Impact of Paternal Exposure to Chemotherapy on Offspring in the Rat

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Paternal exposure to chemotherapeutics may have adverse effects on offspring. In the rat, chronic low-dose paternal exposure to the anticancer drug cyclophosphamide increased pre- and postimplantation loss and malformations. The effects of paternal drug treatment on progeny were influenced by the stage when germ cells were first exposed. Chronic cyclophosphamide treatment resulted in a dramatic decrease in the expression of stress response genes in pachytene spermatocytes and round spermatids but not in elongated spermatids; reduced gene expression may allow damage to accumulate. Exposure for 9 weeks, but not for 6, increased the incidence of aneuploidy. DNA strand breaks were maximal 3 weeks after short-term or acute treatment, during spermiogenesis. Cyclophosphamide-exposed spermatozoa imparted DNA damage to the fertilized embryo. Total RNA synthesis was higher in one-cell embryos sired by drug-treated fathers than in controls, and the expression of specific genes was altered. Thus, in the rat, paternal exposure to an anticancer drug altered germ cell quality, disrupting embryo development and dysregulating zygotic gene activation. [J Natl Cancer Inst Monogr 2005:34:28–31]

INTRODUCTION

There is concern that paternal exposure to chemotherapeutic agents may have adverse effects on offspring. Although the extent to which paternal exposures contribute to human infertility and to pregnancy loss is unknown, epidemiological data show that paternal occupations (such as welder, painter, auto mechanic, or fireman) involving exposures to metals, combustion products, solvents, or pesticides are associated with an increase in male infertility and adverse progeny outcomes [reviewed elsewhere (1)]. These adverse outcomes include spontaneous abortions, birth defects, and childhood cancer. In humans, it has proven difficult to identify a specific chemical as causative for male-mediated adverse effects on progeny because exposures are usually to a plethora of chemicals and because pregnancy loss is not unusual. Animal studies have been pivotal in elucidating the mechanisms by which paternal exposures adversely affect progeny outcome.

The alkylating agent cyclophosphamide has been studied as a model drug to determine the impact of paternal exposure on progeny outcome in the rat. One mechanism by which drugs may affect progeny outcome is their presence in seminal fluid. Cyclophosphamide was found in seminal fluid after systemic administration to male rats and was transmitted to the female during mating; an increase in early (preimplantation) embryo loss was observed in females mated to males treated with an acute high dose of cyclophosphamide (2). Alternatively, cyclophosphamide may alter progeny outcome via a direct effect on male germ cell number or quality. The treatment of male rats for 2 weeks with chronic low doses of cyclophosphamide that had only minor effects on germ cell numbers caused an increase in postimplantation death; this embryo loss rose dramatically to plateau at a level that was dependent on drug dose after 4 weeks of treatment and was reversed within 4 weeks of the end of treatment (3). Thus, cyclophosphamide-induced postimplantation loss was associated with germ cells first exposed during spermiogenesis. In contrast, an increase in external malformations and growth retardation was produced in progeny sired by germ cells first exposed to cyclophosphamide as spermatogonia. Significantly, the increase in postimplantation loss and malformations, as well as some of the behavioral abnormalities caused by paternal cyclophosphamide treatment, persisted to the F2 generation (4). Thus, the susceptibility of the germ cells to insult is stage specific during spermatogenesis in the testis and maturation in the epididymis (Fig. 1).

Damage in Male Germ Cells After Exposure to Chemotherapeutic Agents

Spermatogenesis is a highly ordered and regulated process (5). Spermatogonia undergo several mitotic divisions; upon completing these divisions, germ cells, now referred to as spermatocytes, initiate the two meiotic divisions that will render them haploid. The haploid cell, or spermatid, undergoes a number of cellular rearrangements during spermiogenesis. Some of these include dramatic restructuring and condensation of the nucleus with replacement of most histones with protamines, formation of an acrosome and a tail, reorganization of the mitochondrial matrix into a sheath below the nucleus, and shedding of most of the cytoplasmic elements. It is possible to separate pachytene spermatocytes and round and elongated spermatids, three key points in spermatogenesis, by unit gravity sedimentation (6). Using gene expression profiling, we demonstrated that similar numbers of genes were expressed in pachytene spermatocytes and round spermatids but that only about half were expressed in elongating spermatids (7). Furthermore, the expression of genes involved in stress response mechanisms, such as heat shock proteins-chaperones, DNA repair, and oxidative stress, was differentially regulated during germ cell development (7).

To establish whether treatment with cyclophosphamide altered gene expression in germ cells and whether such effects were specific to the phase of spermatogenesis, we examined the consequences of both an acute high-dose treatment and a chronic low-dose treatment in different phases of spermatogenesis. Acute treatment with cyclophosphamide affected gene

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See “Note” following “References.”

DOI: 10.1093/jncimonographs/lgi028

Journal of the National Cancer Institute Monographs, No. 34, © Oxford University Press 2005, all rights reserved.
expression in all cell types but most dramatically in round spermatids. Increased transcript levels were observed for 15% of the genes on our arrays in round spermatids, whereas only 3% were affected in pachytene spermatocytes and 1% in elongating spermatids. The expression of genes involved in apoptosis, DNA damage recognition, and transcriptional activation was most affected by cyclophosphamide in round spermatids. The magnitude of the response of round spermatids to acute high-dose exposure to cyclophosphamide suggests that this cell type may be highly susceptible to the damaging effects of this drug.

Chronic low-dose cyclophosphamide treatment resulted in effects on the expression of stress response genes in male germ cells dramatically different from those of an acute high dose. Chronic cyclophosphamide treatment reduced the number of transcripts detected in all germ cell types. The predominant effect of this treatment was to decrease the expression of the genes studied in pachytene spermatocytes (34%), round spermatids (29%), and elongating spermatids (4%). The expression profiles of genes involved in DNA repair, posttranslational modification, and antioxidant defence in male germ cells were altered by this treatment. Interestingly, in elongating spermatids, despite the tight chromatin packing, which results in transcriptional inactivation, drug treatment increased the expression of 8% of the genes studied. The overall reduction in expression of stress response genes in male germ cells may decrease the ability of cells to respond to insult. Thus, cyclophosphamide may exert its maximal effects on elongating spermatids and spermatozoa (postmeiotic germ cells) because these cells have lost the ability to repair DNA and to undergo apoptosis.

We postulated that the cyclophosphamide-induced changes in gene expression in germ cells during spermatogenesis were a reflection of damaged chromatin. Several approaches are available today to assess chromatin damage. These include FISH analysis for aneuploidy, comet assay, sperm chromatin structure assay, in situ nick translation assay, PCR amplification of specific genes, and template function tests. We and others [reviewed by Anderson et al. (12)] have shown that cyclophosphamide treatment can result in DNA cross-links and strand breaks in germ cells. The rat sperm Y-4 FISH assay was used to assess the induction of spermatzoal disomy, nullisomy, or diploidy involving chromosomes Y and 4. The overall frequency of numerically abnormal spermatzoa was elevated about twofold after 9 weeks of cyclophosphamide treatment; the frequency of spermatzoa with chromosome 4 disomy and nullisomy was increased significantly compared to that found in corresponding controls. Thus, cyclophosphamide disrupted meiotic events prior to pachynema during spermatogenesis.

The phase specificity of the susceptibility of spermiogenic germ cells to genetic damage induced by cyclophosphamide was investigated further using the comet assay. Adult male rats were given cyclophosphamide for 4 days; to capture germ cells exposed to cyclophosphamide during late, middle, and early spermiogenesis, caudal epididymal spermatozoa were collected 14, 21, and 28 days later, respectively. A dose-related increase in DNA damage was observed; this damage was greatest on day 21, reflecting an increased susceptibility of step 9–14 spermatids. Thus, the DNA damage induced by cyclophosphamide was germ cell phase specific (Fig. 1). The most damaging effects of cyclophosphamide occurred during a key point of sperm chromatin remodeling (histone hyperacetylation and transition protein deposition). We speculate that strand breaks disrupt chromatin remodeling, hence affecting chromatin structure. The next question, then, was how the qualitative defects incurred in male germ cells after exposure to an anticancer drug like cyclophosphamide affect embryo development.

**EFFECTS OF PATERNAL CHEMOTHERAPY ON EMBRYO DEVELOPMENT**

Evidence that spermatozoa with etoposide-induced chromosomal abnormalities could fertilize an oocyte and support zygotic development emphasized the need to elucidate the link between paternal drug exposure and adverse effects on early embryogenesis. After fertilization, the spermatozoa undergo extensive remodeling such that the paternal chromatin becomes competent to synthesize DNA and RNA. The first event is sperm nuclear decondensation, characterized by proteome disulfide bond reduction, degradation, and replacement by histones to form the male pronucleus. The time dependence of sperm nuclear decondensation in hamster oocytes is directly related to disulfide bond content; nuclei with low disulfide content decondense more rapidly and form male pronuclei earlier. Cyclophosphamide administration for 6 weeks significantly altered rat spermatozoal decondensation patterns and decreased reducible sulfhydryl content in vitro. Similarly, using in vitro fertilization of hamster oocytes with spermatozoa from either control or cyclophosphamide-treated males, the formation of the male pronucleus was significantly advanced from 3 to 6 hours post-fertilization in zygotes fertilized by treated spermatozoa. Thus, DNA damage introduced into the zygote via spermatozoa may alter the coordinated events of pronuclear formation and disrupt the development of both the paternal and the maternal pronuclei. Indeed, Marcelli et al. recently tested six mutagens and showed that embryonic fate was established by the end of G1 of the first cell cycle. The presence of paternally transmitted chromosomal aberrations in the zygote was predictive of abnormal embryonic development; however, preimplantation development proceeded to implantation regardless of the presence of genetic instability.

The delivery of an intact sperm nucleus during fertilization is required for normal development, and subtle alterations

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**Fig. 1.** Impact of paternal exposure to cyclophosphamide on offspring is dependent on the extent and specificity of damage to the male germ cell. These are determined, at least in part, by the phase of spermatogenesis when germ cells are first exposed to the drug.
are sufficient to disrupt the contribution of sperm DNA to the embryo (23). Exposure of male germ cell DNA to cyclophosphamide when the chromatin structure was in an open conformation (6 weeks of chronic treatment, first targeting germ cells during meiosis) reduced in vitro DNA template function; in contrast, exposure when the chromatin was condensed (1 week of treatment, first exposing epididymal spermatozoa) had no effect on in vitro template function (11). Altered template function may disturb events that are important in early post-fertilization reprogramming and zygotic genome activation. Replication of paternal and maternal DNA is believed to be one of the first functional tasks exerted by the pronuclei [reviewed by Schultz (24)]. In the bovine species, it has been suggested that the onset of DNA replication in both pronuclei is directly regulated by the paternal component (25). Furthermore, spermatozoa may contain transcripts that are indispensable for the first embryonic division; the paternal genome is actively transcribed soon after fertilization (26). The assessment of total RNA synthesis ([32P]UTP incorporation) in embryos fathered by cyclophosphamide-treated males revealed constant levels of RNA synthesis from the one- to eight-cell stage, while in embryos sired by control males, RNA synthesis was dramatically induced to peak at the four-cell stage (19). Bromouridine triphosphate (BrUTP) incorporation and Sp1 transcription factor immunostaining were nuclear in two-cell embryos sired by control males; however, in embryos fathered by cyclophosphamide-treated males, the staining was dramatically increased and spread to the cytoplasm, suggesting a defect at the level of the transcriptional machinery (19). Furthermore, paternal cyclophosphamide treatment altered the expression profiles in embryos of a number of genes with well-defined roles in preimplantation development as early as the one- and two-cell stages (27, 28). Alterations in the expression of these genes, in addition to past observations of the specific and heritable effects of paternal cyclophosphamide exposure in the F1 progeny that can be further propagated to successive generations (4, 29), suggest that defects persist in the germ line of offspring. In this context, it is important to note that increased mutation rates have been observed in second-generation offspring following paternal exposure to irradiation in mice (30).

CONCLUSIONS

The male-mediated developmental toxicity and transgenerational inheritance observed following chronic cyclophosphamide exposure lead us to hypothesize that this alkylating agent, and potentially other chemotherapeutic drugs, may produce adverse progeny effects through two mechanisms: 1) targeted alterations in specific genes or chromosomes and 2) epigenetic alterations that are responsible for the temporal and spatial control of gene activity in the early embryo. Both of these mechanisms are currently under investigation in our lab to further examine the manner(s) by which paternal drug exposure temporally and spatially disrupts rat zygotic gene activation. Can we extrapolate from rats to humans? Should we be concerned about progeny outcome after the exposure of humans to chemical agents? The studies that exist show no increase in birth defects or genetic disease among the progeny of humans who have been treated with cancer therapies (31). Of necessity, the human studies have been limited in size and have not closely monitored either those adverse effects on progeny outcome that would be manifested only early (early spontaneous abortions) or late (functional deficits such as incidence of cancer, reproductive or immune functions, or postnatal alterations in behavior). Nevertheless, increased incidences of aneuploidy have been reported in the sperm of men after treatment with cancer chemotherapeutic agents (32–34); based on the available animal data, it is anticipated that increased sperm chromosomal aberrations will be associated with an increase in chromosomal defects in offspring and abnormal reproductive outcomes (21). There is a need for well-designed, comprehensive, and prospective studies to assess the extent to which paternal exposures, especially to agents such as the anticancer drugs that possess clear mutagenic potential, may affect offspring.

REFERENCES


NOTE

The Studies from our laboratories were funded by the Canadian Institutes of Health Research.