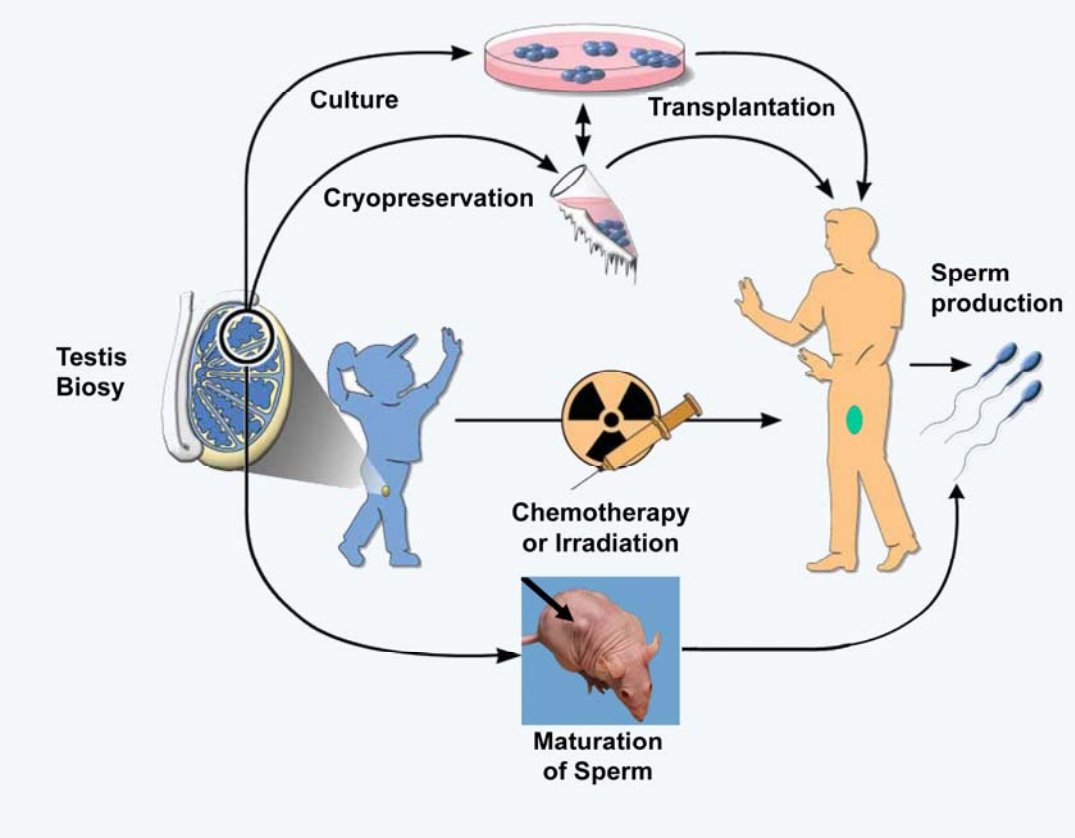
Thesis for doctoral degree (Ph.D.)

Preservation of Male Fertility in Childhood Acute Leukemia:

An experimental study addressing novel strategies and putative risks

Mi Hou



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Department of Woman and Child Health

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An experimental study addressing novel strategies and putative risks

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Cover photo
Strategies for preservation of fertility in boys treated for cancer.
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To my family:

Yiteng, Yiwen and Dawei

ABSTRACT

Although treatment of cancer in young boys with cytotoxic agents has long been known to cause infertility in the survivors, the mechanisms underlying drug-induced damage to the immature testis remain unclear. A number of clinical issues associated with transplantation of testicular germ cells or tissues also require clarification. The present investigation was designed to address some of these questions.

First, we examined how a cytotoxic drug causes damage to immature testes. Following a single intraperitoneal injection of doxorubicin and, in some cases, additional administration of amifostine to 6-, 16- and 24 day-old rats, the frequency of apoptosis in microsegments of seminiferous tubules was evaluated. Gonocytes and migrating spermatogonia were identified as the targets for doxorubicin, which caused an 8-fold increase in the apoptosis of these cell types in the youngest rats. Activation of p53 and caspase-8 were the key initial events involved in germ cell apoptosis. Amifostine was incapable of protecting germ cells from doxorubicin-induced apoptosis **(Paper I)**.

In order to explore transplantation of testicular germ cells as a strategy for preservation of fertility, acute T-lymphoblastic leukemia (ALL) induced experimentally in rats was employed as a model for children with ALL. When testicular cells isolated from the testes of leukemic rats were transplanted into the seminifereous tubules of syngenic healthy rats, all of the recipients died from donor-derived leukemia, indicating a high risk of reseeding tumor cells into patients. The development of novel strategies for elimination of tumor cells and/or of animal models for detection of contaminated leukemic cells is thus required **(Paper II)**.

For this purpose, two purification procedures based on surface markers, the flow cytometric sorting (FACS) and immunomagnetic cell sorting (MACS), were evaluated. First CD4/MHC-Cl I and Ep-CAM, were identified as appropriate markers for leukemic and germ cells, respectively Labeling these cells with fluorescent monoclonal antibodies against these markers allowed positive selection of germ cells and/or negative depletion of leukemic cells. A combination of these procedures yielded testicular cells free from leukemic cells, but the efficacy and safety of this approach are inadequate for clinical use. Variations in the immunophenotypes and other forms of heterogeneity among testicular leukemic cells,

together with aggregation of leukemic and germ cells, were the critical factors that prevented efficient purification of germ cells by FACS **(Paper III)**.

The efficiency of MACS in removing leukemic testis cells labeled with microbeads conjugated with antibody against CD4 was then evaluated. With this procedure, contamination of the germ cell preparation obtained was only reduced 30% (as determined by FACS) and after injection of this preparation into healthy animals, all of the recipients died of leukemia. Clearly, MACS is incapable of eliminating leukemic cells from germ cell preparations **(Paper IV)**.

Finally, fresh or cryopreserved testicular tissues obtained from leukemic rats were grafted subcutaneously into syngenic PVG rats or nude mice to determine whether mature sperm can be produced by such grafts contaminated with leukemic cells as well as to evaluate the possibility of using nude mice for the detection of contaminated leukemic cells. All of the auto- and xenografted animals developed local tumors at the site of injection and/or generalized leukemia. The seminiferous tubules were destroyed by the leukemic cells and no gametes were retrieved. Thus, although testicular grafts contaminated with leukemic cells do not produce gametes, xenotransplantation of testicular tissue into nude mice may provide a valuable biological indictor of leukemic cell contamination in testicular biopsies **(Paper V)**.

Our data provide highly relevant preclinical information concerning cytotoxic damage to the immature testis and transplantation of testicular cells and testicular tissue following treatment for leukemia, and should help in the design of strategies for preservation of fertility in boys treated for cancer. The xenograft model offers a clinical tool for assessing tumor cell contamination in testicular biopsies from boys with cancer designated for use in restoring fertility.

Keywords: Childhood cancer, chemo- or radiotherapy, apoptosis, testis, infertility, Roser´s leukemia, germ cell transplantation, purification, flow cytometry, immunomagnetic sorting, xenograft, nude mouse.

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LIST OF PUBLICATIONS

The present thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I. **Hou, M.,** Chrysis, D., Nurmio, M., Parvinen, M., Eksborg, S., Söder, O., and Jahnukainen, K. Doxorubicin induces apoptosis in germ line stem cells in the immature rat testis and amifostine cannot protect against this cytotoxicity. *Cancer Research.* 2005, **65**:9999-10005.

II. Jahnukainen, K., **Hou, M.,** Petersen, C., Setchell, B., and Söder, O. Intratesticular transplantation of testicular cells from leukemic rats causes transmission of leukemia. *Cancer Research.* 2001, **61**:706-10.

III. **Hou, M.,** Andersson, M., Zheng, C.Y., Sundblad, A., Söder, O., and Jahnukainen, K. Decontamination of leukemic cells and enrichment of germ cells from testicular samples from rats with Roser´s leukemia by flow cytometric sorting. *Reproduction,* in press.

IV. **Hou, M.,** Andersson, M., Zheng, C.Y., Sundblad, A., Söder, O., and Jahnukainen, K. Immunomagnetic separation of normal rat testicular cells from Roser´s T-cell leukemia cells is ineffective. *International Journal of Andrology,* in press.

V. **Hou, M.,** Andersson, M., Eksborg, S., Söder, O., and Jahnukainen, K. Transplantation of testicular tissue into nude mice can be used for detecting leukemic cell contamination. *Human Reproduction.* 2007, **22**:1899-906.

CONTENTS

LIST OF ABBREVIATIONS

ABC	Avidin-biotin complex
AHST	Autologous hematopoietic stem cell transplantation
ALL	Acute lymphoblastic leukemia
Apaf-I	Apoptotic-protease activating factor-I
CD	Cluster differentiation
DAB	3, 3´-Diaminobenzidine
DMSO	Dimethyl sulfoxide
Ep-CAM	Epithelia cell adhesion molecule
FACS	Fluorescence-activated cell sorting (flow cytometry)
FasL	Fas ligand
FCS	Fetal calf serum
FSC^{-high}	Forward-scatter high
FSH	Follicle stimulating hormone
GnRH	Gonadotropin-releasing hormone
HSCT	Hematopoietic stem cell transplantation
ICSI	Intracytoplasm sperm injection
IL	Interleukin
ip.	Intraperitoneal
LCs	Leydig cells
LH	Luteinizing hormone
MAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting (or immunomagnetic cell sorting)
MDM 2	Murine double minute 2
MHC-Cl I	Major histocompatibility complex class I
PCNA	Proliferating cell nuclear antigen
PGCs	Primordial germ cells
PVDF	Polyvinylidene difluoride
PVG rat	Pieblad variegated rat
RT	Room temperature
sc	Subcutaneous

SCs	Sertoli cells
SD rat	Sprague dawley rat
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SRY	Sex determining region on Y chromosome
SSC^{-high}	Side-Scatter high
SSC^{-low}	Side-Scatter low
SSCs	Spermatogonial stem cells
TBI	Total body irradiation
TdT	Terminal deoxynucleotidyl transferase
TFT	Tubular fertility index
TNF	Tumor necrosis factor
TNF-R	Tumor necrosis factor receptor
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nich-end labeling
WBCs	White blood cells

1. INTRODUCTION

1.1 Preface

Almost every day one child in Sweden is diagnosed with cancer (300 such cases annually, according to Swedish Children´s Cancer Foundation). Modern regimens for cancer treatment, involving intensive chemotherapy and/or radiotherapy, allow 80% of these cases to survive for at least 5 years and the number of long-term survivors of childhood cancer is increasing accordingly. It is estimated that by 2010, 1 out of every 250 employed individuals between the age of 15 and 45 will have suffered from cancer as a child (1). Unfortunately, half of those survivors have at least one long-term side-effect of their cancer treatment, of which one is infertility. This severe sequelae is especially common among boys and greatly diminishes their quality of life. Prepubertal boys, who do not have the ability to produce sperm for semen banking, are in urgent needs of novel strategies for preserving their fertility.

In recent years, several such novel approaches have been developed. For instance, testicular tissues transplanted into an ortotopic or ectopic site in the patient (or immunodeficient mice), can, once revascularized, generate sperm (2). Alternatively, spermatogonial stem cells isolated from a testicular biopsy prior to cancer therapy can be re-transplanted following treatment into their own environment in the seminiferous tubules, which they re-colonize to ultimately generate mature germ cells in a process referred to as autologous germ cell transplantation (3). However, the safety of such novel approaches to clinical restoration of fertility has not yet been determined.

For example, what is the risk that transplantation of autologous germ cells or testicular tissues derived from patients with hematopoietic malignancies reintroduces tumor cells? The possibility of eliminating leukemic cells from such testicular samples by fluorescence- (FACS) or magnetic-activated cell sorting (MACS) or of retrieving pure gametes from testicular grafts contaminated by leukemic cells requires examination. It would also be of clinical value to know whether xenotransplantation of testicular tissues into nude mice can be employed for effective detection of contamination by leukemic

cells. Furthermore, the mechanisms by which anticancer drugs damage the immature testis remain unknown.

In the present study, we first examine the mechanism underlying the damage caused by anticancer drugs to the immature rat testis. Subsequently, we performed autologous germ cell transplantation in a rat with acute T-cell lymphoblastic leukemia (ALL), a model for the clinical situation of childhood with ALL, to evaluate possible risks associated with this procedure. Thereafter, the potential for elimination of the contamination of testicular samples by leukemic cells employing FACS or MACS was studied. And, finally xenotransplantation of testicular tissues was utilized to determine whether gametes can be retrieved from leukemic testicular grafts or whether transplantation of such tissues into nude mice can be used effectively to detect contaminated leukemic cells.

1.2 The testis

1.2.1 Formation and function

The **testis** is obviously a highly important reproductive and endocrine organ in male mammals, performing two fundamental functions, i.e., the generation of sperm and production of testosterone, that ensure the continuity of life from one generation to the next. As expected, formation of this organ during ontogeny is genetically controlled. Expression of the *SRY* gene (i.e., the sex-determining region on Y chromosome) directs the embryonic gonads into the pathway leading to the development of testes. By the fourth week of gestation in humans, the primordial germ cells (PGSs) derived from pluripotent cells of the embryonic epiblast proliferate intensively and migrate from the endoderm of the yolk sac into the undifferentiated gonad (gonadal ridge), which becomes morphologically distinct approximately 13.5 day postcoitum (dpc) in the rat and during the seventh week of gestation in humans. The somatic cells present in the differentiating gonad give rise to Sertoli cells (SCs), Leydig cells (LCs), peritubular cells and other types of interstitial cells (4).

When the PGCs have developed, they are enclosed by differentiating Sertoli and peritubular cells to form testicular cords (5), which later become the seminiferous tubules, the site of sperm production following puberty. At this point, the PGCs are called gonocytes, since they are present in the gonad and differ morphologically from primordial germ cells (6). Mitotic proliferation of gonocytes continues for 2-3 days after their entry into the gonad and is then arrested in the G0/G1 phase of the cell cycle until birth (7). Histological development of the testis is largely completed by the end of the third month of gestation and the descent of the testes from the abdominal cavity into the scrotum is finished by the seventh month of gestation or shortly after birth in the human (Griffin and Wilson 2003, Williams textbook of Endocrinology, Saunders, Pennsylvania, USA).

1.2.2 Postnatal development

Postnatal development of the rat testis can be divided into six distinct periods: the neonatal period (the first 6 days of postnatal life), the infantile period (postnatal days 6-20), the juvenile period (days 20-34), the early pubertal period (days 34-44), the pubertal period (days 44-59), and finally, adulthood (8, 9). In the rat the end of the neonatal period overlaps with the beginning of puberty, whereas in humans, the long delay between birth and onset of pubertal development allows these two periods to be easily distinguished.

At birth, the seminiferous tubule of the rat is solid with gonocytes in the centre, while immature SCs and a monolayer of peritubular cells lies side-by-side along the basal membrane. Fetal Leydig cells, macrophages, lymphocytes, nerves, and blood and lymph vessels are present in the interstitial compartment between the tubules (Fig.1A).

Migratory gonocytes restart mitotic division on postnatal day 4 and continue this process actively until postnatal days 6-9 (6). These proliferating gonocytes make direct contact with the basement membrane, where they provide a pool of reserve stem cells. This relocation to the basement of membrane is critical for the survival of the gonocytes and establishment of a pool of germ-line stem cells, since cells which remain in the centre of the lumen eventually undergo apoptosis (10). When they resume proliferation, the

gonocytes give rise to type A spermatogonia, the starting point of spermatogenesis, at 4 to 6 days of postnatal age in the rat. Disturbance of gonocyte proliferation or migration at this critical time-point may result in impaired fertility later in life.

The adult mammalian testis contains several types of germ cells. Spermatogonial stem cells reside at the luminal side of the basal membrane, where they undergo successive mitotic divisions to become differentiated spermatogonia and primary spermatocytes. Subsequently, two meiotic divisions produce haploid spermatids, from which, via a morphogenetic process, spermatozoa are finally formed (Fig. 1B). These immature sperm are thereafter transferred through different ducts into the epididymis, where they mature and are made ready for ejaculation.

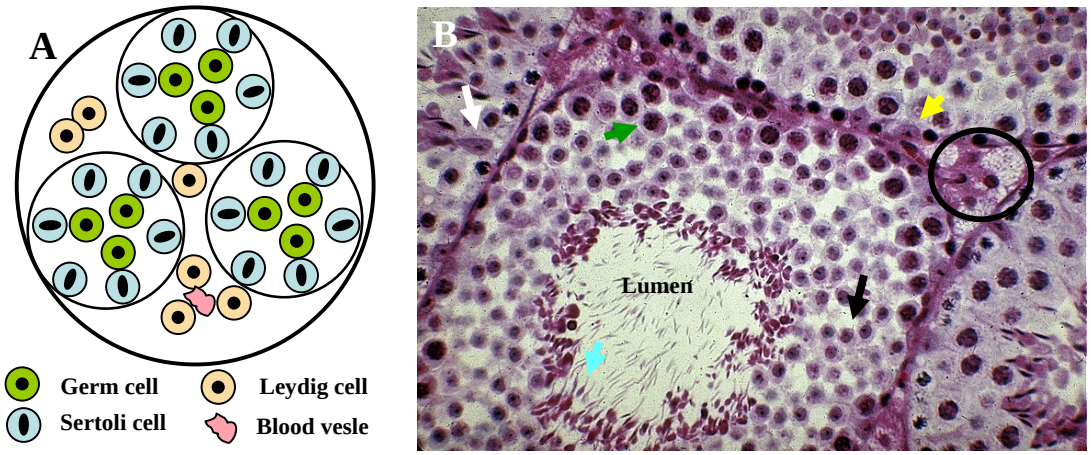

Figure 1. Morphology of immature and adult rat testis
(**A**)The immature testis is composed of an interstitial compartment and seminiferous cords enclosed by a capsule. Following birth, the seminiferous tubule becomes filled with gonocytes in the center and Sertoli cells located at the basal membrane; while Leydig and immune cells, nerves and blood and lymphoid vessels are situated between these tubules.
(**B**) In the adult testis, the lumen of the seminiferous tubules contains fluid and spermatozoa that have been released (the blue arrow). Spermatogonial stem cells (yellow arrow) and Sertoli cells (white arrow) reside on the basement membrane, while more highly differentiated spermatocytes (green arrow) and round spermatids (black arrows) are seen in the lumen. Leydig cells and other interstitial cells (the circle) are located between the seminiferous tubules.

Fetal Leydig cells, which originate from mesenchymal cells, secrete the testosterone and other androgens required for the development of internal and external male genitalia during fetal life. In the rat the number of fetal Leydig cells increases

slightly during the first postnatal week of life (11), following which precursors of adult-type Leydig cells proliferate, differentiate into progenitors and finally produce a population of mature Leydig cells. During this transition, the number and size of Leydig cells are significantly enhanced as is their capacity to secret testosterone (12). In the case of humans, fetal LCs are still present at birth, but disappear by the age of 3-6 months. Very few LCs can be detected in the human testis at 1-8 years of age, but when puberty arrives, adult-type LCs appear (13, 14). Consequently, significant levels of serum and testicular testosterone are present in boys up to 6 months of age, at which point these levels are greatly reduced and then remain low until puberty (13).

During the final week of fetal development in the rat, the Sertoli cells begin to divide rapidly and this mitotic activity continues until postnatal day 15 (12). As puberty approaches, the nuclei of the Sertoli cells become elongated and their cytoplasm stretches across the entire height of these cells, which extend into the lumen of the seminiferous tubules and physically support germ cells at various stages of differentiation. The tight junctions formed between the Sertoli cells establish the so-called blood-testis barrier, which, actually, does not separate the blood from testicular tissues, but rather divides the seminiferous epithelium into two distinct compartments: the basal compartment containing the spermatogonia, leptotene and zygotene spermatocytes; and the adluminal compartment with meiotic pachytene and secondary spermatocytes, haploid spermatids and spermatozoa (15). This blood-testis barrier protects the germ cells from the host´s own immune system (16) and restricts the entry of harmful substances from the blood and extracellular fluid of the interstitial compartment. In addition to physical support, the Sertoli cells nurture germ cells by secreting seminiferous fluid containing nutrients and growth factors, which in turn, results in the transformation of testis cords into seminiferous tubules possessing a lumen (Fig. 1B).

In higher primates, including human, two distinct periods of Sertoli cell proliferation have been described, the first beginning during fetal life and continuing throughout the prepubertal period and the second begins at puberty in association with initiation of spermatogenesis and continues until 15 years of age (17). This proliferation requires gonadotropin; is most rapid during infancy, when secretion of gonadotropin is

extensive (17); and produces a 6-fold increase in testicular volume during the first year of postnatal life (18). Establishment of an adequate number of SCs during childhood is crucial, since each SC can only sustain a certain number of germ cells. Accordingly, the number of SCs present in the testes determines the numbers of sperm produced in adulthood (19).

Testicular development and function are under positive control by the hypothalamus-pituitary-testicular axis. The hypothalamus secretes gonadotropin-releasing hormone (GnRH), which stimulates the pituitary gland to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In response to LH LCs produce the testosterone necessary for the development of secondary sexual characteristics and provision of an appropriate tubular microenviroment. Interaction of testosterone with the androgen receptor (AR) expressed on Sertoli cells allows entry into meiosis and maintenance of spermatogenesis. At the same time, FSH accelerates the rate of proliferation of SCs, thereby strongly influencing the final number of these cells attained. This hormone also indirectly regulates spermatogonial DNA synthesis (20), proliferation and differentiation, probably by inducing expression of the Steel factor by SCs (21, 22). Release of LH and FSH from the pituitary gland is regulated through a negative feedback loop, through which release of LH is inhibited by the testosterone produced by LCs and secretion of FSH attenuated by inhibin, which is produced by SCs.

1.2.3 Physiological germ cell apoptosis

Apoptosis, also known as programmed cell death, is a controlled form of cellular suicide that can be evoked either by physiological or toxic signaling (23), allowing organisms to eliminate unnecessary or damaged cells or structures. Thus, apoptosis removes cells that are potentially dangerous, such as aged, injured or mutated cells, and cells produced in excess, such as germ cells in the testis. Apoptotic cells display typical morphological features, including disruption of intracellular membranes cytoskeletal rearrangement, nuclear condensation and fragmentation of nucleosomal DNA (24).

Physiological apoptosis occurs commonly among germ cells, regulating the initial round of spermatogenesis, which, in rats, takes place between the first and sixth weeks of

postnatal life, with maximal apoptotic cell death during week 3 (6). Type A spermatogonia and spermatocytes are especially prone to apoptosis (25), in a fashion dependent on cell density (26, 27). Such germ cell apoptosis in the immature testis appears to be necessary for the normal maturation processes associated with spermatogenesis, most probably by maintaining the correct ratio of Sertoli cells to germ cells.

Apoptosis also plays an important role in connection with normal sperm production in the adult testis. It has been estimated that no more than 25% of the theoretical maximal number of prelepototene spermatocytes is produced from the original population of spermatogonia (28, 29). Furthermore, death of selected spermatocytes and spermatids is a common aspect of normal spermatogenesis (28), probably reflecting a quality control mechanism. Accumulation of spermatogonia and spermatocytes as well as infertility, are exhibited by "knock out" mice which do not express the pro-apoptotic Bax protein (30); while transgenic male mice expressing elevated levels of the anti-apoptotic Bcl-x_L or Bcl-2 protein in their germ cells are also infertile and demonstrate abnormal spermatogenesis (31). The infertility of these animals appears to result from the prevention of the early wave of spermatogonial apoptosis that normally occurs in the prepubertal rodent testes (31). In contrast, excessive germ cell death caused by gonadotoxic drugs or irradiation employed in cancer treatment gives rise to temporary or permanent azoospermia and infertility (32).

1.2.4 Pathways of germ cell apoptosis

Different pathways, involving numerous different genes and regulators are involved in the apoptotic process. In general, apoptosis is divided into two pathways, namely, the intrinsic, mitochondria-dependent pathway and the extrinsic, mitochondria-independent pathway (Fig. 2).

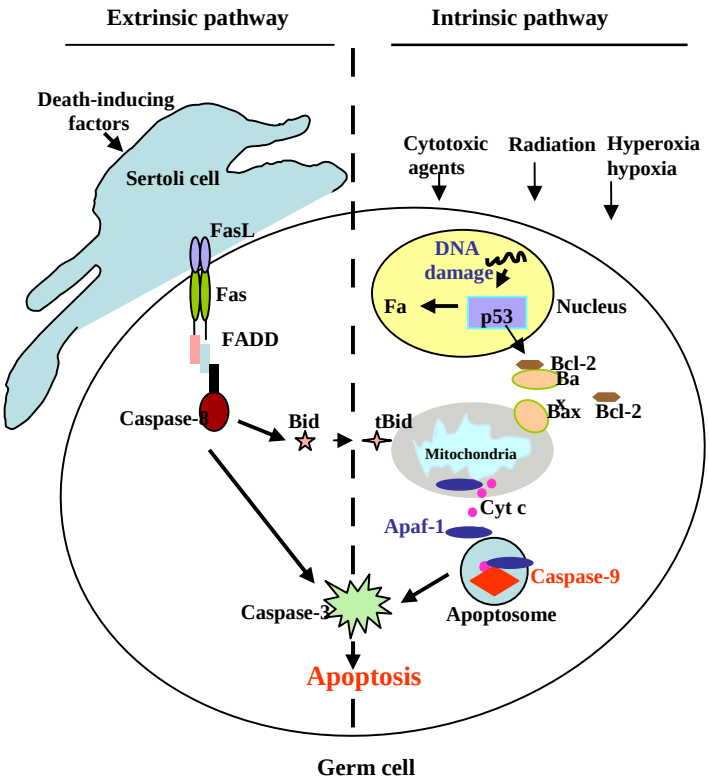

Figure. 2, The pathways of germ cell apoptosis
Apoptotic signals can be transduced either through the intrinsic (right side of the dotted line) or extrinsic (left side) pathway. In the case of the intrinsic pathway, P53 is up regulated in response to damage and, in turn, enhances the expression of Bax, a pro-apoptotic member of the Bcl-2 family of proteins. Cell death is initiated by insertion of Bax into the outer mitochondrial membrane to facilitate the release of cytochrome c into the cytoplasm, where it activates apoptosomes. In the case of extrinsic pathway, a damaged Sertoli cell up-regulates expression of Fas ligand, which binds to its receptor on the germ cell to recruit several apoptotic proteins and activate caspase-8. Both of these pathways converge in the activation of caspase-3, which then activates additional downstream caspases, triggering the process that results in cell death.

In the case of the intrinsic pathway, the tumor suppressor protein p53 and the Bcl-2 family of regulatory proteins play crucial roles in connection with programmed cell death in response to DNA damage. p53 is closely associated with the nuclear membrane and, upon up-regulation, is translocated into the nucleus, where it acts as a transcription factor. Such up-regulation induced by DNA damage caused by cytotoxic agents or ionizing radiation leads to cell-cycle arrest to allow DNA repair or apoptosis. The elevated levels of p53 bind to the promoter region of the Bax gene and activate its transcription (33).

As a consequence, changes in the ratio of heterodimerization of Bax with Bcl-2 (see below) leads to translocation of Bax from the cytoplasm to the outer membrane of mitochondria, which causes release of cytochrome c into the cytoplasm. This cytochrome binds to apoptotic protease-activating factor-1 (Apaf-1) and procaspase-9 to form an apoptosome, which then activates caspase-9. Activated caspase 9 activates, in turn, caspase-3 by partial proteolysis, thereby trigging activation of other downstream caspases

and nucleases, which catalyze fragmentation of internucleosomal DNA, along with degradation of other cellular components.

The MDM2 (murine double minute 2) protein is encoded by an oncogene and exerts potent inhibition of p53. MDM2 binds to the domain of p53 that activates transcription, thereby blocking its ability to up-regulate target genes and exert anti-proliferative effects (34, 35).

The Bcl-2 family of proteins constitutes a major class of intracellular regulators of apoptosis (24). This family can be divided into three subgroups: Members of group 1, including Bcl-2, Bcl-$x_{L,}$ and $Bcl_{-w,}$ generally exert anti-apoptotic effect, whereas Bax, Bak, Bid and Bad are considered to be pro-apoptotic. The key function of members of the Bcl-2 family appears to be regulation of the release of pro-apoptotic factors from mitochondria. Accordingly, oligomerisation of Bax or Bak that has been inserted into the outer membrane of mitochondria facilitates the release of cytochrome, whereas heterodimerization of the anti-apoptotic Bcl-2 and pro-apoptotic Bax prevents this event (24, 36).

Thus, the intracellular ratios of Bax/Bcl-x and Bak/Bcl-x may determine the survival of Sertoli cells, spermatogonia and spermatocytes. In the testis Bcl-w, (which can also form complexes with Bax and Bak) Bax, Bak, and Bad mRNA, as well as the corresponding proteins have been detected in Sertoli cells, spermatogonia, spermatocytes and Leydig cells. The ratios of Bax/Bcl-x and Bak/Bcl-x are significantly elevated in association with induction of germ cell apoptosis either by withdrawal of testosterone, or by blockade of signaling through the stem cell factor/c-kit with antibody or treatment with methoxyacetic acid.

In the case of extrinsic pathway, activation of Fas or TNF-R (the tumor necrosis factor receptor) by their ligands, leads to the recruitment of several cytoplasmic adaptor proteins and procaspase-8, which is the key initiator of the death receptor pathway. The partially cleaved and activated caspase-8 in turn activates the downstream caspases-3, 6, and 7, resulting in effective commitment by the cell to undergo apoptosis. If only a small amount of active caspase-8 is formed, the signal is amplified by caspase-8-catalyzed

cleavage of Bid to yield tBid, which activates the mitochondrial portion of the intrinsic pathway (see Fig. 2).

FasL (the Fas ligand) has been detected in the seminiferous epithelium of the mouse and rat, as well as in human Sertoli cells; while Fas itself is expressed by germ cells in both the rat and human testis. Exposure of experimental animals to radiation causes injury exclusively to germ cells with concomitant up-regulation of Fas; whereas damage to Sertoli cells enhanced production of FasL (37). These observations suggest that (1) the Fas system mediates apoptosis in the testis (2) Up-regulation of Fas is commonly associated with initiation of germ cell death. Fas upregulation is a common step in the initiation of germ cell death (3) and the FasL produced by injured Sertoli cells then induces apoptosis in the Fas-positive germ cells.

1.2.5 Vulnerability of the developing testis to gonad toxic agents and radiotherapy

In humans, the phase of testicular development during which gonocytes resume proliferation, migrate to the basement membrane, and give rise to spermatogonia continues until 4-5 years of age (13, 38). An additional period of gonocyte proliferation and the appearance of spermatogonia located pericentrally occurs in 8-9 years-old boys (39). In contrast to rodents, the juvenile period (from birth to the onset of puberty) in humans does not overlap with the pubertal period (as mentioned above). During this prepubertal period SCs and spermatogonia proliferate intensively and the male gonad triples in volume.

The SCs are functionally active, producing large amounts of anti-Müllerian hormone during the entire prepubertal period. The functional maturity of SCs, LCs and germ cells in the marmoset testis during this same period is also reflected in their expression of sulphated glycoprotein-2, the androgen receptor, 3β-hydroxysteroid dehydrogenase and the proliferating cell nuclear antigen (PCNA) (40). The development of and organization of spermatogenesis in the marmoset testis are similar to the corresponding process in the human testis (41) Because of this steady turnover of germ cells, Sertoli cells and Leydig cells, the developing testis is vulnerable to the deleterious effects of both chemo- and

radiotherapy during this prepubertal period and (40) and cancer therapy at this time can thus seriously disturb differentiation and result in infertility later in life.

1.3 Testicular function following treatment of childhood cancer

1.3.1 Treatment of Childhood cancer

The worldwide incidence of childhood cancer, which is at present 110-130 new cases per million people each year (1), increased during the period of 1960-1998 (42). Leukemia is the most common form of childhood cancer, accounting for 30% of all pediatric malignancies and demonstrating a maximal incidence at 2~4 years of age. Two-thirds of all childhood leukemias are acute lymphoblastic leukemia (ALL). The central nervous system is the second most common site for cancer in children.

In general, treatment of childhood cancer involves surgery, chemotherapy and/or radiotherapy. Chemotherapy with a combination of different drugs is the strategy most frequently used, in attempt to obtain maximal rates of cure with minimal side-effects. Autologous hematopoietic stem cell transplantation (HSCT) is used to replace the patient´s own hematopoietic stem cells damaged by high-dose chemotherapy. Alternatively, stem cells from an unrelated health donor (allogenic) transplantation can restore hematopoietic function in connection with high risk leukemia or leukemia in relapse.

1.3.2 Survival rate

In the 1950´s few victims of childhood cancer survived and even as late as 1970, only 5% of children suffering from ALL survived. Fortunately, during the past few decades treatment of childhood cancer has improved remarkably and the rate of 7-year, event-free survival for patients with ALL in the Nordic countries now approaches 80% (43). As reported by the Swedish Children´s Cancer Foundation, similar improvements have also been achieved in the treatment of lymphoma and renal tumors. Currently, 75% of all children diagnosed with cancer become long-term survivors, and it has been

estimated that by year 2010, 1 out of every 250 employed people at 15-45 years of age will have had cancer during a childhood period (1).

1.3.3 Long-term side-effects

Cytotoxic drugs and irradiation destroy not only cancer cells but also damage and kill normal cells of the body, giving rise to short- and long-term side-effects of cancer therapy. Unfortunately, more than half of the survivors of childhood cancer encounter at least one such side-effect as adults (44). These effects include increased mortality (45, 46), problems with the central neural system, serious toxic effects on various organs (47) and growth and endocrine dysfunctions (48), as well as attenuated or lack of fertility (49), and such late sequelae diminish markedly the quality of the individual´s life. Thus, the primary challenge faced by pediatric oncologists today is to maintain excellent states of survival rates while minimizing side-effects.

1.3.4 Testicular function in long-term survivors of childhood cancer

Spermatogenesis originates with the spermatogonial stem cells, which possess a unique potential for both self-renewal and the production of differentiated daughter cells (50). In primates including humans only A_{dark} and A_{pale} spermatogonia have been defined (Fig. 3), the former supposedly functioning as reserve stem cells, while the latter are considered to be the active stem cells. Humans exhibit only one B spermatogonial generation, in contrast to the six observed in non -primates. A_{dark} spermatogonia are quiescent and proliferate actively only in response to loss of germ cells. Repopulation by surviving stem cells results in the formation of a new population of A_{dark} spermatogonia (51).

The testis is highly susceptible to the toxic effects of radiation and chemotherapy at all stages of life. The prepubertal testis is also vulnerable to such toxic insults, due to its constant turnover of early germ cells and somatic activity (see above). Low-doses of cytotoxic drugs or irradiation destroy A_{pale} and B spermatogonia in the testes of prepubertal boys, while A_{dark} spermatogonia are unaffected (Fig. 3A). These latter cells continue to proliferate and differentiate, ensuring normal testicular function in the future.

However, high-dose chemo- and/or radiotherapy during childhood can destroy all A_{dark}, A_{pale} and B spermatogonia, leading to infertility in adulthood (Fig. 3B).

Moreover, low-dose chemo- and/or radiotherapy can deplete differentiating spermatogonia in both the pubertal and adult testis, while less sensitive spermatogonial stem cells survive and spermatocytes and spermatids continue their maturation and finally exit the testis as spermatozoa (51). This can lead to temporary oligospermia (i.e., a sperm density in the ejaculate of $<20x10^6$/ml) or even azoospermia (no sperm at all in the ejaculate) (Fig. 3C). If the damage is severe (e.g., as a result of high-dose treatment), the spermtogonial stem cells die or fail to differentiate; damaged Setoli cells are unable to support the spermatogonial stem cells; and/or the pool of spermatogonial stem cells is completely depleted and seminiferous tubules contain Sertol cells only (SCO) (52). In this case, the patient becomes permanently infertile (Fig. 3D).

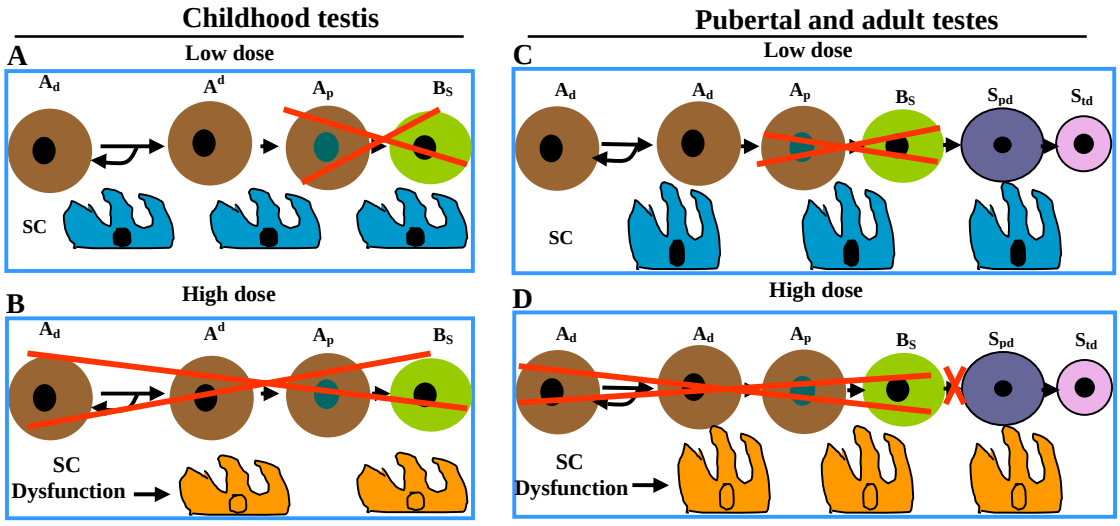

Fig. 3 Schematic illustration of effects of low- and high-dose cancer treatments on the renewal, proliferation and differentiation of spermatogonial stem cells in the epithelia of the childhood, pubertal and adult testis
(**A**) The effects of low- and (**B**) high-does chemotherapy or radiation on the childhood testis.
(**C**) and (**D**) The corresponding effects on the pubertal and adult testis.
Ad=A_{dark} spermatogonia; Ap=A_{pale} spermatogonia; Bs=B spermatogonia; Spd= Spermatocytes; Std=Spermatids; SC=Sertoli cells.

The extent of cytotoxic damage to the testis depends on the nature of the drug administered, the does and the fraction of the total dose that accumulates in this organ

(53). Since chemotherapy usually involves combination of drugs that often exert synergistic toxicity, it can be difficult to determine the specific contribution of each individual agent to the overall toxicity. In addition, lack of appropriate methodology for detecting early cytotoxic damage to the immature testis makes it hard to detect permanent tubular damage before puberty arrives and shows absence of testicular growth and increases in serum levels of gonadotrophins and absence of initiation of sperm production (54, 55).

Infertility was recognized as late sequelae of successful cancer treatment several decades ago. For example, chemotherapeutic treatment of Hodgkin´s disease with the MOPP combination (i.e., mechlorethamine, vincristine, procarbazine and prednisolone) has been reported in a number of studies to result in permanent azoospermia in more than 90% of male patients. Administration of cyclophoshpamide at doses > 7.5g /m^2 is also associated with a high risk for male infertility (50). Current management of childhood ALL involves low-dose treatment with cyclophosphamide, which, according to present opinion, is not associated with a high risk for infertility.

The degree and permanency of radiotherapy-induced testicular damage depends on the area irradiated, the total does and the regimen of fractional doses (56, 57). Total doses as low as 0.1-1.2 Gy damage differentiating spermatogonia, as reflected in abnormal cellular morphology, resulting in temporary oligoazoospermia. With total doses of 1, 2-3 or 4 Gy, spermatogenesis is reestablished in 9-18 months, 30 months and ≥ 5 years following irradiation, respectively (58, 59). Irradiation of the testis of pre-pubertal boys with doses greater than 20 Gy is associated with Leydig cell dysfunction that leads to delayed puberty, testosterone deficiency and later permanent sterility. Radiotherapy with several smaller doses that together give a total body irradiation (TBI) of 12 Gy, employed for conditioning prior to transplantation of hemotopoietic stem cells, causes permanent long-term infertility, but does not normally affect androgen production or the timing of puberty.

1.3.5 The testis provides a sanctuary site for leukemic cells and is a common site for leukemia relapse

Despite the dramatic improvements in cancer treatment, testicular relapse continues to pose a serious clinical problem. Thus, relapsing ALL is the fourth most prevalent childhood malignancy (with an incidence of 0.9/100 000). Although initial remission of ALL is achieved equally well in males and females (60, 61), testicular relapse renders the prognosis for boys worse (60). The incidence of occult-isolated testicular tumor cells is 21% at the time of diagnosis of ALL (62), 1-30% during the treatment period (63), 3-13% after cessation of therapy, and 13% even in patients experiencing more than 5 continuous years of complete remission (64).

The testis is an immunologically privilege organ, which means that the germ cells are protected from autoimmune attack. Multiple factors, both physical and immunological, contribute to the establishment and maintenance of immunotolerance in the testis. In addition to the blood-testis barrier, the elevated local concentration of testosterone plays a role in down-regulating the expression of proinflammatory cytokines such as interleukin-1(IL-1), IL-6 and tumor necrosis factor-α (TNF-α), as well as enhancing production of anti-inflammatory cytokines, such as IL-10 (15).

Furthermore, the leukocytes of the testis also play an essential role in the maintenance of testicular immune privilege. Testicular macrophages participate in Leydig cell development, regulation of adult steroidgenesis and balancing the production of pro- and anti-inflammatory cytokines (15). Moreover, the dendritic cells (DCs) in the testis not only activate lymphocytes, but also render T cells tolerant to antigens, thereby minimizing immunological responses (65).

The immune privilege of the testis provides leukemic cells with a sanctuary from attack by the immune system. Consequently, autotransplantation of testicular tissues obtained by biopsy prior to the initiation of cancer treatment into the cured patient involves a potential risk of re-seeding malignant cells (see also below). In fact, any malignant diseases which spreads via the circulation may give rise to intravascular and testicular involvement.

1.4 Approaches to protecting the testis from damage during cancer treatment and novel strategies for ensuring future fertility following cure

1.4.1 Selection of cytotoxic anti-cancer drugs that exhibit relatively low testicular toxicity and removal of the testes from the field of radiation

Reconstitution of fertility following life-saving cancer treatment is a curative approach, whereas selection of cytotoxic drugs that demonstrate relatively little testicular toxicity or appropriate modification of the administration is designed to prevent or minimize testicular side-effects. In fact, several such regimen modifications have been reported to allow survivors of childhood cancer to father children following spontaneous recovery of spermatogenesis (66, 67). For example, treatment of Hodgkin´s disease with a modified regimen, involving neither alkylating agents nor procarbazine results in temporary azoospermia in 33% and oligoazoospermia in 21% of the patients but full recovery within 18 months, in contrast to the 90% incidence of azoospermia observed with the routine MOPP protocol (see also above). Obviously, shielding the testis or removing this organ from the field of irradiation is an efficient way to avoid infertility, but, at the same time this approach may reduce the efficacy of treatment.

1.4.2 Hormonal manipulation

In rats, suppression of the hypothalamic-pituitary-gonadal axis with an agonist or antagonist of GnRH induces testicular quiescence and thereby render germ cells less vulnerable to cytotoxic effects, and furthermore, promotes recovery of spermatogenesis (68). However, analogous hormonal manipulation of male patients receiving gonadotoxic therapy has thus far failed to provide any beneficial effects, since while the survival of spermatogonia may be partially gonadotrophin-dependent, the proliferation of surviving spermatogonia is largely or entirely independent of this hormone. As the function of stem cells are also thought to be independent of gonadotropin, protection of germ cells with GnRH antagonists is unlikely to be effective (40).

1.4.3 The cytoprotective drug: amifostine

Amifostine (also known as WR-2721) is an organic thiophosphate originally developed to selectively protect normal tissues from the toxic effects of chemo- and radio-therapy without loss of antitumor efficacy. At present, this drug is widely administrated in connection with chemotherapy of lymphomas and solid tumors. Following dephosphorylation by tissue alkaline phosphatase, amifostine exerts its

protective activity by scavenging the oxygen free radicals produced by ionizing radiation and certain anti-cancer agents. Moreover, the free thiol formed by dephosphorylation provides a target for alkylating or platinum agents, which might otherwise react with DNA or RNA. The selective cytoprotection of normal tissues is assumed to be related to distribution and absorption of amifostine, as well as higher level of alkaline phosphatase activity in normal than in malignant tissues (69, 70).

In experimental animals amifostine protects against a variety of toxic effects evoked by chemotherapeutic agents, including the cardiotoxicity caused by doxorubicin. Moreover, this compound protects variety of animal species from lethal doses of radiation. However, along with the protective activity of this drug against doxorubicin-induced testicular toxicity in the infant rat, the rate of mortality with doses of 100-200mg/kg was actually elevated (71).

1.4.4 Transplantation of testicular germ cells

Mammalian spermatogenesis is a continuous, highly organized process involving sequential steps of cell proliferation and differentiation. This process begins with the spermatogonial stem cells, which possess the potential for both self-renewal and the production of differentiated daughter cells that ultimately form spermatozoa (72). These spermatogonial stem cells can be used for transplantation.

In 1994, Brinster and colleagues (73) demonstrated that transplantation of germ cells from fertile donor mice into the testes of infertile recipient mice resulted in donor-derived sperm production by the recipient animals. Moreover, the sperm arising from these transplanted germ cells were capable of participating in fertilization, both *in vivo* and *in vitro* (3, 74). Subsequently, transplantation of germ cells from rats or hamsters to mice and vice-versa has been performed successfully (75-77). In addition, even spermagonial stem cells cryopreserved for prolonged periods of time can reestablish spermatogensis in the recipient testes (78).

These findings motivated ultrasound-guided infusion of suspensions of germ cells into the testis of monkeys and humans (79), resulting in complete restoration of spermatogenesis in X-irradiated moneys (80). Other observations indicate that when human testicular cells harvested prior to initiation of cancer treatment and cryopreserved

are reintroduced into their own niche in the testis, the spermatogonial stem cells present will re-colonize the seminiferous tubules and ultimately generate mature germ cells. A clinical test of this hypothesis in adult men is currently in progress: 11 men with cancer have so far had samples of their testicular tissues harvested and cryopreserved in single-cell suspension prior to treatment (81, 82) and five of these have now completed successfully treatment and had this material injected back into their testes. The results of their semen analyses are awaited with considerable interest (83).

Such transplantation of autologous germ cells might allow long-term reestablishment of the fertility of survivors of prepubertal cancer, who cannot produce semen for cryopreservation prior to cancer therapy. Spermatogonial stem cells are present in the testis at all ages and the kinetics of colony expansion from each individual stem cell are the same regardless of the age of the donor (84, 85).

1.4.5 Transplantation of testicular tissue

Grafting entire samples of testicular tissue containing spermatogonial stem cells in their proper environment into an ortotopic or ectopic site in the patient recipients is another promising approach to preservation of fertility. Testicular tissue injected into an immuno-deficient mouse (xenologenic transplantation) or re-injected into the patient´s own body (autologous), can, once re-vascularized, generate sperm capable of fertilizing an egg *in vitro.* Xenotransplantation between species can be considered as a special form of tissue culture, while grafting into an ectopic site provides a bio-incubator for the grafted tissue.

Complete spermatogenesis and steroidgenesis by testicular grafts can be attained stimulation of the host with gonadotropin. Xenografting of testicular tissue from newborn pigs or goats into mice results in the production of functional sperm by the host. Testicular tissue that is cryoprserved prior to grafting retains its potential for maturation (2, 86, 87). Injection of sperm recovered from allografts (mouse-to-mouse) or xenografts (monkey-to-mouse) into oocytes results in fertilization (88) following embro transfer, mouse sperm from allografts produce normal, fertile progeny (88).

Prepubertal testicular tissues xenografted from the pig or Rhesus macaque monkeys into a castrated adult mouse undergoes accelerated pubertal maturation (2, 87, 89). Following grafting of adult human testicular tissues into mice, the spermatogonia survived for 195 days but spermatogenesis in the grafts degenerated (90). In contrast, ectopic grafting of primate prepubertal testicular tissues into an immuno-deficient mouse leads to differentiation of the stem cells, initiation of spermatogenesis and production of sperm (89).

Thus, transplantation of testicular tissues offers a potential approach to preservation of fertility, but the clinical risks associated with this procedure remain to be elucidated.

1.5 Problems associated with autologous transplantation of germ cells or testicular tissues

1.5.1 Risks associated with autologous transplantation of germ cells

Infiltration of leukemic cells into the testis is typical phenomenon in children with ALL, partially due to the immunoprivileged status of this organ (as described earlier). Actually, most solid tumors that develop in children and all hematopoietic malignancies can potentially release malignant cells into the bloodstream, thereby causing a significant risk for contamination of the testis by these cells. This risk is especially great if the testicular tissue is removed prior to the initiation of cancer treatment. Transplantation of suspensions of testicular cells obtained by enzymatic digestion containing tumor cells into a patient who has been cured will lead to recurrence of the disease. Therefore, it is of the most importance to develop procedures for purging germ cells or eliminating tumor cells from suspensions of testicular cells before transplantation of such cells can be used for clinical purposes.

1.5.2 Risks associated with autologous grafting of testicular tissues

Ectopic grafting of the patient´s own testicular tissue into his body e.g., under the skin on an arm or leg following cure is an alternative approach to preservation of fertility. Intracytoplasm sperm injection (ICSI) of mature sperm retrieved from mouse testicular grafts into oocytes leads to conception (88). Grafting of testicular fragments requires

much less biological material than transplantation of germ cells, but involves the same potential high risk for recurrence of the cancer (see above).

1.5.3 Xenografting of testicular tissue

Through grafting human testicular tissue into an intermediate host, a process referred to as xenografting e.g., an immunodeficient animal (nude mouse) in order to mature sperm for in vitro fertilization reseeding of malignant cells into the patient can be avoided. However, to date all investigations concerning xenografting of testicular tissues have involved healthy or, at least, non-leukemic donors. Consequently, preclinical studies designed to explore whether xenografting of leukemic testicular tissues allows efficient generation of gametes, as well as to identify any possible negative effects on the immuno-deficient, xenogenic host are required (91). If such xenotransplantation dose causes tumors in the host animals, this phenomenon might be employed to detect contamination of gonadal tissue by malignant cells. An unexplored issue concerning xenografting is the potential transmission of viruses or other contagious agents via gametes generated in the xenografts, which might lead to incorporation of foreign DNA into the donor sperm, and, thus, induction of tumors, change in genome of the offspring and/or viral infections.

2. AIMS OF THE STUDY

The general aims of investigation were to examine the mechanisms by which anticancer drugs damage the immature testis and to explore novel strategies and putative risks associated with the use of auto- and xenotransplantation of testicular germ cells or testicular tissues for preservation of fertility. The specific aims were as follows:

1) to identify the cell types in the spermatogenic epithelium of the immature rat testis in which doxorubicin induces apoptosis, as well as to examine the underlying mechanisms and potential cytoprotective effect of amifostine (**Paper I**).

2), to assess the risk of reseeding leukemic cells into a healthy syngenic host by transplanting contaminated testicular cells from a leukemic donor (**Paper II**).

3), to evaluate the efficacy of eliminating leukemic cells from testicular samples designed for autologous transplantation of germ cells by FACS or MACS sorting and to identify the factors that complicate purification of germ cells on the basis of surface markers (**Papers III and IV**); and

4), to investigate the possibility of retrieving male gametes from testicular grafts of a leukemic donor, as well as the potential usefulness of transplantation of such tissue into nude mice for detecting contamination of testicular biopsies by leukemic cells (**paper V**).

3. METHODOLOGICAL CONSIDERATION

3.1 Experimental animals

Male Sprague-Dawley rats 6, 16 and 24 days of age were used in the study designed in **Paper I**. In the case of **Paper II-V**, an acute lymphoblastic leukemia referred to as Roser´s leukemia was introduced into male piebald variegated (PVG) rats, which were then utilized as donors of testicular cells and testicular tissues samples for transplantation e.g., into syngenic PVG rats for evaluation of the risk associated with this procedure (**Papers II and V**), as well as for investigating the capacity for cell purification by FACS and MACS sorting (**Papers III and IV**). In addition, 6-week-old male, homozygous outbred Nu/Nu mice (Nu-*Foxn*1^{nu} mice) were utilized as xenogenic recipients in **Paper V**.

3.2 Methods for detection of apoptotic cells and identification of molecules involved in apoptotic pathways (Paper I)

3.2.1 Dissection of and sequential squash preparation from single, long seminiferous tubules

In order to assess doxorubicin-induced apoptosis in segments of seminiferous tubules, a single, long tubule, selected at random from one testis of 6-day-old rats, was examined. In the case of the 16- and 24 day-old animals, the seminiferous tubules were microdissected into segments representing stages II-VI, VII-VIII and IX-XI of spermatogenesis. Apoptotic cells were counted under microscope before or after squashing or following TUNEL staining.

3.2.2 In situ 3´- end-labeling of DNA (TUNEL staining)

Activation of endonucleases, a biochemical event that occurs uniquely in apoptotic cells, results in the cleavage of cellular DNA into fragments 200 base pairs in length. Such DNA fragmentation in individual cells is therefore as a reliable diagnostic indicator of apoptosis. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) was used here to detect such fragments in apoptotic testicular cells.

3.2.3 Phase contrast light microscopy and electron microscopy

Apoptosis is associated with well-defined morphological changes, including reduction in cell volume, blebbing of the plasma membrane, chromatin condensation, the formation of apoptotic bodies and phagocytosis of the bodies by neighboring cells. The occurrence of these characteristic changes in segments of seminiferous tubules and preparation of testicular tissue was examined here employing both phase-contrast light and electron microscopy.

3.2.4 Western blotting

Caspase 8 is an initiator of the extrinsic apoptotic pathway, medicated via a death receptor, a G-protein receptor on the plasma membrane. Doxorubicin damages the immature testis by up-regulating the expression of the Fas ligand (FasL) by SCs. Germ cell apoptosis is trigged by binding of FasL to its receptor on the surface of these cells and subsequent activation of caspase-8 leading, in turn, to activation of downstream caspases and cell death. At the same time, the DNA damage in the germ cells reduces the expression of MDM2, which normally down-regulates p53. The elevated level of p53 thus obtained triggers the intrinsic pathway of cell death via mitochondria, as well as directly up-regulating the expression of Fas by germ cells, thereby triggering apoptosis through the extrinsic pathway. In this investigation we examined testicular levels of the MDM2 and p53 proteins and of cleaved caspase-8 by Western blotting.

3.3 Elimination of leukemic cells from cell suspensions prepared from leukemic testicular tissue (Papers III and IV)

3.3.1 Immunocyto- and histochemical analyses

Immunocyto- and histochemistry were employed in conjunction with flow cytometry to identify surface markers that might allow efficient purification of germ cells.

3.3.2 Identification of surface markers for cell sorting and assessment of cell purity employing flow cytometry

Flow cytometry can be used to analyze the levels of expression of various protein antigens on the surfaces of cells. In order to determine which proteins are expressed at the highest levels on the surfaces of leukemic and normal germ cells, for use in purging germ cells by FACS and MACS, the expression of a set of lymphoid and spermatogonial surface markers were analyzed by FACS. In addition, cell purity after sorting was also assessed by FACS.

3.3.3 Fluorescence-Activated Cell Sorting (FACS)

With FACS, a heterogeneous population of cells in suspension can be characterized and separated on the basis of the intensity of the fluorescence they emit while passing single-file through an illuminated tube. The detailed FACS protocols employed are described in Paper III and protocol 4 is illustrated in Figure 4. Monoclonal antibodies directed against CD4 or MHC-Cl I (leukemic cell markers) or Ep-CAM (a marker for germ cell) and tagged with the fluorescent dye PE (phycoerythrin) or Alexa, respectively, were allowed to bind to the leukemic (red) and germ cells (green) in a cell suspension, and this suspension was then injected into the cell sorter (Fig. 4). As the cells pass single-file through a laser beam of appropriate wavelength, some of the light is scattered, while the rest excites the fluorescent dye, so that the tagged cells emit fluorescent light on the basis of the scattered and fluorescent light, the computer determines how each cell will be sorted and, when a drop of liquid containing a cell is formed at the end of the stream, an appropriate electrical charge is applied and the charged drop will by deflected to the left or right by charged electrodes into sample tubes. Drops containing no labeled cells are not charged and, thus, collected in the run off tube. The final result is three tubes containing germ cells, leukemic cells and non-labeled cells (Fig. 4).

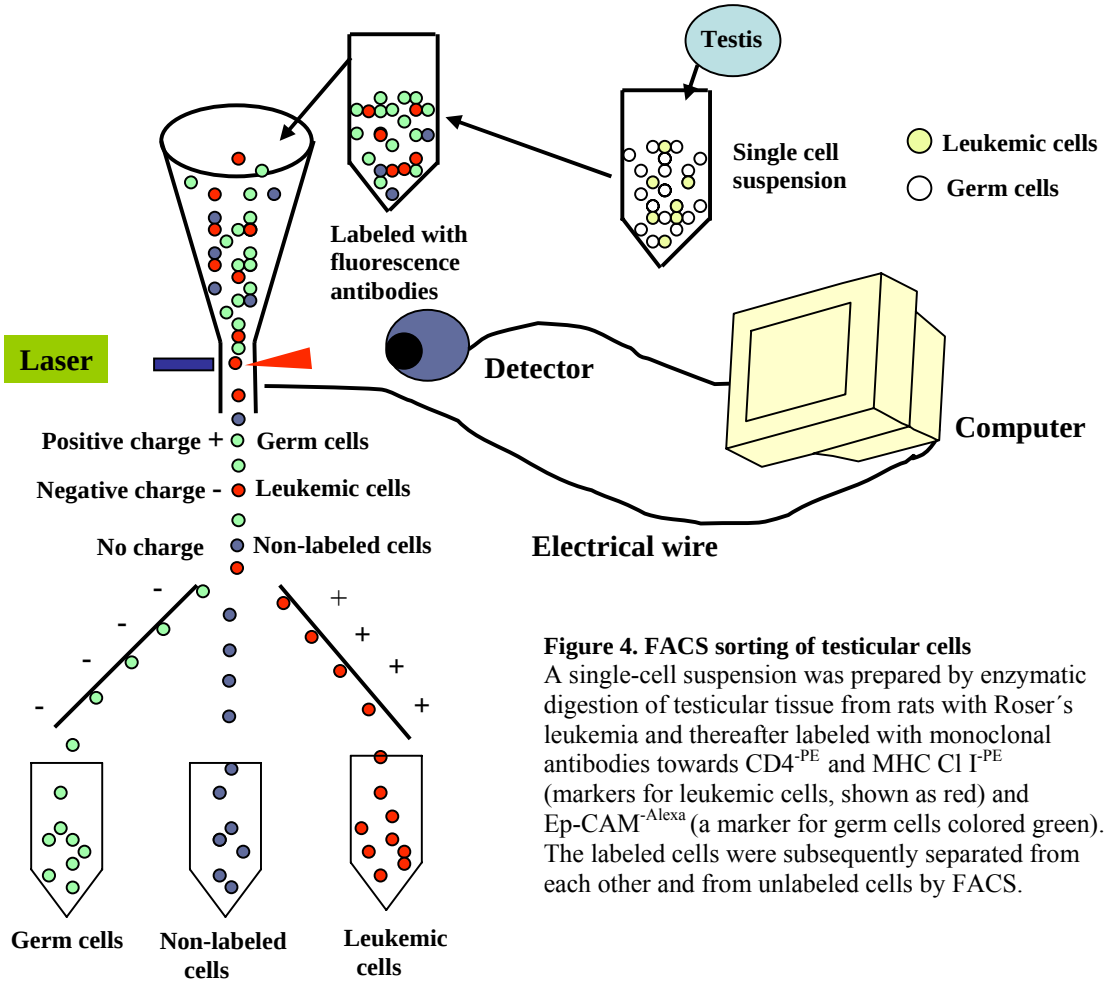

Figure 4. FACS sorting of testicular cells
A single-cell suspension was prepared by enzymatic digestion of testicular tissue from rats with Roser´s leukemia and thereafter labeled with monoclonal antibodies towards $CD4^{-PE}$ and MHC Cl I^{-PE} (markers for leukemic cells, shown as red) and Ep-CAM^{-Alexa} (a marker for germ cells colored green). The labeled cells were subsequently separated from each other and from unlabeled cells by FACS.

3.3.4 Magnetic-Activated Cell Sorting (MACS or immunomagnetic cell sorting))

One of the most frequently employed procedure for sorting cells on the basis of their surface markers, MACS is rapid and simple to perform, which has led to its broad use in clinical practice and in connection with basic research in hematology, cell biology and immunology. This approach has also recently been applied to isolate and enrich spermatogonia from rodent testes (92). Here (Paper IV) negative selection of testicular cells was performed in order to remove leukemic cells from suspension of testicular cells prepared from rats with Roser´s leukemia, as illustrated in Figure 5.

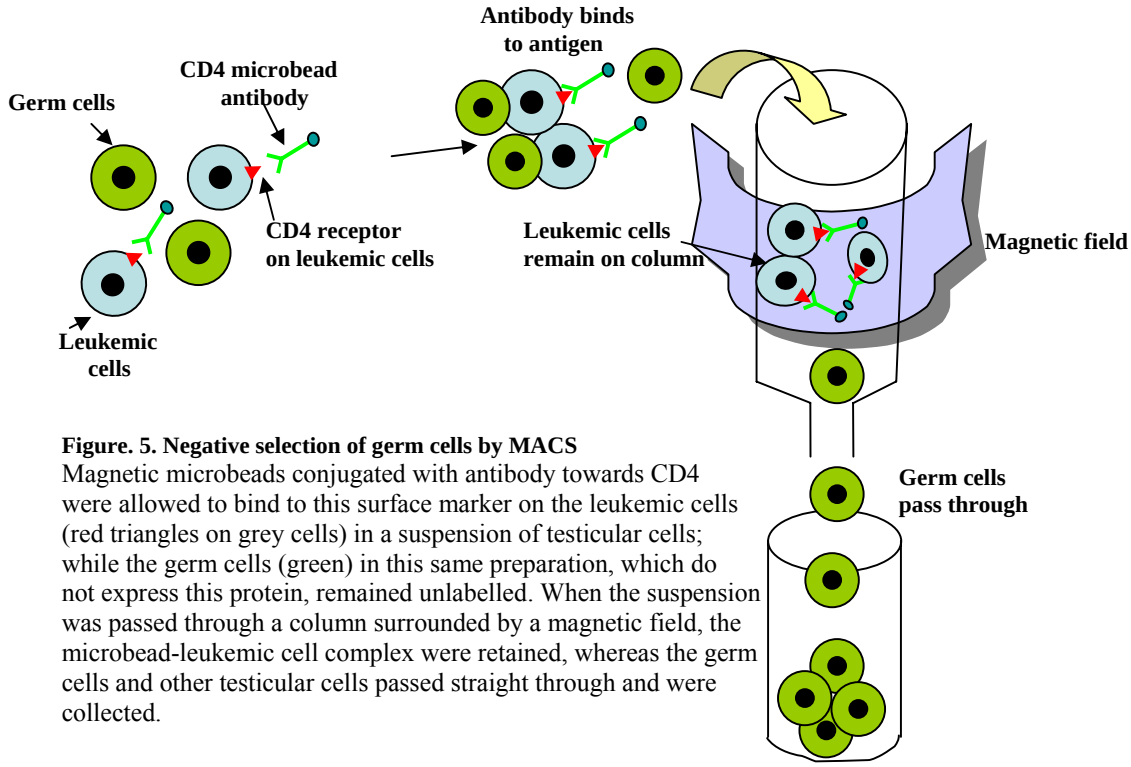

Figure. 5. Negative selection of germ cells by MACS
Magnetic microbeads conjugated with antibody towards CD4 were allowed to bind to this surface marker on the leukemic cells (red triangles on grey cells) in a suspension of testicular cells; while the germ cells (green) in this same preparation, which do not express this protein, remained unlabelled. When the suspension was passed through a column surrounded by a magnetic field, the microbead-leukemic cell complex were retained, whereas the germ cells and other testicular cells passed straight through and were collected.

3.4 Auto- and xenografting of testicular tissues into PVG rats and nude mice (Paper V)

With regards to the potential retrieval of gametes from leukemic testicular grafts, the influence of such grafts on the host animals and the possibility of utilizing nude mice as an indicator of contamination of testicular tissue by leukemic cells were evaluated. Fresh or cryopreserved leukemic testicular tissue was implanted subcutaneously into nude mice or syngenic PVG rats and the growth of these grafts at each site of injection and the general health status of the host animals monitored.

4. RESULTS

4.1 Doxorubicin induces apoptosis in germline stem cells in the immature rat testis and amifostine cannot protect against this cytotoxicity (Paper I)

Forty-eight hours following treatment of 6-day-old rats with doxorubicin, the number of apoptotic germ cells in the testes of these animals (as determined by light microscopy and TUNEL staining) was enhanced 8-fold. Migrating spermatogonia and gonocytes, located in the vicinity of the basement membrane were the major target cells for this cytotoxic effect, whereas the mean number of apoptotic gonocytes located pericentrally was unattached. Under the same experimental conditions, the statistically significant, physiological increase in the number of Sertoli cells that occurred in untreated animals by postnatal day 8 was delayed in the doxorubicin-treated rats.

This elevated germ cell apoptosis and reduction in the total number of Sertoli cells in the youngest rats 48 hours after treatment were trigged by up-regulation of p53, activation of caspase-8 and a lowering of the level of the MDM2 protein. Moreover, administration of a single dose of amifostine 15 minutes prior to doxorubicin treatment exerted no protective effect.

In contrast, doxorubicin caused no significant change in the physiological, stage-specific germ cell apoptosis occurring in the testes of 16- and 24-day-old rats.

4.2 Intratesticular transplantation of testicular cells from leukemic rats into healthy recipients results in transmission of leukemia (Paper II)

All animals injected with either fresh or cryopreserved leukemic testicular cells died from donor-derived leukemia. Cryopreservation caused a 3~6-day delay in the development of the terminal phase of this leukemia. Furthermore, when 20 leukemic cells were mixed with normal testicular cells and transplanted into the testes of synsgenic rats, 3 of the 5 recipients developed leukemia. Moreover, the time required for developing leukemia was directly proportional to the number of leukemic cells injected.

These findings indicate that transplantation of testicular germ cells involves a considerable risk of reseeding leukemic cells into the recipients. In a clinical setting, this means that testicular tissues obtained from a child with cancer prior to the initiation of treatment cannot later be reintroduced into to the patient without first removing the malignant cells. Procedures for the removal of leukemic cells from testicular samples must therefore be developed.

4.3 Combined positive and negative flow cytometric sorting allows purification of spermatogonial stem cells from rats with Roser´s rat T-cell leukemia (Paper III)

Both FACS and immunocytochemical analysis revealed that leukemic lymphoblasts of Roser´s leukemia express high levels of CD4 and MHC Cl I on their surface. Of the antigens towards which the antibodies tested were directed, Ep-CAM was the only surface marker on spermatogonia that was absent from the leukemic cells. Accordingly, for FACS purification of germ cells, CD4 and MHC Cl I were chosen as the markers for leukemic cells and Ep-CAM as the marker for germ cells.

The results of the four different protocols employed for FACS sorting of testicular cells from leukemic donors are summarized in Table 1. With protocol 1, the number of leukemic cells was reduced 96%, while the relative proportion of spermatogonial stem cells was similar to that present in the leukemic testicular cells prior to sorting. In this case, only one recipient of the sorting cells survived. With protocol 2, all animals that received sorted testicular cells died 25-27 days after injection and the proportion of spermatogonial germ cells was reduced. The poorest outcome was obtained with protocol 3, which reduced the relative number of leukemic cells by 99%, after which but 0.2% of the cells expressing spermatogonial markers remained. Moreover, none of the recipients of testicular germ cells sorted according to protocol 3 survived.

Table 1. Representative FACS sorting of testicular cells from leukemic donors

			Leukemic testicular cells			
			Pre-sorting	Post-sorting		
Immunological phenotype	Cell type	Normal testicular cells(%)		**Protocol 1:** Ep-CAM$^{-}_{Alexa}$ (%)	**Protocol 2:** EpCAM+2nd Ab^{-PE} (%)	**Protocol 3:** CD4^{PE}+MHC-Cl I^{-PE} (%)	**Protocol 4:** EpCAM^{Alexa}+CD4^{PE}+MHC-Cl I^{-PE} (%)
CD90^{+}+Ep-CAM^{+}	Spermatogonial stem cells	0.5	1.0	1.1	0.8	0.2	0.6
CD90^{+}+CD4^{+} +MHC-Cl I^{+}	Leukemic cells and normal testicular somatic cells	7.7	12.3	0.5	ND	0.1	0

ND: Not detected

Only combined positive and negative sorting according to protocol 4 allowed purification of testicular cells free from leukemic contamination. All recipients of these cells survived without developing leukemia and no leukemic cells were detected in the sorted fraction upon FACS analysis. However, 99.5% of all of the testicular cells were lost and no enrichment of putative spermatogonial stem cells could be detected (Table 1).

In addition, the poor specificity of the spermatogonial surface markers employed here, as well as aggregation of germ and leukemic cells proved to be serious obstacles to positive selection of germ cells; while, at the same time immunophenotypic variation among the lymphoblastic leukemia cells prevented their adequate removal. Although our present observations demonstrate that with presently available procedures, purification of germ cells from a leukemic donor by flow cytometry is possible, but exhibits insufficient efficacy and safety for clinical use.

4.4 Normal rat testicular cells cannot be separated effectively from Roser´s T-cell leukemia cells by immunomagnetic approaches (Paper IV)

After CD4-negative cells (i.e., testicular cells) obtained by MACS sorting were injected interstitially into syngenic PVG rats, all of the recipients died 15 days later,

which was only one day later than the terminal phase of leukemia developed in animals that received unsorted testicular cells. On the average, only a 30% reduction in the proportion of leukemic cells could be achieved by MACS separation as determined by FACS analysis of the sorted cells. This reduction was improved from 27% to 49% by optimization of the concentration of antibody, but the cell yield decreased simultaneously. Variations in the size and expression of surface antigens on testicular leukemic cells (Fig. 6) were shown to be the major obstacles to effective removal of leukemic cells from leukemic testicular cells, preparations by MACS. As expected, cell sorting with this procedure does not prevent the transmission of leukemia to syngenic PVG rats via transplantation.

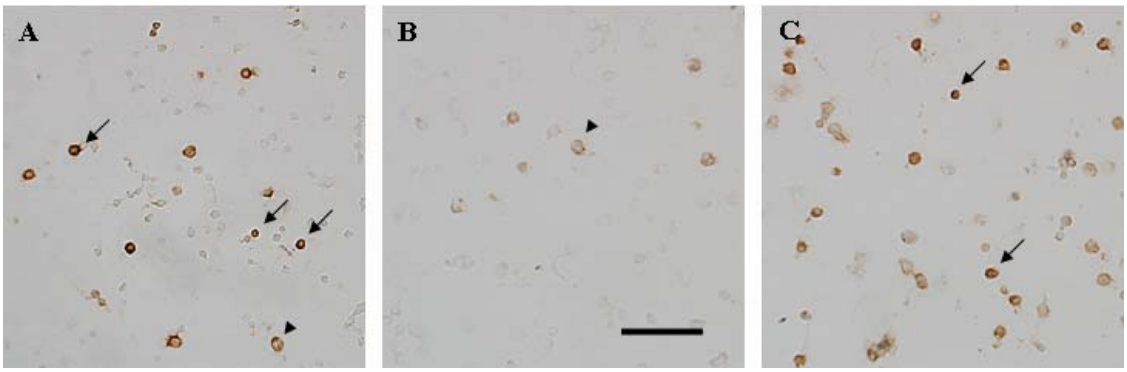

Fig. 6 Selective removal of small cells that were immunostained intensely for CD4 by MACS

In photographs show cytospin slides of testicular cells stained immunocytochemically with antibody directed towards CD4 before and after separation by MACS (**A**). Prior to sorting, the infiltrating lymphoblasts of various sizes stained positively for CD4 (brown color). There were variations in the intensity of labeling and some of the small cells stained very heavily (arrows). (**B**) Following separation by MACS, some large, weakly CD4-positive cells (arrowhead) were still observed in the germ cell fraction; whereas cells which expressed CD4 strongly and/or were small-to-intermediate in size were detected in leukemic cell fraction (**C**).

4.5 Xenotransplantation of testicular tissues into nude mice can be used for the detection of contaminated leukemic cells (paper V)

Following injection of known numbers of leukemic cells into nude mice, 70% of the animals that received more than 200 leukemic cells each developed terminal-phase

leukemia, while mice injected with 20 leukemic cells survived. All of the leukemia in mice originated from the donor, as confirmed by their expression of the rat CD90 marker.

Furthermore, all mouse and syngenic PVG rat hosts developed either local tumors at each site of injection and/or generalized leukemia following implantation of fresh or cryopreserved leukemic testicular tissues, regardless of the number of grafts implanted. The seminiferous tubules of these grafts were destroyed by proliferating leukemic cells and no spermatogenesis was detected nor were any donor-derived gametes retrieved from any of grafts.

These findings reveal that ectopic transplants of leukemic testicular tissue do not produce mature sperm, but rather local tumor formation due to contamination by leukemic cells. The pronounced susceptibility of nude mice to the formation of such local tumors makes this system a sensitive indicator of contamination of testicular grafts by leukemic cells. This indictor could be utilized to evaluate the purity of clinical biopsy material.

5. DISCUSSION AND FUTURE DIRECTION

The observations described in **Paper I** indicate that the primary deleterious effect of doxorubicin on the immature rat testis is damage to and the consequent induction of apoptosis in the pool of germline stem cells. Since these precursors of the spermatozoa are damaged, reproductive function in adulthood will be permanently impaired (6, 93, 94). These cells are most sensitive to doxorubicin-induced apoptosis during initiation of spermatogenesis, since no alteration on the frequency apoptosis in response to doxorubicin was detected in rats undergoing and late puberty. In general, these findings confirmed that the immature testis is highly susceptible to damage by cytotoxic drugs and suggest that reproductive capacity later in life can be impaired by treatment of cancer early childhood.

In the present study, Sertoli cells were seen to be totally resistant to doxorubicin-induced apoptosis. However, in the 6-day-old rat doxorubicin dose inhibit the physiological proliferation of immature Sertoli cells, which nurture the spermatogonial stem cells. Indeed, this effect on Sertoli cells may contribute to the enhanced apoptosis occurring among spermatogonial stem cells, as well as the capacity of these cells to support germ cells later in life.

The pronounced sensitivity of the immature rat testis to the cytotoxicity of doxorubicin may reflect the fact that the multiple-drug resistance systems in this organ are not yet fully developed. P-glycoprotein mediates the efflux of cytotoxic drugs out of cells and the presence of higher testicular and lower serum concentrations of doxorubicin in 6-day-old than in the older rats suggests that the immature testis expression relatively low levels of this glycoprotein and /or other defense system. Clearly, more research on this question is called for.

The quantity and quality of the donor spermatogonial stem cells and of the environment in the testis of the recipient exert a profound influence on recolonization and reinitiation of spermatogenesis following transplantation of spermatogonial stem cells. Survivors of childhood cancer have usually been subjected to multidrug regimens, which may exert synergistic cytotoxicity on the testis, and it is critical to determine whether

testes exposed to a combination of anticancer drugs can provide the proper environment for transplanted spermatogonial stem cells. When rats were treated with busulfan on postnatal days 8-12, 40% of the animals were unable to support endogenous or grafted stem cell spermatogonia and became infertile (85). On the other hand, successful spermatogenesis occurs in the irradiated testes of monkeys following autologous germ cell transplantation (95).

Recently, Zhang and colleagues (96) demonstrated that the blockage of spermatogonial differentiation caused by irradiation of rodent reflects damage to the somatic testicular environment, not to the germ cells themselves. This observation is agreement with our present finding that the somatic environment provided by the testis is also damaged by treatment of the immature rat with cytotoxic drugs. Thus, successful transplantation of spermatogonia into testes exposed to chemotherapy at an early age may be limited by the somatic damage caused and simultaneous hormone treatments and/or transplantation of somatic elements may be required to restore normal spermatogenesis. More research on primates could reveal whether similar toxic damage to the somatic environment in the testis affects autologous transplantation of germ cells in humans as well.

The observations described in **Paper II** indicate that autologous transplantation of germ cells together with leukemic testicular cells involves a considerable risk for recurrence of the disease. This may be particularly true for patients with blood-bone malignancies, lymphoma or any form of cancer that exhibits hematologinous metastasis. Upon transfer into SCID (severe combined immunodeficient) mice, a single leukemic stem cell can cause tumors development, i.g., only a minimal number of contaminating cancer cells are required for relapse (97). It would appear that new procedures for totally eliminating cancer cells from mixtures of testicular cells and/or completely novel approaches involving tissue transplantation into xenogenetic animals and designed to retrieve donor-derived gametes must be developed in order to restore the fertility of patients who have been cured without simultaneously risking cancer relapse.

Small biopsies of testicular tissue contain only a limited number of spermatogonial stem cells. Appropriate procedure for culturing the cells in such biopsies *in vitro* might not only multiply the number of spermatogonia available, but might also eliminate contaminating malignant cells. Transplantation of cultured testicular cells into rodents has already been shown to result in initiation of spermatogenesis (98). Clearly, development of appropriate system for culturing human testicular cells would be of considerable value in connection with attempts to preserve the fertility of survivors of childhood cancer.

At present, the most efficient procedures for eliminating malignant cells from clinical bone samples are FACS and MACS. Recently, FACS was also successfully used to isolate and enrich gonocytes and spermtogonia from rodent testes. In the **Papers III** and **IV** we took advantage of the high-quality performance FACS and MACS to attempt to decontaminate testicular samples taken from rats with Roser´s leukemia. In the case of sorting by FACS, neither positive (protocols 1 and 2) nor negative (protocol 3) selection of germ cells could successfully eliminate leukemic testicular cells. These leukemic cells aggregate with the germ cells, thereby limiting the efficacy of such positive selection by FACS. Although combined positive and negative selection did yield testicular cells free of leukemic cells (protocol 4), 99.5% of all testicular cells were lost and no enrichment of putative spermatogonial stem cells was observed with this approach. Accordingly, combined positive and negative flow cytometric isolation of germ cells from testicular samples from leukemic rats is possible, but inefficient and unsafe.

At least four different factors should be considered in connection with the purification of germ cells. Firstly, there is the yield with our successful protocol 4 200-300x10^6 testicular cells obtained from 6-10 testes of 40-day-old rats were required for FACS sorting. Morphological studies indicate that one of the testes of 1-year-old boy contains no more than approximately 13x10^6 germ cells. Only very small testicular biopsies (0.3-0.4 cm^3) can be taken and these may contain, at most, only 0.018~0.024x10^6 spermatogonial stem cells (3x30x0.02%). In practice, cryopreservation and subsequent isolation of germ cells employing several enzymatic digestions result in significant loss of these cells. Furthermore, purification by FACS yields only 0.23% of

the original testicular cells. According to previous reports, the rate of colonization of a recipient testis by transplanted germ cells can be expected to be less than 4% (99). Thus, the unattainably large number of spermatogonia stem cells required, is the major factor that restricts clinical application of testicular germ cell transplantation. Approaches designed to expand the number of human spermatogonial stem cells available and/or enhance their capacity for colonization are needed.

A second factor of significance in this context is the purity. With the exception for Ep-CAM all of the spermatogonial surface markers examined here were also present on the leukemic cells. Ep-CAM is known to be expressed on carcinoma cells, which may restrict its usefulness as a selection marker for spermatogonia in procedures involving solid tumors. There is definitely a need for the identification of novel, more specific surface markers for spermatogonia.

Thirdly, although injection of $0.1x10^6$ testicular cells obtained by combined positive and negative FACS sorting did not lead to transmission of leukemia, would this also be the case if the number of cells were increased 10- to 100-fold? The capacity of a single leukemic stem cell to cause cancer relapse (97), together with the relatively limited capacity of FACS to detect residual malignant cells (one in 10^4-10^5 cells) (100) seriously restrict the usefulness of FACS for clinical purposes.

Finally, it should be remembered that reports concerning efficiency of FACS sorting for elimination of tumor cells from mixture of testicular cells have been contradictory (101-103). Employing a mouse leukemia induced by a murine leukemic cell line and mixtures of human testicular cells and leukemia or lymphoma cell lines, Fujita and colleagues (101, 102) found that FACS based on CD45 and MHC Cl I as selective surface markers could completely remove malignant cells from the testicular cells. Accordingly, these investigators propose that FACS offers a potential clinical application for restoration of human fertility following anticancer therapy. In contrast, using very similar approach, Geens and coworker (103) could not eliminate cancer cell lines mixed artificially with normal testicular cells by FACS. In that case, 0.58% of the malignant

cells remained in 10 of the 11 samples sorted by FACS using combined positive and negative selection with CD49 as the germ cell marker and HLA marker for somatic cells. These findings are in good agreement with our own.

At present, it can be concluded that the various purification strategies involving FACS that have been described to date have not proven effective in connection with most autologous transplantation of hematopoietic stem cells (104). The findings by both Geens and coworkers and us strongly support a similar conclusion with respect to testicular cells. However, the model system involving cell lines employed by Geens, Fujita and their coworkers do not allow examination of testicular factors that might prevent effective purification.

Using experimentally induced leukemia in rat, where leukemic cells infiltrate testicular tissues naturally, we were able to demonstrate here that aggregation of leukemic and germ cells, variations in the immunophenotype and size of testicular leukemic cells, and the heterogeneity of testicular leukemic cell populations are the major factors that limit the ability of FACS and MACS to eliminate cancer cells. Since immunophenotypic variation and the formation of subsets of leukemic cells also occur regularly in connection with the development of human acute leukemias (105, 106), similar problems is expected to be encountered in the clinic. We conclude that at present, FACS is not a reliable or efficient procedure for the purification of germ cells for autologous transplantation.

In comparison to sorting by FACS, MACS was even less effective in reducing the number of leukemic cells that contaminate testicular samples. On an average, only 30% of the leukemic cells present could be removed by MACS. Although this reduction could be enhanced from 27% to 50% by increasing the concentration of antibody, this also caused major loss of testicular cells. Again, intrinsic variations in the levels of surface antigens and size of leukemic cells render MACS unable to effectively eliminate these cells from testicular samples.

All procedures designed to decontaminate testicular samples that are based on surface markers may prove unsuccessful since variations in antigen expression and physical characteristics are common features of all tumor cells (**Papers III** and **IV**).

Xenotransplantation of testicular tissue into immuno-deficient mice to obtain donor-derived gametes is a potential means of avoiding cancer relapse. However, as confirmed by the data presented in **Paper V**, leukemic cell infiltration also prevents production of gametes by such testicular grafts. Donor-derived local tumors or generalized leukemia developed in all of the recipients and the architecture of the seminiferous tubules was destroyed by rapidly proliferating leukemic cells. These observations indicate that contamination of testicular biopsies by tumor cells produces a considerable risk for cancer development even in the case of xenotransplantation. On the other hand, formation of local leukemic tumors at each site of injection suggests that nude mice may function as a valuable tool for detecting contamination of testicular biopsies by leukemic cells (**Paper V**).

Cryopreservation of spermatogonial stem cells remains as the main option for preservation of fertility for prepubertal boys diseased with cancer. Despite the risk for contamination by cancer cells, testicular biopsies can be taken and cryopreserved as a germ line bank, either in single-cell suspension or as intact testicular tissue. In order to assess the risk for cancer recurrence and evaluate the stem cell potential of this cryopreserved testicular tissue, a single graft from the testicular biopsy can be transplanted into nude mouse. If no tumor transmission to the nude mouse occurs and spermatogenesis is initiated, the remaining tissue can be transplanted into the patient. Evaluation of this clinical strategy is the one of our future priorities.

6. CONCLUSIONS AND SUMMARY

We have demonstrated here that a single dose of doxorubicin selectively induces apoptosis in germline stem cells in the immature testis of 6-day-old rats. This apoptotic process was triggered by down-regulation of MDM2, enhanced expression of p53 and activation of caspase-8. In a subsequent series of preclinical experiments involving rats with T-cell leukemia, we addressed the risks associated with autologous transplantation of germ cells or testicular tissues and explored the possibility of utilizing FACS or MACS to eliminate leukemic cells from testicular samples. The potential for retrieval of donor-derived gametes from xenografts and the possibility of using nude mice as an *in vivo* bio-indicator of contamination of testicular grafts by tumor cells were evaluated as well.

On the basis of our present findings, we draw the following conclusions:

1. The initiation phase of spermatogenesis is highly sensitive to the cytotoxicity of doxorubicin. Gonocytes and early spermatogonia are the targets of the apoptosis induced by this drug, which is triggered by caspase-8 and p53. Amifositine fails to protect against this cytotoxicity (**Paper I**).

2. Autologous transplantation of germ cells without prior removal of contaminating cancer cells involves a high risk of reseeding cancer cells into a patient who has been cured. Procedures for the purification or enrichment of spermatogonia and/or passage through a xenogenic host in order to detect contaminated cancer cells must be developed (**Paper II**).

3. Employing a combination of negative and positive selections, flow cytometric purification of germ cells free from contamination by leukemic cells is possible, but shows insufficient efficacy and safety for clinical use. Aggregation of germ and leukemic cells, variations in the immunophenotype and size of leukemic cells and the heterogeneity of testicular leukemic cells are the major factors that prevent deletion of leukemic cells by this approach. (**Paper III**).

4. Immunomagnetic cell sorting cannot be used to eliminate leukemic cells from testicular samples. (**Paper IV**).

5. Testicular xenografts contaminated with leukemic cells cannot produce gametes, but do transmit leukemia to the recipients. Accordingly, xenotransplantation of testicular tissue into nude mice may be of value for detecting contamination of testicular biopsies by leukemic cells. (**Paper V**).

In summary, our present data provide preclinical information of direct relevance to efforts to preserve the future fertility of prepubertal boys treated for cancer. We believe that continuous progress in reproductive technology and cell biology will offer such patients reliable and safe options for fathering their own child in the future.

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