonrepopulating tubules were counted because it is more difficult to iden-

tify A spermatogonia in repopulating tubules and, in such tubules, many of the A spermatogonia would be differentiating. In the nonrepopulating tubules, we recently showed that the clones of the proliferating A spermatogonia almost exclusively consisted of 4 cells at most (Shuttlesworth et al, 2000). Because this clonal size is typical of the undifferentiated but not the differentiating spermatogonia (Huckins, 1971), these A spermatogonia must be earlier than the A_1 stage. The Abercrombie correction, which accounts for the likelihood of finding nuclei of a given diameter whose center may be outside the cross-section (Abercrombie, 1946), was applied to the counts using the previously measured diameters (Kangasniemi et al, 1996). The number of A spermatogonia was then normalized to the number of Sertoli cells. The mitotic index of A spermatogonia was determined by dividing the total number of mitoses observed by the number of mitoses plus A spermatogonia.

The other testis was weighed after the tunica albuginea was removed. The tissue was homogenized, an aliquot was removed and sonicated (Meistrich and van Beek, 1993), and sperm heads were counted in a hemacytometer.

Hormone Measurements

Blood was collected by cardiac puncture at the time the rats were killed. Serum luteinizing hormone (LH) concentration was measured by an immunofluorometric assay (Haavisto et al, 1993), and FSH concentration was measured by a double-antibody radioimmunoassay (Clayton et al, 1980). The remainder of the testicular homogenate was used to determe intratesticular testosterone (ITT) concentrations with coated-tube radioimmunoassay (RIA) kits (DSL 4000, Diagnostic Systems Laboratories, Webster, Tex; Meistrich and Kangasniemi, 1997). The data presented are either from a single assay or several assays done consecutively with the same reagents.

Data Analysis

The means and the standard errors of the mean of sperm counts, serum LH, and ITT were obtained on log-transformed data. In such cases, the standard errors are not symmetric around the mean. Statistical analyses of the increases or declines in spermatogenic characteristics at different times after irradiation were done using a nonparametric Mann-Whitney test. SPSS for Windows, version 7.5 (SPSS Inc, Chicago, Ill) software was used.

Results

Recovery of Spermatogenesis After Irradiation

In $LBNF_1$ rats that received irradiation alone, spermatogenesis showed a transient initial recovery at 6 weeks after irradiation, as evidenced by repopulation of the tubules with differentiated germ cells, but then declined (see Figure). Testis weights also declined significantly over time. The small increase in sperm head counts between 10 and 20 weeks after 3.5 Gy of irradiation was not significant. The lack of decline of sperm head counts despite a decline in RI was attributable to an increase in the proportion of repopulating tubules that had reached the condensed spermatid stage, which was 27% at 20 weeks, compared with 6% at 10 weeks. This does not reflect an increase in potential for spermatogenic recovery but merely the greater time after irradiation, which permitted the appearance of later stages.

In contrast to the decline in spermatogenic recovery observed after 6 weeks with radiation alone, when the rats were treated with T + E before irradiation, there was either a maintenance of the level (after 6 Gy) or a progressive recovery of spermatogenesis (after 3.5 Gy; see Figure). Significant increases in testis weights and RI occurred in rats that had been pretreated with T + E between 6 and 10 weeks and between 10 and 20 weeks (RI increased from 99.5 ± 0.1% to 100 ± 0.0%) and in sperm head counts between 10 and 20 weeks after irradiation with 3.5 Gy. After 6 Gy of irradiation, there were significant increases in testis weights and RI between weeks 6 and 10, and there was an increase in sperm head count between weeks 10 and 20, but there were no significant changes in testis weights or RI between weeks 10 and 20. Overall, we conclude that in contrast to the steady decrease observed in these characteristics after radiation alone, there is maintenance or an increase in spermatogenesis when the hormone treatment is given before irradiation.

A Spermatogonia in Nonrepopulating Tubules

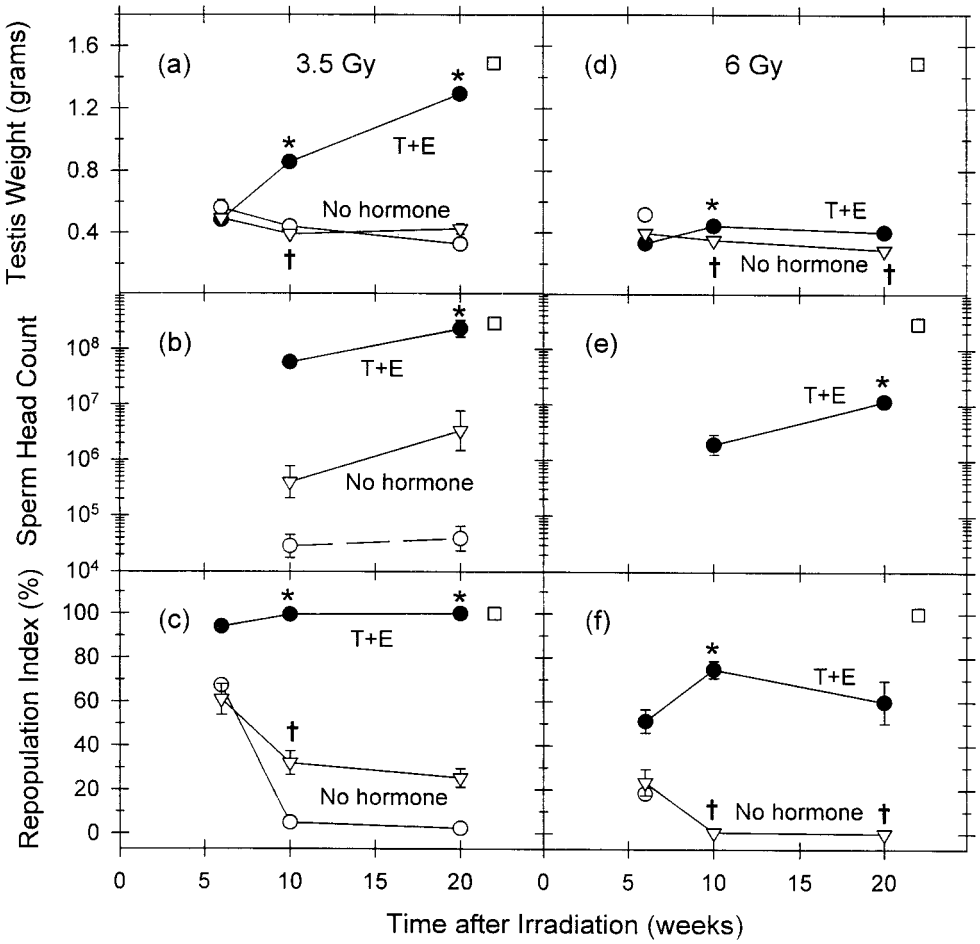
We previously observed that there were proliferating A spermatogonia in nonrepopulating seminiferous tubules after irradiation (Kangasniemi et al, 1996). Similarly, in the nonrepopulating tubules in the T + E-pretreated rats, there were also proliferating A spermatogonia (Table 1). At the earliest time analyzed in this study, 6 weeks after irradiation, these tubules contained similar numbers of both total and mitotic A spermatogonia as those of rats that had not been treated with T + E. Thus, it appeared that the hormone treatment did not increase the survival or proliferation of A spermatogonia.

Hormone Levels

T + E treatment markedly reduced the levels of LH and ITT but produced no significant reduction of FSH (Table 2). In contrast, irradiation increased the levels of all 3 hormones within 6 weeks after irradiation as an indirect consequence of the loss of germ cells (Table 2). Similarly, in the irradiated rats that were pretreated with T + E, the levels of FSH, LH, and ITT were also increased from those observed at the end of T + E treatment and were also above those in untreated rats.

Discussion

The data indicating that stem spermatogonial numbers after irradiation are unaffected by T + E pretreatment is



Effect of hormone pretreatment on the time course of recovery of spermatogenesis from surviving stem cells after irradiation with 3.5 Gy **(a, b, c)** or 6 Gy **(d, e, f)** as measured by testicular weights **(a, d)**, sperm head counts **(b, e)**, and repopulation indices **(c, f)**. Error bars (±1 standard error of the mean) are shown when they are larger than the symbols used to indicate the data points. Sperm head counts at 6 weeks after irradiation were not included because these were derived from cells that were differentiating at the time of irradiation. No sperm heads were seen at 10 and 20 weeks after 6 Gy in animals not treated with T + E. ● indicates 6-week treatment with T + E implants before irradiation; ○, cholesterol implant for 6 weeks before irradiation; ∇, no implants (taken from other experiments; n = 4–15); □, unirradiated rats (no hormone treatment; n = 9); *, statistically significant ($P < .05$) increase compared with preceding time point; †, statistically significant decrease compared with preceding time point.

further evidence against the concept that hormone pretreatment enhances recovery of spermatogenesis by protecting stem cells from being killed by the cytotoxic agent. Rather, hormone pretreatment appears to act by preserving the subsequent ability of stem spermatogonia to differentiate. At 6 weeks after irradiation, RI was already higher in the hormone-treated animals than in those not treated with hormones, and the difference in repopulation between the 2 groups increased with time.

This increasing difference with time in repopulation between the 2 groups was not noted in previous studies from our laboratory because the recovery of spermatogenesis had always been assessed at 9 or 10 weeks after irradiation or treatment with procarbazine (Kurdoglu et al, 1994; Meistrich et al, 1994; Wilson et al, 1999). However, there is 1 report that testis weights and sperm counts declined between 11 and 14 weeks after procarbazine treatment, but when the rats were pretreated with hormones, the tes-

Table 1. *A spermatogonia in nonrepopulating tubules of rats at 6 weeks after irradiation with 6 Gy, with or without T + E pretreatment*

Hormone treatment	Number of rats	Sertoli cells per tubule cross-section*	A spermatogonia per 100 Sertoli cells*	Mitotic index of A spermatogonia (%)*
None	4	15.7 ± 4.3	2.6 ± 1.0	7.7 ± 2.7
Cholesterol	2	9.8, 10.0	1.6, 3.0	5.0, 12.1
T + E	4	11.1 ± 0.9	3.2 ± 0.1	8.9 ± 2.3

*Values are means ± SEM, except for the cholesterol-treated group's values, which are the individual values.

Table 2. *Hormone levels unirradiated and irradiated rats, with or without T + E treatment.*

Hormone pretreatment	Radiation (Gy)	Weeks postradiation	Number of rats	FSH (ng/ml; mean ± SEM)	Mean LH (ng/ml)†	Mean ITT (ng/g-testis)†
None	None	n/a	15–27‡	22 ± 1	0.18 (0.15–0.22)	34 (29–39)
T + E	None	n/a§	4–7‡‖	20 ± 2	<0.04	0.9 (0.7–1.2)
None	3.5	6	5‡	27 ± 1	0.64 (0.54–0.77)	144 (125–164)
Cholesterol	3.5	6	2‖	25 ± 0	0.63 (0.47–0.85)	109 (102–115)
T + E	3.5	6	4‖	26 ± 1	0.35 (0.25–0.49)	102 (65–160)
None	6	6	5–8‡	34 ± 2	0.34 (0.29–0.40)	193 (162–231)
Cholesterol	6	6	2‖	24 ± 1	0.46 (0.36–0.58)	122 (103–145)
T + E	6	6	4‖	30 ± 1	0.27 (0.24–0.31)	60 (54–67)

*Different numbers of rats from other experiments were used depending on how many were assayed at the same time as the ones in this experiment.
†Numbers in parentheses are the 1-SEM range of values.
‡Rats from other experiments.
§Rats were killed at the end of a 6-week hormone treatment.
‖Rats from this experiment.

tis weights and sperm counts increased during this time (Velez de la Calle and Jegou, 1990). Other studies may have failed to show differences in the effect of hormone treatment on the kinetics of spermatogenic recovery after procarbazine, either because recovery was already maximal at the shorter time in the hormone-pretreated rats (Ward et al, 1990) or because there was no repopulation at all in rats that were not treated with hormone (Delic et al, 1986).

The mechanism by which hormone treatment given before irradiation affects the ability of the testis to support the differentiation of spermatogonial stem cells many weeks after the end of hormone treatment and irradiation is not known. In a previous study, we proposed that the apparent protection effect appeared to be better correlated with the degree of suppression of spermatogenesis at the time of chemotherapy or radiation treatment than the degree of suppression of ITT (Meistrich et al, 1996). However, later studies demonstrated that suppression of ITT immediately or even several months after cytotoxic injury can restore the differentiation of spermatogonia (Meistrich and Kangasniemi, 1997; Meistrich et al, 1999). Suppression of the intratesticular levels of testosterone and its androgenic metabolites appear to be the most important factors for the recovery of spermatogenesis.

It is still difficult to find a unified mechanism by which suppression of testosterone either before or after cytotoxic treatment can similarly preserve the subsequent ability of spermatogonia to differentiate. One possibility may be the persistence of reduced ITT produced by the hormone pretreatment even after the cytotoxic insult. Depot forms of GnRH agonists (Ward et al, 1990; Kangasniemi et al, 1995) may continue to exert suppression after their nominal period of action is over (Okada et al, 1994). In the current experiment, the T + E implants were removed 1 day after irradiation, and ITT levels were already above control levels 6 weeks later. Other studies showed that testosterone production by the testis was restored between 1 and 2 weeks after removal of T + E implants (Keeney et al, 1990). Because a 3-week suppression of ITT after irradiation was not sufficiently long enough to stimulate recovery of spermatogenesis (Meistrich and Kangasniemi, 1997), we therefore conclude that a possible continued suppression of testosterone for 1 to 2 weeks after cytotoxic insult is not likely to be sufficient to stimulate recovery of spermatogenesis and is not the mechanism by which hormonal pretreatment acts.

We suggest the following hypothesis by which both hormonal pretreatment and hormonal posttreatment can preserve or stimulate the recovery of spermatogenesis. The failure of recovery of spermatogenesis may be a result of persistent radiation-induced alterations in gene expression (Keyse, 1993; Chiang et al, 1997) by a somatic, androgen-responsive cell. These alterations may reduce the production of a paracrine factor for spermatogonial differentiation or they may up-regulate a factor that causes apoptosis of spermatogonia as they prepare to differentiate. Hormonal pretreatment renders the target cell insensitive to this damage and hence enhances the ability of spermatogenesis to recover after cytotoxic insult. However, even after this damage has occurred and spermatogonial differentiation ceases, it is possible to reverse this epigenetic phenomenon by temporarily suppressing testosterone and reinitiating appropriate gene expression. Our results are consistent with this hypothesis, but further studies, including identifying such paracrine factors and the target cells, are necessary to evaluate its validity. The Sertoli cell is most likely the target for both the hormonal effects and the damage, but the Leydig cell, which produces paracrine factors that act upon the Sertoli cells or spermatogonia, should also be considered.

The preservation of spermatogenesis following radiation or chemotherapy would be clinically important. The studies that have been performed have all tried to protect

the recovery of spermatogenesis by hormonal treatment before and during cytotoxic therapy (Morris and Shalet, 1990). Our observation of A spermatogonia in the non-repopulating tubules in rats pretreated with T + E indicates that it may be possible to further stimulate recovery by hormone posttreatment. Although it is not known whether hormonal treatment will stimulate spermatogenic recovery in humans, varying the regimens by continuing the hormonal treatment after cytotoxic therapy has been completed should improve any possible chances of stimulating the recovery of sperm production.

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