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CANCER THERAPY-RELATED TOXICITY IN THE IMMATURE TESTIS

by

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ABSTRACT

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Cancer therapy-related toxicity in the immature testis

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Infertility is a common late effect of childhood cancer treatment. Testicular toxicity can clinically be first detected after the onset of pubertal maturation of the patients when the testis does not grow, spermatogenesis does not initiate and serum levels of gonadotrophins rise. Improved prognosis for childhood cancer has resulted in a growing number of childhood cancer survivors with late effects.

In our study, we developed novel tools for detecting cancer therapy-related testicular toxicity during development. By using these methods the effects of the tyrosine kinase inhibitor imatinib mesylate, chemotherapy agent doxorubicin and irradiation on testicular development were investigated in rat and monkey.

Patients with chronic myeloid leukemia and some patients with acute lymphoblastic leukemia have fusion gene BCR-ABL which codes for abnormal tyrosine kinase protein. Imatinib mesylate (Glivec®) inhibits activity of this protein. In addition, imatinib inhibits the action of the c-kit and PDGF –receptors, which are both important for the survival and proliferation of the spermatogonial stem cell pool. Imatinib exposure during prepubertal development disturbed the development and the growth of the testis. Spermatogonial stem cells were also sensitive to the toxic effects of doxorubicin and irradiation during the initiation phase of spermatogenesis.

In addition, the effect of the treatment of acute lymphoblastic leukemia on germ cell numbers and recovery of reproductive functions after sexual maturation was investigated. Therapy for childhood acute lymphoblastic leukemia seldom results in infertility.

The present study gives new information on the mechanisms by which cancer treatments exert their gonadal toxicity in immature testis.

Key words: testis, germ cell, fertility, imatinib, doxorubicin, irradiation, leukemia

TIIVISTELMÄ

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Infertiliteetti on yksi tavallisimmista syöpähoitojen jälkivaikutuksista. Syöpähoitojen haitalliset kivesvaikutukset ilmenevät vasta puberteettikehityksen käynnistyessä. Vaurioitunut kives ei kasva, spermatogeneesi ei käynnisty ja aivolisäkkeen erittämien gonadotropiinien tasot veressä kohoavat. Parantuneen ennusteen myötä on huomattu näiden hoitojen pitkäaikaisvaikutusten lisääntyneen.

Tutkimuksessamme olemme kehittäneet uusia menetelmiä, joiden avulla kivekseen kohdistuvia syöpähoitovaikutuksia voidaan tutkia jo aiemmin lapsuuden aikana. Näitä menetelmiä käyttäen olemme tutkineet tyrosiinikinaasi-inhibiittori imatinib-mesylaatin, sytostaatti doksorubisiinin ja säteilytyksen vaikutuksia kehittyvään kivekseen rotalla ja apinalla.

Kroonista myeloista leukemiaa ja joidenkin akuuttia lymfoblastileukemiaa sairastavien potilaiden BCR-ABL-geeni tuottaa virheellistä tyrosiinikinaasiproteiinia, jota kemoterapiaan käytettävä imatinib-mesylaatti (Glivec®) estää. Imatinib estää lisäksi c-kit- ja PDGF –reseptoreita, jotka molemmat ovat tärkeitä kiveksen spermatogeneettisten kantasolujen jakautumisessa ja eloonjäämisessä kehittyvässä kiveksessä. Työssämme olemme todenneet prepubertaalivaiheessa tapahtuvan imatinib-altistuksen häiritsevän kiveksen kehitystä ja kasvua. Spermatogeneettiset kantasolut (spermatogoniot) osoittautuivat myös herkiksi doksorubisiinin ja säteilyn toksisille vaikutuksille heti lääkealtistuksen jälkeen spermatogeneesin käynnistymisen vaiheessa.

Tutkimuksessa selvitettiin lisäksi leukemiahoidon vaikutusta kiveksen kantasolujen määriin ja löydöksen yhteyttä potilaiden todelliseen fertiliteettiin aikuisiällä. Akuutin lymfoblastisen leukemian hoidon huomattiin vain harvoin aiheuttavan hedelmättömyyttä.

Tutkimuksemme on tuottanut uutta tietoa mekanismeista, joilla syöpähoidot vaurioittavat kehittyvää kivestä.

Avainsanat: kives, lisääntyminen, hedelmällisyys, imatinib, doksorubisiini, säteily, leukemia

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ABBREVIATIONS

Ad	A dark
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
Ap	A pale
ATP	adenosine triphosphate
BCR-ABL	fusion gene of the BCR ("breakpoint cluster region") gene from chromosome 22 to Abl gene on chromosome 9
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumine
CML	chronic myeloid leukemia
CNS	central nervous system
cpm	counts per minute
DAB	3,3'-diaminobentzedine
DNA	deoxyribonucleic acid
FSH	follicle stimulating hormone
GIST	gastrointestinal stromal tumors
GnRH	gonadotropin releasing hormone
Gy	gray
INSL3	insulin-like factor 3
ISOL	in situ oligo ligation
LH	luteinizing hormone
MM	mouse monoclonal
MOPP	mechlorethamine, vincristine, prednisone and procarbazine
mRNA	messenger ribonucleic acid
PDGF	platelet-derived growth factor
PAD	patholgical anatomical diagnosis
PARP	poly-(ADP-ribose)-polymerase
PAS	periodic acid-Schiff's reagent/Gill's hematoxylin
PFA	paraformaldehyde
RTK	receptor tyrosine kinases
RP	rabbit polyclonal
SCF	stem cell factor
SCO	Sertoli cell only
SEM	standard error of mean
TBS	tris phosphate saline
VEGF	vascular endothelial growth factor

LIST OF ORIGINAL PUBLICATIONS

This study is based on the following publications, which are referred to in the text by their Roman numbers I-V.

I. **Nurmio M**, Toppari J, Zaman F, Andersson AM, Paranko J, Söder O, Jahnukainen K. (2007) Inhibition of tyrosine kinases PDGFR and C-Kit by imatinib mesylate interferes with postnatal testicular development in the rat. *Int J Androl*. 30: 366-76.

II. **Nurmio M**, Kallio J, Toppari J, Jahnukainen K. (2008) Adult reproductive functions after early postnatal inhibition by imatinib of the two receptor tyrosine kinases, c-kit and PDGFR, in the rat testis. *Repro Tox.* 25: 442-6.

III. **Nurmio M**, Toppari J, Hou M, Kallio J, Söder O and Jahnukainen K. (2009) In vitro assessment of doxorubicin induced cytotoxic damage in maturing postnatal rat testis. *Repro Tox*. 27: 28-34.

IV. Jahnukainen K, Ehmcke J, **Nurmio M**, and Schlatt S. (2007) Irradiation causes acute and long-term spermatogonial depletion in cultured and xenotransplanted testicular tissue from juvenile nonhuman primates. *Endocrinology*. 148:5541-8.

V. **Nurmio M,** Keros V, Lähteenmäki P, Salmi TT, Kallajoki M and Jahnukainen K. (2009) Effect of childhood acute lymphoblastic leukemia therapy on spermatogonia populations and future fertility. *J Clin Endocrinol Metab*. Accepted for publication.

1 INTRODUCTION

Improved long-term prognosis for childhood cancer has resulted in a situation where the majority of patients survive. A growing number of childhood cancer survivors have long-term side effects as a result of such treatments; more than half of these patients have at least one medical problem in adulthood (Stevens *et al*. 1998). Improved treatment results have partly been achieved at the cost of an increased number of late sequelae, such as infertility.

Postnatal testicular development is believed to play an important role in the formation of the male germ line stem cell pool and in determining final testicular size. Therefore, appropriate postnatal development of the testicular cells is required for the normal function of the adult testes. Cancer therapy may damage both the germ cells and the somatic cells (Sertoli and Leydig cells) (Wallace *et al*. 2005). Permanent damage results if Sertoli cells are unable to support stem cell spermatogonia or if the entire stem cell spermatogonia population is depleted. While the effects of cancer treatment become immediately detectable in the adult testes, the consequences of such treatments in immature boys are hidden until puberty. Currently, gonadal damage can not be detected before this.

Chemotherapy and irradiation often target dividing cells and, therefore, both the prepubertal and pubertal testes are sensitive to the effects of these treatments. In contrast, novel tyrosine kinase inhibitors aim to modulate specific signalling events targeting only the tumor cells, avoiding many of the common toxicities associated with conventional cancer therapy. However, some signalling pathways, although upregulated in tumor cells, also have critical functions in normal cells, especially during development. Inhibition of these signalling pathways during development could potentially cause adverse effects in growing children.

Novel tools for detecting cancer therapy-related testicular toxicity are needed. The present study is an approach to developing new methods for detecting gonadal toxicity in the immature testis. By using these methods the effects of the tyrosine kinase inhibitor imatinib mesylate, chemotherapy agent doxorubicin and irradiation on the testicular development were investigated.

2 REVIEW OF THE LITERATURE

2.1 DEVELOPMENT OF THE POSTNATAL TESTIS

Development of the postnatal testis involves highly organised paracrine communication that controls proliferation, differentiation and apoptotic death of germ cells, Sertoli cells, myoid cells and interstitial Leydig cells. Appropriate development of testicular cells is required for the normal function of the adult testes.

2.1.1 The Germ cells

At birth, the gonocytes of the rat are located centrally in the seminiferous cords and are mitotically quiescent until they resume mitosis on postnatal days 3-8 and begin to migrate to the basement membrane (Beaumont & Mandl 1963, Novi & Saba 1968, McGuinness & Orth 1992). Relocated gonocytes form the spermatogonial stem cell pool and differentiate further into type A spermatogonia. Proliferation of type A spermatogonia indicates the beginning of spermatogenesis.

In rats, the end of the neonatal period overlaps with the beginning of puberty. In humans, there is a long delay between birth and onset of puberty. This prolonged prepubertal period is not quiescent although some developmental events remain clinically unnoticed. As in rodents, human fetal gonocytes are located at birth in the central part of the cord and migrate towards the basement membrane. During childhood (0-10 years) the number of germ cells, tubular length and testis size increases (Muller & Skakkebaek 1983). At the beginning of puberty germ cells, now called spermatogonia, begin to proliferate at a much higher rate (Chemes 2001).

The adult testis contains stem spermatogonia, differentiating spermatogonia, primary and secondary spermatocytes and spermatids. Spermatogonia are located next to the basement membrane and in progressing towards to tubular lumen spermatogonia develop into spermatocytes and finally spermatids. This developmental process can be divided into three phases; spermatogonial multiplication under which spermatogonia go through a series of mitotic division, meiosis where first meiotic division forms secondary spermatocytes and the second meitotic division during which the spermatids are formed and spermiogenesis where spermatids transform into spermlike mature spermatids. In rats, the duration of spermatogenesis takes 52 days (Clermont & Harvey 1965), in monkey 40 days (de Rooij *et al*. 1986, Rosiepen *et al*. 1997) and in human 74 days (Heller & Clermont 1964, Amann 2008). Spermatogenic cells are arranged in distinct cellular associations that follow each other in the tubulus in a wave-like fashion and cyclically in time. These are known as the stages of the seminiferous epithelial cycle. In the rat, there are fourteen stages (Leblond & Clermont 1952), in rhesus monkey 12 (de Rooij *et al*. 1986) and in humans 6 stages (Clermont 1966). Each of these stages has a unique composition of developing germ cells.

2.1.2 The Somatic cells

The Sertoli cell has a supportive role in the development process of germ cells by providing nutrients and growth factors for them. Each Sertoli cell can support the development of a certain number of germ cells. Since the number of Sertoli cells is known to correlate closely with testicular size and sperm output (Orth *et al*. 1988), the number of these cells is important for future fertility.

Sertoli cells divide actively after birth, but cease dividing in the rat at postnatal days 15-18 (Clermont & Perey 1957, Steinberger & Steinberger 1971). Thereafter, the number of Sertoli cells is considered to be constant. Postnatally, Sertoli cells are first distributed unevenly in the testicular cords but as development proceeds they become more organized, columnar and acquire features of the adult Sertoli cells. In rats, by postnatal day 35 Sertoli cells have gained all the characteristics required for adult Sertoli cells (Ramos & Dym 1979).

In primates, infancy has been classically described as a quiescent period during which the size of the testis or the number of Sertoli cells do not increase (Nistal *et al*. 1982, Paniagua & Nistal 1984). However, when using variables that take into account the increase in the length of the seminiferous cord and volumetric growth of the testis, the active proliferation and differentiation of Sertoli cells during this period was revealed (Cortes *et al*. 1987, Rey *et al*. 1993). Higher primates have been reported to have two periods of Sertoli cell proliferation. The first begins during fetal life and continues through the prepubertal period and the second begins at puberty (Cortes *et al*. 1987, Marshall & Plant 1996). It is important to notice that until puberty testicular growth results mainly from increased tubular length as Sertoli cells proliferate. During puberty, testis growth is mainly due to an increase in the number of germ cells. The diameter of seminiferous tubules increases as germ cells proliferate and the tubular lumen begins to develop.

Leydig cells secrete testosterone and insulin-like factor 3 (INSL3) required for development and maintenance of the male phenotype. There are two generations of Leydig cells in mammals; fetal- and adult-type Leydig cells. In rats, the fetal-type Leydig cells appear on embryonic day 15.5 (Roosen-Runge & Anderson 1959, Magre & Jost 1980). The origin of these cells remains uncertain but it has been suggested that they originate from mesenchymal-like cells which have migrated into the developing testis from the mesonephros or gonadal ridge (Haider 2004). Adult-type Leydig cells begin to differentiate and replace fetal-type Leydig cells after the second postnatal week (Lording & De Kretser 1972). It is still under debate if adult-type Leydig cells are derived from the same pool as the fetal-type Leydig cells or if they actually differentiate from fetal-type Leydig cells (Haider *et al*. 1995, Haider 2004). The number of these adult-type Leydig cells increases until the age of 5 weeks (Lording & De Kretser 1972) and testosterone production follows a similar pattern, although in a slightly delayed manner (Lee *et al*. 1975).

Leydig cell development in humans has long been described to be a biphasic developmental phenomenon. However, testosterone production in humans clearly shows a triphasic pattern. The first peak occurs during week 14-18 of fetal life, the second 2-3 months after

birth and the third one lasts from puberty throughout adulthood (Prince 2001). It has been suggested that the first population of Leydig cells is referred to as fetal Leydig cells, the second, as neonatal Leydig cells and finally as adult Leydig cells. As in rats, in humans there is also uncertainty whether fetal Leydig cells regress or if they serve as a precursor population for the next developing Leydig cell population.

Mesenchyme originated peritubular myoid cells surround the seminiferous tubules in all mammalian species. In rats, mice and hamsters it is a uni-cellular layer, but in humans it can be formed from one up to six layers (Bustos-Obregon & Holstein 1973). Peritubular myoid cells are contractile and their movement facilitates the transport of spermatozoa and testicular fluid in the seminiferous tubules. In rats, peritubular myoid cells begin to contract around postnatal day 15 (Kormano & Hovatta 1972). In addition to their role in structural support and movement, myoid cells also actively participate in the regulation of the seminiferous epithelium. Many factors secreted by peritubular myoid cells affect Sertoli cell function and it has been suggested that communication between these two cell types is required for the proper formation of basal lamina during postnatal development (Tung *et al*. 1984, Skinner *et al*. 1985). Proliferation of peritubular myoid cells is also known to coincide with Sertoli cell proliferation during the growth phase of postnatal testis in rats (Palombi *et al*. 1992), which further supports the idea of their close interaction during development.

2.1.3 Endocrine regulation of spermatogenesis

Follicle stimulating hormone (FSH) and luteinizing hormone (LH) are the key regulators of spermatogenesis. Pituitary secretion of these hormones is regulated by hypothalamic gonadotrophin hormone-releasing hormone (GnRH).

FSH binds to G-protein coupled receptors located on Sertoli cells (Huhtaniemi & Toppari 2005) and controls spermatogenesis through Sertoli cell function. FSH has an important role in the proliferation of Sertoli cells during postnatal period (Arslan *et al*. 1993, Marshall & Plant 1996, Griswold 1998) and it also regulates the secretion of a number of Sertoli cell products such as transferrin, androgen binding protein, testibumin, Müllerian inhibiting substance, stem cell factor (SCF) and inhibin B (Jegou 1992, Huhtaniemi & Toppari 2005). Inhibin B consists of α- and β-subunits (De Kretser & McFarlane. 1996) and immature Sertoli cells secrete both these subunits (Andersson *et al*. 1998a). At least in humans, at puberty localization of the expression of inhibin β-subunit changes; Sertoli cells cease to produce inhibin β and germ cells from pachytene spermatocytes to round spermatids begin to secrete inhibin β. Sertoli cells also continue to secrete inhibin α-subunit in adulthood. This maturational change makes inhibin B a good marker for germinal epithelial function (Andersson *et al*. 1998a). FSH production is regulated via inhibin B by negative feedback (Huhtaniemi & Toppari 2005).

LH binds to G-protein-coupled receptors located in Leydig cells and stimulates the secretion of testosterone. Testosterone in turn has both paracrine and autocrine effects and it binds to nuclear androgen receptors, which is expressed in Leydig, Sertoli and peritubular myoid cells. LH levels are regulated via testosterone by negative feedback.

In rats, plasma levels of FSH decline after birth reaching the lowest point around postnatal day 5-15 (Miyachi *et al*. 1973, Ketelslegers *et al*. 1978). Then FSH levels begin to rise again, and it peaks on postnatal day 25-45 in rats (Swerdloff *et al*. 1971, Ketelslegers *et al*. 1978, Piacsek & Goodspeed. 1978, Culler & Negro-Vilar 1988). Thereafter, FSH levels fall again and reach a plateau from postnatal day 50 onwards (Swerdloff *et al*. 1971, Ketelslegers *et al*. 1978). Changes in the plasma levels of LH are less marked during the postnatal period; LH levels are quite constant and have a tendency to increase as the rat matures (Swerdloff *et al*. 1971, Ketelslegers *et al*. 1978).

In humans, levels of FSH and LH increase directly after birth and reach a peak during the first three months (Winter *et al*. 1975, Forest *et al*. 1976, Andersson *et al*. 1998b). Thereafter, levels of these hormones decline and reach the level at which they remain during childhood (Sizonenko 1978). During puberty secretion of LH and FSH increases (Sizonenko 1978). The level of testosterone follows the pattern of LH, suggesting that the immature testis responds to LH stimulation (Forest *et al*. 1973, Forest *et al*. 1976). Gonadotropins are secreted in a pulsative manner both in prepubertal and adult humans (Sizonenko 1978).

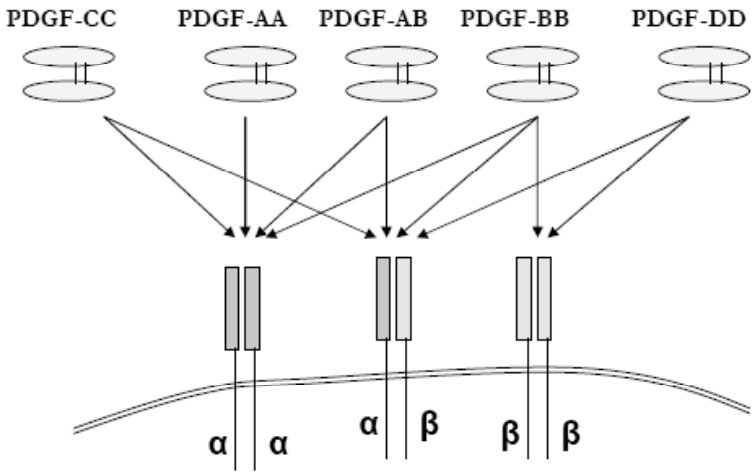
2.1.4 Paracrine regulation of spermatogenesis

Local paracrine factors secreted by Leydig, Sertoli, germ and peritubular myoid cells have an important role in the regulation of spermatogenesis (Jegou 1992). Platelet-derived growth factor (PDGF) and SCF are two of the key regulators in postnatal testis development (Rossi *et al*. 2000, Mariani *et al*. 2002).

2.1.4.1 PDGFs in the postnatal testis

PDGFs belong to the PDGF-vascular endothelial growth factor (PDGF-VEGF) family of growth factors. There are two isoforms of PDGF receptors (PDGFR-α and PDGFR-β) and four isoforms of their ligands (PDGF-A, PDGF-B, PDGF-C and PDGF-D) (Mariani *et al*. 2002). PDGFs are dimeric molecules that bind two receptor tyrosine kinases simultaneously and dimerize receptors upon ligand binding (Heldin & Westermark 1999). The capacity of different receptor dimers to bind ligands varies greatly; PDGFR-αα can bind PDGF-AA, PDGF-BB, PDGF-AB and PDGF-CC; PDGFR-ββ binds PDGF-BB and PDGF-DD; and PDGFR-αβ binds PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD (Figure 1).

PDGFs and their receptors are expressed in the rat testis during prenatal and postnatal life in a time- and space-dependent manner (Mariani *et al*. 2002). The levels of these factors are especially high during the postnatal period, reaching a peak on postnatal day 5 and declining thereafter. Sertoli cells have been shown to be the main cell types that express PDGF ligands and peritubular myoid cells possess PDGF receptors (Gnessi *et al*. 1995, Basciani *et al*. 2008). PDGF receptor expression has also been reported in part of the gonocytes during the first postnatal days (Li *et al*. 1997, Basciani *et al*. 2008). During the pubertal period only minimal positive staining of PDGF ligands is seen in Sertoli cells and receptor expression continues to be in peritubular myoid cells. In adult rat the expression patterns change; Leydig cells are the only cell type expressing PDGF-A, PDGF-B, PDGFR-α and PDFGR-β (Gnessi *et al*. 1992, Gnessi *et al*. 1995).



The expression patterns of PDGFs in human testis are less known. An expression of PDGF-A, PDGF-B, PDGFR-α and PDGFR-β has been reported in the sex cords of the human fetus (Burton *et al*. 1993, Basciani *et al*. 2002b). In the adult testis, the expression of messenger ribonucleic acids (mRNA) of both ligands have been reported (Peltomaki *et al*. 1991, Bergsten *et al*. 2001). PDGF-A is localized in adult human Sertoli cells, peritubular myoid cells, Leydig cells, leptotene primary spermatocytes and round and elongated spermatids (Basciani *et al*. 2002b) and PDGF-B is expressed in peritubular myoid cells and Leydig cells. Leydig cells express both PDGF ligands and receptors (Basciani *et al*. 2002b). This is in agreement with findings in rodents as well as the observation that peritubular myoid cells seem to lose their PDGFR expression as they mature functionally. All this indicates that PDGF signalling also plays a role in human testicular function.

In rats PDGF has been shown to be a chemoattractant *in vitro* for peritubular myoid cells (Gnessi *et al*. 1995). Peritubular myoid cells migrate towards Sertoli cells and this migration can be inhibited by blocking the PDGF receptor function (Gnessi *et al*. 1995). This emphasizes the importance of PDGF signalling during the postnatal recruitment of peritubular myoid cells to form tubule structures in co-operation with Sertoli cells. In addition, PDGFs stimulate gonocyte proliferation and migration and peritubular myoid cell proliferation (Gnessi *et al*. 1993, Li *et al*. 1997, Basciani *et al*. 2008). PDGFs also enhance the testosterone production of Leydig cells (Loveland *et al*. 1993, Risbridger. 1993).

In rodents, the interaction between the PDGF ligand and its receptor-α plays a role in the commitment of the precursors of the adult Leydig cells to differentiate (Mariani *et al*. 2002). The testicular phenotype of Pdgfa-/- mouse highlights the importance of the PDGF-A ligand to Leydig cell development showing progressive reduction in testicular size and lack of adult type Leydig cells (Gnessi *et al*. 2000). Spermatogenesis is also arrested in these animals due to progressive reduction of testosterone. Because of the perinatal lethality of the Pdgfb or

Pdfgrb knockout animals, there is no data on the development of the reproductive organs of postnatal animals (Leveen *et al*. 1994, Soriano 1994, Basciani *et al*. 2008).

2.1.4.2 C-kit receptor in the postnatal testis

The receptor tyrosine kinase c-kit and its ligand SCF are encoded at the *White spotting* and *Steel* loci, respectively. Mutations at these regions cause varying degrees of anemia, defects in melanogenesis and sterility (Russell 1979, Dubreuil *et al*. 1990, Besmer 1991, Besmer *et al*. 1993). Sterility results from the failure of primordial germ cells to increase in number and thereafter from their defective migration to the genital ridge (Mintz & Russell 1957).

C-kit receptor is widely expressed in the postnatal rodent testis. Messenger RNA has been found in spermatogonia, primary spermatocytes and Leydig cells (Clermont & Perey 1957, Manova *et al*. 1990). The protein for the c-kit receptor has been found in both immature and adult Leydig cells (Yoshinaga *et al*. 1991, Manova *et al*. 1993, Yan *et al*. 2000a). Expression of c-kit receptor in germ cells is at first postnatally faint but then around postnatal day 6 expression significantly increases and begins to appear strongly in differentiating spermatogonia (Yoshinaga *et al*. 1991). In the adult testis, c-kit protein is detected in differentiating type A, intermediate and type B spermatogonia and in the earliest preleptotene spermatocytes (Yoshinaga *et al*. 1991).

There are two forms of SCF expressed in the testis: the soluble SCF precursor and the membrane SCF. The mature soluble SCF factor undergoes proteolytic cleavage to generate the final form of this ligand. Both forms of SCF are produced at all ages by Sertoli cells and they are both able to interact with c-kit receptor (Rossi *et al*. 1991, Manova *et al*. 1993). Immunoreactive SCF has also been found in various types of germ cells and Leydig cells (Manova *et al*. 1993, Munsie *et al*. 1997). However, it has been suggested that only Sertoli cells produce SCF and these other cell types show binding of SCF.

In rat, the interaction between tyrosine kinase receptor c-kit and SCF is thought to play an role in the gonocyte migration process (Orth *et al*. 1996, Orth *et al*. 1997). Relocated germ cells at the basement membrane turn c-kit negative and form the spermatogonial stem cell pool that differentiates further into type A spermatogonia (Schrans-Stassen *et al*. 1999). C-kit expression reappears in differentiating A spermatogonia that start to proliferate (Schrans-Stassen *et al*. 1999). The importance of SCF in the regulation of the proliferation and survival of spermatogonia has been shown in several studies (Yoshinaga *et al*. 1991, Rossi *et al*. 1993, Packer *et al*. 1995, Yan *et al*. 2000b). Lack of c-kit/SCF signalling during the postnatal period has been studied by using monoclonal anti-c-kit antibody ACK-2 to block binding of SCF to its receptor. ACK-2 administration to mice *in vivo* disturbs proliferation of differentiating type A spermatogonia and increases the apoptosis of these cells (Yoshinaga *et al*. 1991, Packer *et al*. 1995).

SCF/c-kit interaction functions as a survival signal for mature Leydig cells (Yan *et al*. 2000a). In addition, studies on Leydig cell repopulation after ethylene dimethane sulfonate treatment has shown that adult-type Leydig cell development requires SCF (Yan *et al*. 2000a).

Less is known about the expression of c-kit and SCF in humans. In adults spermatogonia, spermatocytes, spermatids and Leydig cells show positive reaction for c-kit protein and SCF expression is localized in Sertoli (Strohmeyer *et al*. 1995, Bokemeyer *et al*. 1996, Sandlow *et al*. 1996) and Leydig cells (Sandlow *et al*. 1996).

2.2 TESTICULAR FUNCTION AFTER CANCER TREATMENT IN CHILDHOOD

The worldwide incidence of childhood cancer is low. Age-standardized annual incidence is 70-160 new cases per million children at the age of 0-14 (Stiller 2004). In Finland, about 150 children are diagnosed with cancer each year. Leukemia is the most common form of childhood cancer; 85% of leukemias are acute lymfoblastic leukemia (ALL) and about 10% acute myeloid leukemia (AML). Chronic myeloid leukemia (CML) is a rare form of leukemia in children. Central nervous system tumors are the second most common type of cancer in children and the third most common are lymphomas. Imatinib mesylate is used to treat patients with Philadelphia chromosome positive ALL and patients with CML. Most of the patients that suffer from leukemia or solid tumors are treated with anthracyclines including doxorubicin. Irradiation is needed in the treatment of paediatric brain and some of the solid tumors. In addition, approximately 20 children in Finland every year need allogenic stem cell transplantation and many of these patients are treated with whole body irradiation.

Although cancer incidence is low, improved prognosis has led to an increasing number of survivors. Today 50% of all childhood cancer patients and 85% of childhood leukemia patients are cured. However, more than half of the childhood cancer survivors encounter at least one medical problem that is associated with the cancer treatment in adulthood and 30 % have two or more (Stevens *et al*. 1998). Improved treatment results have partly been reached at the cost of an increased number of late sequelae, such as infertility (Nicholson & Byrne 1993). Other side effects include increased secondary cancer (Hawkins *et al*. 1987, Robertson *et al*. 1994) and serious toxic effects on various organs (Skinner *et al*. 1991).

The severity of treatment toxicity depends on the drug given, the dose of the drug and the age of the patient. In the testis this damage can affect both somatic and germ cells (Wallace *et al*. 2005). Since chemotherapy and irradiation target dividing cells, both the prepubertal and pubertal testis are sensitive to the effects of these treatments. Before puberty, the spermatogonial stem cell population renews itself via slow proliferation and during puberty spermatogenesis is initiated and spermatogonia divide rapidly. Since stem cell spermatogonia rarely proliferate they are more resistant to cancer therapy. Indeed, low-dose chemo- or irradiation therapy depletes only differentiating spermatogonia leaving stem cell spermatogonia undamaged. Higher doses of cytotoxic treatments can kill both differentiating germ cells and stem cell spermatogonia and can even damage the resistant Sertoli cells.

Spermatogenesis can recover if even few stem cell spermatogonia survive and recolonize the seminiferous tubules. Recovery of fully normal spermatogenesis occurs focally in a few seminiferous tubules. Permanent azoospermia results if Sertoli cells are unable to support

stem cell spermatogonia or if the entire stem cell spermatogonia population is depleted. In oligospermia sperm density in ejaculate is <20 x 10^6 /ml and in azoospermia no sperm can be detected in ejaculate.

The mechanisms underlying the Leydig cell damage are unclear, but it has been suggested that irradiation may directly affect the Leydig cells, the vasculature needed indirectly, or the surrounding paracrine regulation of these cells.

The impact of prepubertal cancer treatment on the fertility potential in boys manifests itself first at the onset of puberty when FSH levels begin to increase above normal, inhibin B levels decrease and the testis do not grow (Table 1). At present, gonadal damage can not be detected before puberty. (Wallace *et al*. 2005)

Table 1. Indicators of testicular function.

Dysfunction	Outcome
Seminiferous epithelium dysfunction	Reduced testicular volume
	Elevated FSH
	Low inhibin B
	Impaired spermatogenesis
Leydig cell dysfunction	Reduced testosterone
	Elevated LH
	Delayed or lacking pubertal progress
	Lacking pubertal growth spurt

2.2.1 Effects of chemotherapy on germ cells

Chemotherapeutic agents can be divided into phase- and non-phase specific drugs. Non-phase specific drugs act in all phases of the cell cycle and phase specific drugs act within a specific phase. Since stem cell spermatogonia rarely proliferate, they are sensitive to non-phase specific drugs such as alkylating agents and platinums (Byrne *et al*. 1987, Wallace *et al*. 2005) (Table 2). With phase specific drugs such as antimetabolites, vinca-alkaloids, asparagines, podophyllotoxins, testis damage is uncommon. The exception to the rule is anthracyclines. Although it is a non-phase specific drug it does not seem to harm the human testis as it does the rodent testis. In rats it has been shown to induce apoptosis in male germ cells (Shinoda *et al*. 1999) by causing deoxyribonucleic acid (DNA) damage by intercalating with DNA, inducing DNA breaks and inhibiting topoisomerase II activity (Cummings *et al*. 1991, Hortobagyi 1997). Furthermore, in a rat model the initiation phase of spermatogenesis has been reported to be very sensitive to the toxic effects of this compound (Bechter *et al*. 1987, Hou *et al*. 2005). Administration of doxorubicin at postnatal day 6 causes sterility at adulthood by extensive killing of spermatogonia and partial killing of stem cells in rats. Since chemotherapy usually involves a combination of several drugs that often exert synergistic toxicity, it can be difficult to determine the specific contribution of each individual agent to overall toxicity.

Table 2. Chemotherapeutic agents associated to causation of germ cell damage in humans.

High risk	Medium risk	Low risk
Alkylating agents	**Platinums**	**Vinca alkaloids**
Cyclophosphamide	Cisplatin	Vincristine
Ifosfamide	Carboplatin	Vinblastine
Chlormethine	**Antineoplastic antibiotics**	**Antimetabolites**
Busulfan	Doxorubicin	Methotrexate
Melphalan		Mercaptopurine
Procarbazine		**Antineoplastic antibiotics**
Chlorambucil		Dactinomycin
		Bleomycin

2.2.2 Effects of chemotherapy on Leydig cells

Although proliferating Leydig cells in immature testis are more vulnerable to chemotherapy than non-proliferating adult type Leydig cells, in general Leydig cells are resistant to chemotherapy (Sklar 1999). The majority of treated boys undergo normal puberty and their Leydig cells produce normal levels of testosterone in adulthood. Compensated Leydig cell failure (increased LH, low normal testosterone) has been reported after alkylating agent based therapy for Hodgkin´s disease during adolescence (Sherins *et al*. 1978, Whitehead *et al*. 1982), but otherwise Leydig cell toxicity is rare.

2.2.3 Irradiation

2.2.3.1 Effects of irradiation germ cells

The severity of radiotherapy-induced testicular damage depends on the irradiation target volume, the total dose and the regimen of fractional dosing. Indeed, the adverse effect of irradiation on fertility is inversely related to the distance from the area irradiated (Sklar *et al*. 1990). In the testis, rapidly dividing germ cells are especially sensitive to irradiation. Low doses target differentiating germ cells whereas more resistant stem cell spermatogonia will survive. Studies in adult monkeys show that doses of 0.5-4 gray (Gy) initially decreases the number of type A pale spermatogonia and stem cell A dark spermatogonia remain intact. However, as the stem cell A dark spermatogonia begin to proliferate, the number of both types of spermatogonia decreases. Stem cell spermatogonia are vulnerable to irradiation-induced damage at all ages. However, in adult testis the damage done can be detected after irradiation, whereas the impact of irradiation on immature testis can first be detected after onset of puberty when FSH levels increase above normal and the testis does not grow (Shalet *et al*. 1978).

It is thought that the reason for irradiation-induced infertility is the lack of spermatogonial stem cells. If even a small number of stem cell spermatogonia survive, they have the capacity to slowly recolonize the testis. Speed of recovery is dependent on the dose used; in humans a dose as low as 0.15 Gy has reported to cause impaired sperm production, but germ cell number is shown to improve as time proceeds even with doses up to 1-2 Gy (Rowley *et al*. 1974, Hahn *et al*. 1982). In humans with total doses of 1, 2-3 or 4-6 Gy,

spermatogenesis is re-established in 9-18 months, 30 months or 5 years or more following irradiation, respectively (Rowley *et al*. 1974).

Pediatric patients undergoing total body irradiation for bone marrow ablation therapy prior to hematopoietic stem cell transplantation are at a high risk for testicular dysfunction (Wallace *et al*. 2005). Since hematopoietic stem cell transplantation has been used with success and most of the patients survive their childhood cancer, the number of childhood cancer survivors facing the risk of permanent infertility is rising.

2.2.3.2 Effects of irradiation on Leydig cells

In general, Leydig cells are more resistant to irradiation than germ cells (Sklar 1999, Thomson *et al*. 2002) and Leydig cells of the immature testis are more vulnerable to irradiation-related damage than adult Leydig cells (Thomson *et al*. 2002) due to their proliferation profile. Adult mature Leydig cells are usually preserved from irradiation-caused damage up to 30 Gy (Thomson *et al*. 2002), while lower doses of >20 Gy may cause damage to the Leydig cells in immature testis. Doses greater than 24 Gy on the testis of pre-pubertal boys are associated with Leydig cell dysfunction that leads to delayed puberty, testosterone deficiency, and later permanent sterility (Castillo *et al*. 1990). These patients need testosterone replacement therapy (Blatt *et al*. 1985, Shalet *et al*. 1985, Leiper *et al*. 1986).

2.2.4 Novel molecularly targeted cancer drugs

The aim of the novel targeted therapies is to modulate specific signalling events with a critical role in the survival, proliferation, invasion or metastasis of cancer cells. Receptor tyrosine kinases (RTK) are a group of proteins having an important role in normal cellular processes such as cellular growth, anti-apoptotic signalling, differentiation, metabolism, adhesion and motility. However, RTKs are also involved in oncogenesis. Dysregulation of the RTKs can result from chromosome translocation, gene mutation or abnormal expression of a receptor or ligand (Krause & Van Etten 2005). Targeting tyrosine kinase receptors has become one of the most impressive new approaches to targeted cancer therapy. Theoretically, the molecularly targeted approach has the advantage of hitting or targeting only the tumor cells, avoiding many of the common toxicities associated with conventional cancer therapy. Some signalling pathways, although upregulated in tumor cells, also have critical functions in normal cells, especially during development. Inhibition of these developmental signalling pathways could potentially cause adverse effects in growing individuals. The effects of these drugs in growing children have not yet been studied extensively.

Tyrosine kinases are cell surface receptors which bind growth factors, cytokines and hormones. They can be divided into two classes; receptor and non-receptor tyrosine kinases. Receptor tyrosine kinases have a transmembrane structure which anchors them to the cell membrane. Non-receptor kinases lack this structure and they can be found in the cytosol. In the absence of ligand RTKs are usually monomeric and unphosphorylated. As the ligand binds to the extracellular part of the receptor it induces dimerization of the receptor. Binding causes structural changes that gives free access to adenosine triphosphate (ATP) to

bind to the active site. Finally, autophosphorylation recruits signalling proteins to activate multiple signalling pathways. (Krause & Van Etten 2005)

2.2.4.1 Imatinib mesylate

Cancer drug imatinib mesylate (Glivec®, STI 571; Novartis Pharma, Switzerland) is the first successful small-molecule tyrosine kinase inhibitor. It inhibits the binding of the ATP to its binding site in the BCR-ABL fusion protein and in this way inhibits the action of fusion protein (Figure 2). Imatinib is used to treat chronic myelogenous leukemia and Philadelphia-positive acute lymphoblastic leukemia. It inhibits the action of BCR-ABL fusion protein, which results from translocation between chromosome 9 and 22 (Kalidas *et al*. 2001). In addition, imatinib is used to treat c-kit positive and negative gastrointestinal stromal tumors (GISTs) and many other malignancies, since it potently also inhibits PDGFR-α, PDGFR-β, c-Fms, Arg and c-kit tyrosine kinases (Buchdunger *et al*. 2000, Nishimura *et al*. 2003, Dewar *et al*. 2005, Krause & Van Etten 2005).

In general, imatinib is well tolerated. Most patients have only mild side-effects including oedema, rash, nausea and musculoskeletal pain (Hensley & Ford 2003). Since imatinib is already employed in the treatment of pediatric patients, a better understanding of the possible developmental effects of imatinib is required.

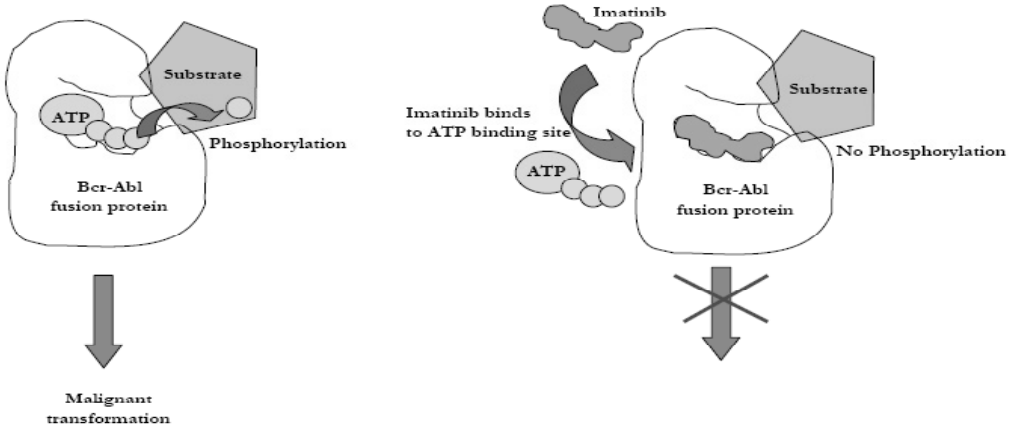


Figure 2. Mechanism of action of imatinib.

2.2.5 Effect of cancer therapies on offspring

Radiation and chemotherapy are potential mutagens and could therefore increase risk of miscarriage, stillbirth, low birth weight, congenital malformations, cancer or early death of the offspring born to childhood cancer survivors. Although animal studies have shown an association between cancer treatment and abnormalities in offspring (Kirk & Lyon 1982, Trasler *et al*. 1985, Nomura. 1988), several studies in human have shown that male survivors' children are healthy (Mulvihill *et al*. 1987, Green *et al*. 1991, Dodds *et al*. 1993, Hawkins. 1994, Sankila *et al*. 1998).

2.3 MODELS TO STUDY CANCER THERAPY-RELATED REPRODUCTIVE TOXICITY

Toxicity testing has been used to investigate the effects of different cancer treatments on reproductive function. The mechanisms of action of different compounds, the safe doses and risks related to reproductive toxicity have been widely studied. Most toxicological studies have been performed either on pregnant or adult animals. Although newborns, infants and juveniles are uniquely vulnerable to exposure, developmental toxicity data of the special groups is often lacking. More information on critical developmental windows during postnatal period is needed and novel tools for detecting cancer therapy-related testicular toxicity are highly warranted.

In vivo animal models are the best approach to study development and organogenesis since the complexity of animals is difficult to mimic *in vitro*. In addition, *in vivo* models are the only way to perform multigenerational studies that produce important information on growth and reproductive capacity in the next generation. Although there are differences in metabolism, absorption and excretion of drugs between human and rodents, animal models can predict toxicological effects.

Cell and tissue cultures have become an important approach in studing the consequences of exposure to different chemicals. These *in vitro* tests can potentially offer many advantages over animal experimentation since they are fast, low-cost and reduce the need to use animals. *In vitro* testing will probably not replace animal testing but it has important value when screening new drugs and mechanisms behind the toxicity of different compounds. Development of *in vitro* methods requires careful analyses to compare key toxicological events to those *in vivo*.

The use of organ culture provides an important advantage over single cell cultures in testis studies; normal structural relationships are maintained and acute loss of spermatogonia during the isolation procedure and early culture period is diminished. However, there are clear disadvantages when using organ culture. Poor access of oxygen to the central part of the cultured tissue can lead to cell death and tested agents may penetrate irregularly into tissue. Organ culture of the postnatal testis has been used in studies to investigate testicular development and the effects of hormones, growth factors (Habert *et al*. 1991, Boitani *et al*. 1993, Olaso *et al*. 1998, Salanova *et al*. 1998, Schlatt *et al*. 1999) and toxicants (Li & Kim 2003).

Xenografting is a new approach to study cancer therapy-related toxicity. Xenotransplantation means transplantation of the cells, tissues or organs between species. Transplanted tissue is called xenografts or xenotransplants and the animals receiving the xenograft provide a bioincubator for the grafted tissue. Recently, the xenografting approach has become a promising model to study spermatogenesis (Honaramooz *et al*. 2002). When immature testicular tissue is transplanted subcutaneously in the back of the castrated immunodeficient mouse, the immature testicular fragment undergoes accelerated maturation. Due to the host's gonadotrophin stimulation complete spermatogenesis and steroidogenesis is achieved.

Fresh and cryopreserved testicular tissue from the immature testis of mouse, hamster, pig, goat and monkey have all been shown to be capable of initiating spermatogenesis (Honaramooz *et al*. 2002, Schlatt *et al*. 2002, Schlatt *et al*. 2003, Honaramooz *et al*. 2004). However, when grafting testis from adult mouse, hamster or human into immunodeficient mice, spermatogenesis was only partially recovered and a number of tubules showed atrophy (Schlatt *et al*. 2002, Geens *et al*. 2006). The better survival of immature testis may be due to its higher capacity for angiogenesis. In addition, immature testicular cells may have more ability for repopulation.

3 AIMS OF THE PRESENT STUDY

The main objective of the present study was to develop novel tools for detecting cancer therapy-related toxicity in immature testis. Special emphasis was put on evaluating how early events correlate to the long-term toxicity of cancer treatment.

The specific aims were:

1) To investigate the acute and long-term effects of molecularly targeted cancer therapeutic imatinib mesylate, conventional chemotherapeutic agent doxorubicin and irradiation on testicular development (I-IV).

2) To validate *in vitro* organ culture and xenograft methods to study cancer therapy related gonadal toxicity in immature testicular tissue (III, IV)

3) To evaluate whether acute testicular toxicity predicts the long-term effects of cancer treatment on testicular endocrine function and fertility among long-term survivors of childhood cancer (IV,V)

4 MATERIALS AND METHODS

4.1 EXPERIMENTAL ANIMALS AND TREATMENTS

4.1.1 Rat (I-III)

Sprague-Dawley rats were housed in plastic cages (Tecniplast, Buguggiate, Italy) in a climate-controlled room at 21 ± 3°C with a relative humidity of 55 ± 15% at the Animal Center of Turku University, (Turku, Finland). Aspen chips (Tapvei Co., Kaavi, Finland) were used as bedding material and animals were maintained on a 12 h light/ 12 h dark cycle (lighted from 07 to 19 h). Animals had free access to tap water and standard laboratory animal feed (Commercial RM3 (E) SQC, Special Diet Service, Witham, UK). All experiments were approved by the Turku University Committee on the Ethics of Animal Experimentation

Imatinib mesylate treatment (I-II). Rats were treated with imatinib mesylate (50 or 150 mg/kg; a generous gift from Novartis; Glivec®) dissolved in water and injected intracavitally into the stomach either once on the fifth day of postnatal life or once daily on postanatal days 5-7. Control animals were injected in the same manner with water alone.

The fertility of the animals was investigated by mating each male at the age of 10 weeks with one healthy, control female for a period of 2 weeks. The fertility index (number of pregnant females / number of females mated), litter size, the live birth index (number of live offspring / number of offspring produced), sex ratio and frequency of survival until the time of weaning were subsequently determined.

Rats were sacrificed by cervical dislocation under CO_2 anesthesia at 24, 48, 72 hours (short-term study) or 80 days (long-term study) following treatment. Testes were collected and either fixed with 5% glutaraldehyde dissolved in s-collidine buffer at room temperature, Bouin or 4% paraformaldehyde (PFA) or snap frozen in liquid nitrogen and stored at -70°C. In the long-term study, body weight and the weight of the testis, epididymis and seminal vesicles were recorded and blood samples collected. Blood samples were obtained by cardiac puncture.

For male puppies in fertility study (II) (second generation) the nose-tail length was measured; the whole body, seminal vesicles, epididymis and both testis were weighed; and blood samples were collected at the postnatal age of 77 days. Testes were collected and fixed either with Bouin, PFA or snap frozen in liquid nitrogen and stored at -70°C.

Doxorubicin exposure in vitro (III). The decapsulated testes of 5-day-old rat pups were dissected into pieces ($1mm^3$) and placed on filters (Millipore, ISO-PORE, Membrane Filters, Carrigtwohill, Ireland) immersed in 1ml of Dulbecco´s Modified Eagle's Medium/F12 medium (1:1) supplemented with 0.1% bovine serum albumine (BSA) and gentamycin (50 µg/ml), maintained at 34°C and balanced with an atmosphere containing 5% CO_2. The filters with these tissue fragments were placed in sterile, four-well plastic culture plates (NUNC™ Brand Products, Denmark) in an incubator in the presence or absence of doxorubicin (Pharmacia Upjohn, Stockholm, Sweden) (0, 40 or 100 ng/ml) for 6, 12, 16, 24, 48 or 72 hours.

In vitro concentrations were selected to correspond to the serum measurements of our previous *in vivo* study (Hou *et al*. 2005) and serum concentrations detected in clinical pharmacokinetical studies (Frost *et al*. 2002). A single dose of 3 mg/kg of doxorubicin used on 6 day old rats (corresponding to a calculated rat surface related dose of 6.7 mg/m^2) has been shown to lead to mean peak serum concentration of 190 ng/ml 20 min after injection (Hou *et al*. 2005). In clinical study, a single doxorubicin dose of 40 mg/m^2 (corresponding to the calculated human weight related dose of 1.3 mg/kg) has been shown to lead to mean peak serum concentrations of 50-70 ng/ml (Frost *et al*. 2002).

4.1.2 Rhesus monkey (IV)

All rhesus monkey experiments were approved by and performed under the guidance of the Animal Care and Use Committee at the University of Pittsburgh, School of Medicine.

Irradiation in vitro. Testicular tissue was obtained from two juvenile male rhesus monkeys (16 and 19 mo of age). Monkey testes were decapsulated and cut into small pieces (0.5-1.0 mm^3). Tissue pieces were exposed to external irradiation by using laboratory irradiation equipment (Gammacell 40, Atomic Energy of Canada Ltd., Kanada, Ontario, Canada). The tissue fragments were exposed to doses of 0, 0.5, 1 and 4 Gy irradiation. Doses were selected to cover the range from minor temporary damage to the permanent severe disruption of spermatogenesis (Rowley *et al*. 1974)

After irradiation, the fragments were put either in short term culture in 35°C or grafted in the immunodeficient male mice.

Short-term culture of irradiated testicular pieces. Twentyfour-well plastic plates (NUNC™ Brand Products, Denmark) were used as culture dishes. Membranes were inserted to face the top of the well (Millipore, ISO-PORE, Membrane filters, Ireland). 1 ml of Leibovitz-L15 medium was added until the inserts floated. Four grafts were placed on each filter and cultured at 35°C and 5% CO_2 for 2, 24 and 48h. 5-bromo-2-deoxyuridine (BrdU) (100 µM) was added to the culture medium one hour before ending of the culture. Short-term cultured grafts were fixed with 4% PFA solution, transferred for storage into 70% ethanol, and embedded in paraffin for sectioning at 2 µm.

Xenografting of irradiated testis pieces. 5- to 7-wk-old intact immunodeficient male Nude mice (Crl:Nu/Nu-nuBR, Charles River Laboratories, Wilmington, MA) were used as recipients. Eight testicular fragments of monkey tissue exposed to either no or the three different doses of irradiation *in vitro* were placed under the dorsal skin on either sides of the dorsal midline by using cancer implant G13 needles (Popper Precision Instruments, Lincoln, RI).

The xenografting experiment was terminated 4 months after transplantation. Mice received an injection of BrdU (ip, 100 mg/kg) 2 h before being killed and then were anesthetized and weighed, and blood was collected by cardiac puncture. The seminal vesicles were dissected and weighed, the back skin was removed and photographed, and the number of visible grafts was recorded. The grafts were dissected from the skin and fixed in Bouin solution for

18–24 h, weighed, transferred for storage into 70% ethanol, and embedded in Technovit (Kulzer, Germany) for sectioning at 2 µm.

4.2 CLINICAL SERIES (V)

This study involved 23 prepubertal boys treated for acute lymphoblastic leukemia at the Department of Pediatrics, Turku University Hospital, during the period of December 5, 1979 - March 31, 1995. In order to examine for the presence of leukemia, 12 and 34 routine bilateral testicular biopsies were performed on these boys following completion of induction therapy and upon termination of treatment, respectively. In addition to these routine biopsies, 5 bi- or unilateral testicular biopsies in connection with suspected testicular relapse and two bilateral biopsies following treatment of testicular relapse were also performed. Five patients were subjected to serial biopsies. This material represents all of the testicular biopsies taken from children with ALL at this hospital unit during this period of time. The National Authority for Medicolegal Affairs in Finland approved the use of this clinical biopsy material for research purposes (DNR 1905-32/300/05).

Treatments. At the time of diagnosis, all of the boys were clinically prepubertal. On the basis of their age, initial white blood cell counts and immunophenotypes, 16 of these patients were considered to be at standard risk and 6 were classified as high-risk patients. The remaining one patient had developed secondary ALL following treatment for Langerhans cell histosytosis. These boys were treated according to different schedules all including induction and maintenance treatment. Induction therapy involved vincristine and prednisolone followed by administration of asparginase. The major difference between the groups in treatment was that the high-risk patients and the patient with secondary ALL received cyclophosphamide, whereas the patients considered to be at standard risk did not. The median cumulative doses of cytotoxic drugs administered prior to testicular biopsy are presented in V: Table 1. In addition, four patients in the high-risk group received prophylactic cranial irradiation (24 Gy) whereas spinal irradiation was not used. Patients experiencing testicular relapse underwent a multi-drug chemotherapic regimen together with testicular and cranial irradiation at a dose of 24 Gy.

Testicular biopsies. All biopsies were performed under general anesthesia. An open wedge of tissue approximately 2x2x2 mm^3 in size was removed from each testis, fixed in formalin, then embedded in paraffin and stored long-term at the Department of Pathology, University of Turku. All complications encountered in connection with testicular biopsy and the original patholgical anatomical diagnoses (PAD) were described in the patients´ medical records.

4.3 HISTOLOGICAL ANALYSIS

4.3.1 Histological assessment of the numbers of germ and Sertoli cells in rats (I & III)

Semi-thin (1-µm) sections prepared from testicular tissue embedded in epoxy resin were stained with toluidine blue and then examined with oil immersion under a light microscope.

In each cross-section of the seminiferous cord, cells exhibiting nuclear morphology typical of gonocytes and type A spermatogonia (Clermont & Perey 1957) were counted as germ cells. The location of these germ cells either in contact with the basement membrane (peripherally located germ cell) or centrally in the seminiferous cord (centrally located germ cell) was noted. The numbers of Sertoli cells present in each cross-section of a seminiferous cord were determined on the basis of their typical nuclear morphology. Cells expressing apoptotic morphology (accumulation of heterochromatin followed by condensation of DNA at the periphery of the nucleus and formation of apoptotic bodies) were also noted.

4.3.2 Histological analysis of monkey xenografts (IV)

All tissue sections were stained with periodic acid-Schiff's reagent/Gill's hematoxylin (PAS) and examined with oil immersion under light microscopy. Xenograft survival was defined by light microscopic observation of the seminiferous tubules in the retrieved grafts. Each cross section was scored for the presence of germ cell types (spermatogonia, preleptotene spermatocytes, pachytene spermatocytes, round and/or elongating spermatids), absence of germ cells (Sertoli cell only (SCO)) or complete hyalinization. The identification of type A dark (Ad) and A pale (Ap) spermatogonia was performed as described earlier (Clermont & Leblond 1959). Tubules with spermatogonia located in the centre of seminiferous tubules and detached from the basement membrane were scored as a separate category named adluminal spermatogonia.

4.3.3 Analysis of the length of seminiferous cords/tubules (I)

In order to calculate the length of the testicular cords and tubules the Bouin-fixed testis sections were stained with hematoxylin-eosin. The diameter and the proportion of the testis made by seminiferous cords/tubules and the intestitial tissue were determined with a morphometric program (Leica IM500 Version: 4.0, Leica Microsystems Imaging Solutions Ltd., Cambridge, UK) and testicular cord/tubular length was calculated with the formula: length of the cord/tubule (m) = (volume of the testis (ml) * cord/tubulus area (%)) / [π x (diameter of the cord/tubule (μm) /2)^2] (Leidl *et al*. 1976).

4.4 IMMUNOHISTOCHEMISTRY (I-V)

4.4.1 Immunohistochemistry (I-V)

After dewaxing and rehydration sections were pretreated in 10 mM sodium citrate in a microwave oven. Slides were allowed to cool and then rinsed with H_2O. After washing, sections were covered with primary antibody diluted in tris phosphate saline (TBS) with 0.1% BSA. After 60 min of incubation at room temperature the antibody was washed away with TBS. Then the slides were incubated for 10 minutes with 3%H_2O_2 in PBS. Subsequently, the sections were incubated with secondary antibody for 45 min. The sections were rewashed with TBS and then treated with avidin–biotin peroxidase complex solution for 30 min. The

sections were washed with TBS and finally the antibody was localized by means of a colour reaction with DAB (3,3'-diaminobentzedine; Sigma Chemical Co., St Louis, USA).

In dual immunohistochemistry following treatment with citrate buffer, the slides were incubated in 2N HCl at 37°C for 30 min, then neutralized in 0.1 M borate buffer for 10 min and finally placed in 0.85% NaCl for 3 min before the incubation with antibodies.

Table 3. Antibodies: targets, species, dilutions, and source.

Antibody	Target	Species raised in	Dilution	Source
cleaved caspase-3	apoptotic cells	rabbit	1:200	Cell Signaling Technology, Inc
MAGE-A4	spermatogonia	mouse	1:200	Kind gift from G. Spagnoli, Switzerland
anti-BrdU	proliferating cells	mouse	1:100	Roche
3βHSD	Leydig cell	rabbit	1:200	Kind gift from I. Mason, UK
CD9	pluripotent germ cells	mouse (H-110)	1:100	Novocastra Laboratories Ltd
GATA-4	Sertoli cells	rabbit (H-112)	1:100	Santa Cruz Biotechnology Inc
WT1	Sertoli cells	rabbit (180)	1:100	Santa Cruz Biotechnology Inc
OCT 3/4	pluripotent germ cells	goat (C-20)	1:200	Santa Cruz Biotechnology Inc
AP-2γ	gonocytes	rabbit (H-77)	1:200	Santa Cruz Biotechnology Inc

4.4.2 Morphometric analysis of the testis and identification of Leydig cells (II)

The proportions of the total area of the samples from normal and treated rats that were occupied by seminiferous tubules, 3βHSD1-positive Leydig cells and interstitial tissue were determined by point-counting of randomly chosen fields, using a 42-point test grid and 25-fold magnification (Baak & Oort 1983).

4.4.3 Immunohistochemical detection of BrdU incorporation (I, III, IV)

The slides were incubated in 2 N HCl for 60 minutes at 37°C and then neutralized with 0.1 M borate buffer (pH 8.5). After washing with PBS, the tissue sections were covered with anti-BrdU antibody (Roche Diagnostics Corp.) diluted (1:100) in PBS with 0.1% BSA. After 60 minutes of incubation at room temperature the antibody was washed away with PBS. Subsequently the sections were incubated with goat anti-mouse IgG monoclonal antibody (1:500, Vector, Vectastain ABC kit, Burlingame) for 45 minutes. The sections were rewashed with PBS and then treated with avidin-biotin peroxidase complex solution for 30 minutes. The sections were washed with PBS and finally the antibody was localized by means of a colour reaction with DAB (Sigma Chemical Co., Germany).

4.5 INCORPORATION OF ^{3}H-THYMIDINE (III)

^{3}H-thymidine (final activity 0.5 µCi) (Amersham Pharmacia Biotech, UK, specific radioactivity 5.0 Ci/mmol) labelled samples were harvested by a Skatron Instrumens Cell Harvester (Skatron, Lier, Norway) and collected on a glass fiber filter paper by rinsing with distilled water. Filter discs containing high molecular weight material including DNA, were punched

out to small plastic tubes and 3 ml of Ready Safe scintillation fluid (Beckman Instruments, Fullerton, CA) was added into each tube. The incorporated radioactivity was measured by a Beckman LS5000CE scintillation spectrometer as counts per minute (cpm).

4.6 IN SITU OLIGO LIGATION ANALYSIS AND DETERMINATION OF APOPTOTIC INDEX (III)

Apoptotic cells were detected with the ApopTag in situ oligo ligation (ISOL) kit using oligo A according to the manufacturer's instructions (Chemicon International, Serologicals, Norcross, GA, USA).

4.7 DETERMINATION OF CASPASE-3 ACTIVITY (I)

Cleaved caspase-3 activity was assayed employing the specific fluorogenic peptide substrate Ac-DEVD-AMC (50 µM; Biomol, Plymouth Meeting, PA), as instructed by the manufacturer.

4.8 WESTERN IMMUNOBLOTTING (I)

The samples were lysed in RIPA buffer (150 mM NaCl, 10 mM Tris (pH 7.2), 0.1% sodium dodecyl sulfate, 1.0% Triton X-100, 1% deoxycholate, and 5 mM EDTA) and the protein concentration was then measured using the Bradford procedure (Bio-Rad Laboratories AB, Sundbyberg, Sweden). Proteins were separated by electrophoresis on 7.5% Tris-HCl or 4–20% gradient acrylamide gels (Bio-Rad Laboratories) and transferred electrophoretically to a Hybond-P polyvinylidene difluoride-transfer membrane. The membranes were blocked with 5% Blotto non-fat dry milk (Santa Cruz Biotechnology Inc., Santa Cruz, CA) in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature. Subsequently, the membranes were probed overnight at 4°C (with gentle shaking) employing antibodies against poly-(ADP-ribose)-polymerase (PARP) (1:1500 dilution); and the corresponding secondary antibody, goat anti-rabbit Ig-horseradish peroxidase (all purchased from Santa Cruz Biotechnology, Inc. Santa Cruz, CA). The antigen-antibody complexes thus formed were detected by chemiluminescence with the ECL^{plus} Western blotting detection system (Amersham Pharmatica Biotech), according to the manufacturer's instructions.

4.9 DNA FLOW CYTOMETRY OF ADULT TESTICULAR SAMPLES (I)

DNA flow cytometry was performed as described earlier (Toppari *et al*. 1988) with some modifications. 1-mm long stage-specific segments of seminiferous tubules were treated with a detergent (0.3 % Nonidet P-40) in phosphate-buffered saline containing 0.2 % of bovine serum albumine (Sigma, St. Louis, MO) and ribonuclease A (5µg/ml; Sigma). The samples were vortexed and incubated at 37°C for 15 min. Propidium iodide (25 µg/ml; Sigma) and 10 µl of diluted fluorescent particle solution (500 beads/µl, TrueCount Beads; Becton Dickinson, Mountain View, CA) were added. The beads served as an internal volume

standard for quantification of the absolute cell number. The samples were filtered though sterile silk tissue with a pore size of 40 µm and counted with BD LSRII (Becton, Dickinson). The excitation wavelength used was 488 nm and 5000 fluorescent impulses were counted from each sample. Data was further analyzed using FACSDiVa Software (Becton Dickinson).

4.10 EXTRACTION OF RNA AND PERFORMANCE QUANTITATIVE RT-PCR (III)

Total RNA was extracted from testicular tissue using the TRIzol reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions and treated with the DNAse I Amplification Grade Kit (Invitrogen, UK). Thereafter, quantitative RT-PCR was performed embloying the DyNAmo SYBR Green 2-Step qRT-PCR Kit (Finnzymes Oy, Espoo, Finland), with the inclusion of controls without reverse transcriptase.

4.11 HORMONE ASSAYS (I, II, III, V)

Rats. Plasma levels of LH and FSH were measured employing immunofluorometric assays as described earlier (Haavisto *et al*. 1993, van Casteren *et al*. 2000). Testicular levels of testosterone were determined by RIA utilizing diethyl ether extracts of samples homogenized in Dulbecco´s PBS, as described in detail earlier (Huhtaniemi *et al*. 1985).

Patients. Blood samples were analyzed at the Department of Growth and Reproduction of Rigshospitalet, Denmark. Serum levels of FSH, LH and testosterone were determined employing a time-resolved immunofluorometric assay (Delfia, Wallac, Turku, Finland), and inhibin-B by a specific two-sided enzyme immunometric assay (Serotec, UK), as described previously (Lähteenmäki *et al*. 1999, Jorgensen *et al*. 2002).

4.12 SQUASH PREPARATIONS (I)

In order to detect apoptosis, fresh squash preparations were prepared from 1 mm-long cord segments as described earlier (Jahnukainen *et al*. 2004). Apoptotic cells with a condensed nucleus appeared as bright phase-negative spheres and were counted per mm of tubular length.

4.13 SPERM COUNTS (I, V)

Rats. The number of spermatozoa in the cauda epididymis was analyzed as described earlier (Pakarainen *et al*. 2005). The tissue was incubated in KSOM + AA medium (catalogue no. MR-121, Specially Media, Phillipsburg, NJ) for 15 min at 37°C and the number of spermatozoa was counted under a light microscope using a Bürger haemocytometer chamber.

Patients. Semen samples were maintained at 37° C until being analyzed in accordance with WHO guidelines (1992), as described earlier (Jorgensen *et al*. 2002). In brief, samples were diluted in an aqueous a solution of 0.6M $NaHCO_3$ and 0.4% (v/v) formaldehyde and the sperm concentration was assessed using a haemocytometer (Improved Neubauer Chamber).

4.14 PHYSICAL EXAMINATION OF PUBIC HAIR DEVELOPMENT AND TESTICULAR SIZE (V)

The Tanner stages of pubic hair development were evaluated by physical examination. For assessment of testicular size, all examiners utilized the same type of wooden orchidometer (Pharmacia & Upjohn, Denmark).

4.15 PRESENTATION AND STATISTICAL ANALYSIS OF THE DATA (I-V)

Parametric data are presented as mean ± SEM and nonparametric data as median, 10th, 25th, 75th, and 90th percentiles. The Mann-Whitney Rank Sum test or t-test was employed for single statistical comparisons of different groups. Multiple comparisons were analysed for statistically significant differences using analysis of variance, ANOVA, followed by the Tukey test for multiple comparisons of independent groups of samples for parametric data and Kruskal-Wallis analysis with Dunn's *post hoc* test for nonparametric data. A P (two-sided) value of less than 0.05 was considered to indicate a statistically significant difference.

5 RESULTS

5.1 THE SHORT-TERM EFFECTS OF POSTNATAL IMATINIB EXPOSURE ON TESTIS DEVELOPMENT (I)

In order to investigate whether imatinib disturbs the development of the spermatogonial stem cell pool, morphological analysis of testicular tissue was performed. In control testis centrally located germ cells migrate to the basement membrane during the first postnatal days and form a spermatogonial stem cell pool. Repeated imatinib dosing on postnatal days 5-8 (150 mg/kg x 3) inhibited this migration of centrally located germ cells to the basement membrane (I: Fig. 2) and consequently, significantly decreased the number of peripherally located germ cells (I: Table 1). In addition, germ cells located centrally continued to proliferate in an adluminal position and the number of centrally located germ cells increased.

Imatinib induced apoptosis in germ cells. Indeed, the number of apoptotic cells per mm of squashed seminiferous cord was significantly increased first at 72 hours after the exposure of 150mg/kg. When tissue sections from the same treatment group were evaluated the cells with apoptotic morphology were located at the periphery of the seminiferous cord and had a cell size compatible to spermatogonia (I: Fig 2B). When the imatinib dose (150 mg/kg) was administered on consecutive days (5, 6 and 7), no increase in apoptosis was observed 72h after the first administration (day 8). The same result was seen by detecting the activation of caspase-3 and cleavage of PARP. Administration of a single dose of imatinib (50 or 150 mg/kg) resulted in the activation of caspase-3 in a time- and dose-dependent manner, and significantly elevated levels of cleaved PARP were observed in the immature rat testis 72 hours after a single injection of imatinib (150 mg/kg) (I: Fig. 3). However, in the case of three injections of imatinib (150 mg/kg), no change in caspase-3 activity was observed 72 hours after first administration (I: Fig. 3).

A physiological increase in Sertoli cell number was detected from the fifth to the eight postnatal day in control animals. Treatment with imatinib did not significantly decrease the number of Sertoli cells (I: Table 2). Instead, the number of Sertoli cells per cord cross section increased slightly (I: Table 2).

Imatinib inhibited the proliferation of the peritubular myoid cells. Seventy-two hours after the initial treatment with imatinib (150 mg/kg x 3) the number of BrdU-positive mesenchymal myoid precursor cells lining the seminiferous cords was reduced significantly (I: Fig. 4). As a result, cord growth was decreased dose dependently (I: Table 1). With the highest repeated dose the growth was totally blocked and the length difference of the seminiferous cord at the age of 8 days between control and the group treated with 3x150 mg/kg of imatinib was -27%.

5.2 THE LONG-TERM EFFECTS OF POSTNATAL IMATINIB EXPOSURE ON THE TESTIS (I & II) AND THE EFFECTS OF IMATINIB EXPOSURE ON THE SECOND GENERATION (II)

To evaluate whether imatinib is capable at causing permanent damage to the developing testis, animals exposed to imatinib on postnatal days 5-7 were investigated after reaching sexual maturation at the age of 80 days. We observed that animals were significantly smaller and their testis, epididymis and seminal vesicle weight was significantly smaller than in controls. When the weight of the reproductive organs was correlated to the total body weight, there was no significant difference in control and imatinib-treated groups. Imatinib-exposed animals had significantly higher plasma levels of FSH, LH and inhibin B than controls. However, intratesticular testosterone levels did not differ significantly from control animals (I: Table 3; II: Fig. 1).

No significant changes in the seminiferous tubule diameter or epithelium thickness were observed but tubular length was decreased 15 % as compared to controls (I: Table 3). There were no significant differences in the proportions of the testis occupied by seminiferous tubules, interstitial tissue and Leydig cells in imatinib-treated and control animals (II: Table 3) and the Leydig cells of treated animals appeared normal under the light microscope. Under our experimental conditions the testicular levels of mRNA coding for *Insl3*, *Hsd3b1*, *c-kit*, *Scf*, *Pdgfra*, *Pdgfrb*, *Pdgfa*, *Pdgfb*, *Lhr*, *Fshr*, *Inhibin α* and *Inhibin ß*b in 77-day-old rats were not influenced by short-term treatment with imatinib (II: Table 4).

In order to analyze whether imatinib exposure had changed the number of spermatogonia, spermatocytes and round spermatids per unit length of seminiferous tubules a flow cytometric analysis was performed. No significant changes were observed. There was slightly higher number of elongated spermatids in stages VII-VIII in the imatinib-treated group as compared to controls. However, the epididymal sperm counts of imatinib-exposed animals were not different from control animals (I: Table 3).

The fertility of imatinib exposed animals was investigated by mating of 20 control and 20 treated male rats with healthy control females for two weeks. Imatinib did not influence the fertility index, the live birth index, the sex ratio or the frequency of survival to the time of weaning but did, however, reduce the litter size significantly (II: Table 2). All the pups were born healthy, no birth defects or other abnormalities were observed. The absolute and relative body and organ weights, as well as the nose-to-tail length of the first-generation male offspring of treated and control rats were not different at the age of 77 days of age (II: Table 3). There were no changes in the plasma levels of FSH and LH or in the level of intratesticular testosterone as compared to controls.

5.3 ORGAN CULTURE OF POSTNATAL RAT AND RHESUS MONKEY TESTIS (III, IV)

To validate the organ culture system for postnatal testicular tissue, control testicular tissue was cultured for different time periods and analyzed histologically. When fragments

(1mm^3) of testicular tissue from 5-day-old rat pups were cultured no signs of cell death or morphological disorganization was observed after 6 or 12 hours. However, following more than 24 hours of organ culture, some of the largest tissue samples began to lose tissue integrity in the middle region. Most of the cultured samples maintained normal tissue morphology throughout the sample (III: Fig. 1). After 48 hour in culture the central region of the tissue had lost its morphological integrity and the surrounding seminiferous tubules stained positively for cleaved caspase-3. A clear margin between the intact and necrotic regions of the tissue was observed (III: Fig. 1). After 72 hours in culture, necrosis also spread into the outer layers of the samples. In the subsequent investigations only the intact regions of samples cultured for 24 or 48 hours were subjected to morphological and immunohistochemical analyses.

The characteristics and limitations of organ culture were examined by comparing the developmental events that occur in such cultures of immature testicular tissue to those known to occur *in vivo*. For 48 hours, the number of germ cells located centrally in the seminiferous cords remained constant (III: Fig. 2C), whereas the number of peripheral germ cells increased significantly (III: Fig. 2C). These observations reflect the initiation of the mitotic proliferation of newly formed spermatogonia, i.e. the beginning of the first round of spermatogenesis *in vitro* occurs in a comparable way as *in vivo* (III: Fig 1C). In addition, the number of Sertoli cells per cord cross-section (III: Fig. 2A) in culture up to 48 hours reflected very precisely the situation *in vivo* (I). In addition, the ratio of Sertoli to germ cells decreased in the control organ cultures with time, indicating that normal longitudinal growth of seminiferous cords and proliferation of germ cells taking place (III: Fig. 2B).

The same short culture window was used when testicular fragments of immature rhesus monkey were cultured (IV). We observed a severe disintegration of morphology in the primate testicular fragments after 48 h in culture. However, a limited culture window of 24 hours was long enough for detect the acute effects of irradiation on the developing monkey testis and the acute effects of doxorubicin on developing rat testis.

5.4 KINETIC EVALUATIONS OF DOXORUBICIN INDUCED-TOXICITY IN IMMATURE RAT TESTIS (III)

The immature testicular fragments of rat were cultured in the presence of doxorubicin to discover the effects of this drug on developing testis. Doxorubicin induced a dose- and time-dependent reduction of the number of germ cells when compared to control samples. At the highest dose used, this reduction was statistically significant in the case of peripherally located (III: Fig. 2C) and the total number (III: Fig. 2D) of germ cells. This decrease after 24 and 48 hours in culture was preceded by a decline in the proliferation and increase in the frequency of apoptosis among germ cells. Proliferation of peripheral and total germ cells declined significantly following 16 hours of culture in the presence of 100 ng doxorubicin per ml (III: Table 1) and after 24 hours, few BrdU-positive germ cells were found in comparison to untreated cultures (III: Fig. 3B). Concurrently, the number of apoptotic germ cells was

significantly elevated following 12 hours of culture in the presence of 100 ng doxorubicin per ml, and this number reached its maximal value after 16 hours in culture (III: Fig. 4A-C).

The number of Sertoli cells per seminiferous cord cross-section was not significantly changed, although there was a tendency towards a decrease after 48 hours of incubation with the higher dose of doxorubicin (III: Fig. 2A). The ratio of Sertoli to germ cells was significantly increased, providing a further indication that during the first 24-48 hours of exposure, doxorubicin exerts its toxic effects primarily on germ cells. These observations are consistent with the finding that the proliferation of Sertoli cells was reduced significantly by doxorubicin only after 24 hours in culture (III: Tables 1, Fig. 3 A and B). Even at this time-point the number of Sertoli cells was not changed (III: Fig. 2A). No damage to the ultrastructural morphology of Sertoli cells (III: Fig. 3C and D) or their expression pattern of specific proteins (WT-1 and GATA-4) in the presence doxorubicin could be detected after culture.

Doxorubicin did not influence the proliferation of peritubular myoid cells (III: Table 1) and it did not alter the release of testosterone by Leydig cells.

5.5 EFFECTS OF *IN VITRO* IRRADIATION ON SPERMATOGONIA AND SERTOLI CELLS IN CULTURED RHESUS MONKEY TISSUE FRAGMENTS (IV)

In order to study the acute effects of irradiation on prepubertal Rhesus monkey testis, irradiated testicular tissue fragments were investigated after short-term culture. At 24h post irradiation, a significant decrease in the numbers of Ad and Ap spermatogonia was detected in fragments irradiated with the highest irradiation dose of 4 Gy (IV: Table 2). The number of Sertoli cells also decreased in fragments irradiated with 4 Gy (IV: Table 2). Exposure to irradiation evoked an increase of apoptotic cell death in the cultured fragments after 24 hours, which reached statistical significance at the highest dose (IV: Fig.3). This apoptotic increase affected both Sertoli and germ cells. Sertoli cells and germ cells behaved differently when their mitotic response was analyzed. Although the decrease in germ cell number did not reach statistical significance (IV: Fig. 3) it was obvious that no BrdU-labelled germ cells were detected after exposure to 1 and 4 Gy of irradiation. BrdU-positive Sertoli cells, however, were encountered in all experimental groups.

5.6 THE EFFECT OF IRRADIATION ON THE XENOGRAFTED IMMATURE MONKEY TESTIS (IV)

To investigate the long-term effects of irradiation, irradiated xenografts were analyzed four months after grafting. In all experimental groups, survival of xenografted monkey testicular tissue was similar. However, the weight of grafts was significantly lower in the group irradiated with the 4 Gy dose when compared to controls. At the time of grafting, spermatogonia were the most advanced germ cell type (IV: Fig. 1A). Four months after grafting, there was a dose dependent decline in the number of grafts containing any type of spermatogonia. At the same time, the number of grafts showing Sertoli cell only patterns increased dose dependently (IV: Fig. 2). No initiation of spermatogenesis was observed after 1 and 4 Gy.

5.7 THE INFLUENCE OF THERAPY FOR CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA ON SPERMATOGONIA AND ON MALE REPRODUCTIVE FUNCTIONS FOLLOWING SEXUAL MATURATION (V)

The effects of childhood ALL treatment on the germ cells were investigated by analyzing the testicular biopsies taken during and after ALL therapy. After induction therapy of childhood ALL, 90% of the seminiferous cords contained MAGE-positive spermatogonia (V: Table 2) and approximately 20% of the seminiferous cords contained spermatogonia that expressed pluripotent germ cell markers OCT4 or CD9.

Following therapy to ALL patients at standard risk, the number of MAGE-positive spermatogonia per cross-section of seminiferous cord was only 50% of the corresponding number after induction treatment (V: Table 2). Although standard-risk therapy did not statically significantly influence the number of OCT4- and CD9-positive germ cells when compared to the numbers after induction therapy, their numbers were also clearly decreased (V: Table 2).

The therapy for high-risk or secondary ALL significantly reduced the numbers of MAGE- and CD9-positive spermatogonia in comparison to the level observed after induction therapy. Only 20% of the seminiferous cords contained MAGE-positive spermatogonia, none contained CD9-expressing germ cells and only a few individual OCT4 –positive cells were detected (V: Table 2).

When the specimens were grouped according to the therapy received prior to overt testicular relapse, several MAGE (18/10 cross-sections), CD9- (1/10 cross-sections) and OCT4- (5/10 cross-sections) positive spermatogonia were observed in the single biopsy taken following standard intensity therapy, whereas no cells expressing spermatogonial markers were detected in the two samples obtained after high risk therapy involving cyclophosphamide. Leukemic cell infiltration localized into the interstitial tissue showed no expression of the studied spermatogonial markers.

All of the 12 long-term survivors were reassessed after reaching sexual maturation (Tanner stage G5P5) in order to analyze the effect of ALL treatment on the male reproductive function in adulthood. Testicular size (mean± SEM: 19.3±1.7 ml), and levels of FSH (3.2±0.5 IU/l), LH (4.2 ±0.9 IU/l), testosterone (19 ±3 nmol/l) and inhibin B (225 ±38 ng/l) were comparable to values among healthy Finnish young men (Jorgensen *et al*. 2002) in all 8 long-term survivors at standard-risk who received ALL therapy not involving cyclophosphamide. The three who provided semen samples had normal mean sperm counts of 61±3 $x10^6$/ml (Jorgensen *et al*. 2002), and two of them had fathered a child.

Of the three long-term survivors of high-risk therapy, one exhibited total recovery of spermatogenesis, as well as normal reproductive and endocrine function (FSH 3.5 IU/l; LH 3.8 IU/l; testosterone 26 nmol/l; inhibin 285 ng/l; testicular size 18ml, sperm count 35 $x10^6$/ml). The other 2 patients were oligo/azoospermic (3 and 0 $x10^6$/ml) and had significantly reduced testicular size (6 and 7 ml). One of them did not receive prophylactic cranial

irradiation and showed elevated serum levels of gonadotrophin (FSH 11.6 IU/l and LH 6.3 IU/l) and an attenuated serum level of inhibin (38 ng/l); while the other, treated with 24 Gy cranial irradiation, had normal gonadotrophin (FSH 2.2 IU/l and LH 2.4 IU/l) and inhibin (105 ng/l) values. Levels of testosterone were normal in both cases (14 and 13 nmol/l). The patient who survived testicular relapse required testosterone replacement therapy, was azoospermic (0 $x10^6$/ml) and had small testes (1ml).

5.8 THE EXPRESSION OF SPERMATOGONIAL MARKERS DURING TESTICULAR MATURATION (V)

In order to evaluate maturation of the testicular germ cell population during treatment for leukemia, the expression of appropriate markers in a patient group younger than 7 years was compared to a group of patients older than 7 years. Only patients that did not receive cyclophophamide or irradiation were included in the evaluation. The numbers of OCT4 positive spermatogonia were found to be strongly correlated to the age of the patient at the time of biopsy: an 8-fold higher number of OCT4 –positive germ cells per cord cross-section was detected in the younger boys and only very few such spermatogonia were present in the biopsies of older patients. Altogether, 25% of the seminiferous cords of the patients younger than 7 years of age exhibited OCT4 –positive germ cells, while the corresponding value for older patients was 5%. The oldest patient demonstrating testicular expression of OCT4 was 11 years. In contrast, the numbers of MAGE- and CD9-positive spermatogonia observed showed no correlation to the age of the patient.

6 DISCUSSION

6.1 THE INITIATION PHASE OF SPERMATOGENESIS MAY BE ESPECIALLY SENSITIVE TO MOLECULARLY TARGETED DRUG IMATINIB MESYLATE

Adult men treated with imatinib have normal sperm counts and several pregnancies among the partners of such men have been documented (Hensley & Ford 2003). In connection with previous clinical trials, this drug has been considered to be non-genotoxic and the spermatozoa produced have been assumed to be qualitatively normal. However, since some of signalling cascades are age and maturational-related, like PDGF and SCF/c-kit signalling in the testis, the pharmacokinetics, the effectiveness and undesirable side-effects of this therapeutic agent in children and adults may be different. Indeed, oligozoospermia and failure in spermatogenesis has been reported (Seshadri *et al*. 2004, Mariani *et al*. 2008).

The United States Food and Drug Administration have approved several kinase inhibitors for use in adult cancers, but currently imatinib is the only tyrosine kinase inhibitor that is labeled for pediatric use. Imatinib was approved to treat children with Philadelphia chromosome positive CML which is uncommon in children. It accounts only for 2% of all leukemia in children. Although imatinib is selective, it targets several pathways. In addition to inhibition of BCR-ABL, imatinib inhibits the c-Kit and PDGFR mediated pathways (Buchdunger *et al*. 2000) and it is, therefore, also used to treat pediatric GIST tumors (Kuroiwa *et al*. 2005).

In the present study, we showed that a 3-day-inhibition of tyrosine kinase receptor c-kit and PDGFR during the first postnatal week has a permanent adverse effect on the development of the rat testis. Imatinib mesylate disturbed c-kit and PDGFR-mediated migration (Orth *et al*. 1996, Orth *et al*. 1997, Basciani *et al*. 2008), proliferation (Yoshinaga *et al*. 1991, Schrans-Stassen *et al*. 1999) and the survival (Yoshinaga *et al*. 1991, Hakovirta *et al*. 1999, Yan *et al*. 2000b) of germ cells. In addition, PDGFR-mediated proliferation of peritubular myoid cells was inhibited which resulted in decreased longitudinal growth of the seminiferous tubules. The slight increase in the number of Sertoli cells per cord cross section can be explained with the fact that Sertoli cells do not express c-kit or PDGFR receptors and, therefore, Sertoli cells continued to proliferate normally. Since the tubular outgrowth was disturbed Sertoli cells were forced to be packed into a smaller space. Although it has been generally believed that the number of Sertoli cells closely correlates with testicular size and sperm output (Orth *et al*. 1988), the present results highlight the role of peritubular myoid cells in determining final testicular size. It is possible that peritubular cell proliferation plays a more critical role in determination of final testicular size than earlier considered. In this connection imatinib also benefited basic research since it provided a tool to explore the biological function of the c-kit and PDGF tyrosine kinase receptors in testicular development.

Imatinib exposure during the first postnatal week permanently increased LH level, but did not affect the levels of testicular testosterone in 80-day-old animals. This suggests Leydig cell failure that is compensated by an increased LH drive. This can be detected following

conventional cancer therapy with a high dose of alkylating agents (Kenney *et al*. 2001a). Infertile men have been shown to be able to maintain normal testosterone levels in the presence of slightly elevated levels of LH (Glass & Vigersky 1980, Stanwell-Smith *et al*. 1985, Giagulli & Vermeulen. 1988, Anapliotou *et al*. 1994). In the present study, serum levels of testosterone and the weight of the seminal vesicles in the treated rats were also unchanged. In addition, increased levels of FSH and inhibin B after sexual maturation suggests that imatinib at the early postnatal age is also able to cause permanent changes also in the hypothalamus–pituitary–testis axis.

Although the litter size and weight of the reproductive organs of the exposed animals were significantly decreased when compared to the control animals, most of the postnatal changes induced by imatinib were reversible. Epididymal sperm counts, other fertility outcomes and testicular morphology returned to normal levels. In addition, transcript levels of ligands and receptors of PDGF and SCF/c-Kit signalling were not significantly changed when compared to the control levels. In addition, reassuring in the present data is that no transgenerational effects of imatinib treatment could be detected and male offspring were born healthy. In conclusion, our findings indicate that short-term blockage of the SCF/c-kit- and PDGF-mediated signalling in the postnatal rat testis does not result in permanent qualitative alterations in spermatogenesis in adult animals.

Although the present study was done by using rat as a model organism, immature human testis can also be vulnerable to the effects of imatinib since c-kit is reported to be expressed in prepubertal human testis (Nicotina *et al*. 2004), and the pattern of expression of the PDGF receptor in fetal and adult human testicular tissue is comparable with that in rats (Basciani *et al*. 2002a). Long-term evaluation of the novel drugs at clinically relevant doses and employing valid animal models are necessary. Although understanding of the function of different tyrosine kinases is increasing, we have to remember the importance of these signalling pathways at the level of system biology.

6.2 TOXICITY OF DOXORUBICIN AND IRRADIATION IN THE DEVELOPING TESTIS

In the present study, significant changes of germ cell numbers were induced in response to irradiation and doxorubicin already after 24 hours in culture. In the case of doxorubicin our observations indicated that germ cells were the primary, most sensitive targets of doxorubicin toxicity and, furthermore the germ cell death induced by this compound is not secondary to alterations in the number of Sertoli cells. In addition, doxorubicin did not influence the proliferation or ultrastructural morphology of peritubular cells under any of the conditions examined here. Furthermore, testosterone production by the organ cultures was not altered by doxorubicin and, as reported previously *in vivo*, no apparent changes in Leydig cell morphology or any signs of Leydig cell hyperplasia (Bechter *et al*. 1987) could be detected in up to 48 hours of exposure. Thus, the toxic effects of doxorubicin on these cell types may require longer time to develop and cannot be characterized using the organ culture system described here.

In rats the initiation of spermatogenesis has been shown to be more sensitive to the toxic effects of doxorubicin than the later stages (Bechter *et al*. 1987, Hou *et al*. 2005). This may be due to differences in the maturation of the blood-testis barrier, which occurs during pubertal development (Kormano 1967). Its major components are Sertoli cells, testicular microvessels and peritubular myoid cells. The blood-testis barrier prevents the entry of potentially harmful compounds into the lumen of the seminiferous tubules (Dym & Fawcett 1970) and affects the conditions to which spermatogonia are exposed. Differences in the time course of maturation of the blood-testis barrier in humans and rats could possibly offer an explanation why doxorubicin is not similarly toxic to humans as it is to rodents.

Irradiation induced depletion of 80% of A spermatogonia via apoptosis 24h post-irradiation with 4 Gy and, in addition, spermatogonia also ceased to proliferate. The effects of irradiation exposure of Sertoli cells at prepuberty has been previously studied (de Rooij *et al*. 2002a). In this study single or fractionated total body irradiation applying doses of 4-8.5 Gy reported a depletion of Sertoli cells leading to low testis weights in adulthood. This is consistent with our finding that in cultured fragments Sertoli cells undergo apoptotic cell death after the highest dose of irradiation 24 hours post irradiation and 4 months later grafts show decreased growth after such exposure. This led us to conclude that irradiation of 4 Gy affects the somatic environment in the juvenile primate testis.

Our observations support the idea that spermatogonia in the immature primate testis are similarly sensitive to irradiation as the adult testis. However, fetal tissue may differ in terms of sensitivity to the irradiation. A recent report describes the high sensitivity of cultured fetal human testicular tissue to germ cell apoptosis after very low irradiation doses 0.1-0.2 Gy (Lambrot *et al*. 2007). These observations suggest that a significant difference in radiosensitivity may exist between fetal and juvenile testes while pre- and postpubertal primate germ cells show similar sensitivity.

6.3 PROS AND CONS OF THE *IN VITRO* ORGAN CULTURE AND XENOGRAFTING METHODS AS TOOLS FOR TESTING THE EFFECTS OF CHEMOTHERAPY ON THE DEVELOPMENT OF THE TESTIS

We validated an *in vitro* organ culture model of developing rat and rhesus monkey testis. The limited duration of the viability of such cultures was an obvious disadvantage. The postnatal testicular development of the rat testis proceeded normally in the viable tissue parts for 48h *in vitro*; germ and Sertoli cell numbers and germ cell migration were comparable *in vitro* and *in vivo*. In addition, the time-dependent reduction in the rate of DNA synthesis in our untreated cultures was similar to what we observed in intact rat pups at the corresponding age.

Necrosis was observed in the central regions of the rat tissue samples after 24 hours and in the monkey tissue samples after 48 hours of culture. This damage was probably due to hypoxia, since it appeared first in the central region of the largest tissue fragments and, as described earlier, appeared by light microscopy to involve necrosis (Schlatt *et al*. 1999).

Judging from earlier reports on organ cultures of fetal human and rodent testicular tissue, such cultures appear to be more resistant to hypoxic damage than were our postnatal cultures. Thus, no sign of necrosis or disorganization has been reported in fetal testicular cultures, even following culture for more than 4 days (Lambrot *et al*. 2006, Lambrot *et al*. 2006, Livera *et al*. 2006). For the reasons mentioned above, we used only viable outer layers of tissue and morphologically normal regions in the cultures of rat testicular tissue in the analyses. These intact areas were found to reliably reflect *in vivo* conditions for at least 48 hours in culture. Following 72 hours in culture, the outer layers of tissue also became necrotic and, therefore after this time-point our testicular organ cultures were no longer a relevant experimental system. In the case of culturing monkey testicular fragments we experienced similar severe disintegration of the morphology also in the primate testicular fragments after 48 h in culture allowing only one examination time-point of 24 hours. Although the limited time-window for evaluation of short-term effects limits the applicability of organ culture, the combination of organ culture and xenografting offered a unique opportunity to explore both the acute and long-term effects of irradiation exposure.

Testicular tissue xenotransplantation of immature non-human primate testicular tissue into adult immunodeficient mouse allows accelerated pubertal development and early initiation of spermatogenesis (Jahnukainen *et al*. 2006). Therefore, xenograft analysis allowed us to detect the long-term effects of irradiation after only 4 months, whereas detection of this outcome *in vivo* in monkeys would have required pubertal initiation and the experiment would have lasted for several years. Indeed, in the clinical situation an estimate of the fertility of an irradiation-exposed boy can only be made after pubertal initiation, when the loss of Sertoli cells and germ cells incurs a diminished growth of the testis and a focal to complete absence of spermatogenesis. Since xenografting and organ culture result in poor outcome after using adult donor testes (Schlatt *et al*. 2006), this promising combination of tools appears to be restricted to the exploration of immature testes.

For ethical reasons, experimental studies exploring the effects of cancer therapy on healthy children cannot be carried out. The best and closest model organisms to obtain clinically relevant data are therefore *in vivo* studies with nonhuman primates. Macaques show close anatomical and endocrinological similarities to men before and after puberty and have therefore been used in previous studies to explore irradiation effects on the testis (van Alphen *et al*. 1988a, van Alphen *et al*. 1988b, de Rooij *et al*. 2002b). However, these experiments are expensive to perform and raise concerns of welfare of the animal.

Xenograft analysis allows toxicological manipulation and evaluation of its consequences on primate spermatogonia and their somatic microenvironment *ex vivo* without treatment of the nonhuman primates or the mouse host. With *in vitro* organ cultures animal exposures is also avoided. These models save animals from the harmful side-effects of the drugs and other exposure. Both models provide novel options for toxicity experiments and can reduce the need for animal experimentation. The number of animals needed for each study is decreased, since one animal provides material for several experiments with different doses and time-points. Since xenograft survival has also been detected for prepubertal human

tissue, similar xenograft methods serve as a tool for toxicity analysis for the prepubertal human testis (Goossens *et al*. 2008, Wyns *et al*. 2008).

6.4 CAN ACUTE TOXICITY PREDICT THE LONG-TERM TERM EFFECT OF CHILDHOOD ALL THERAPY?

The present results highlight the fact that acute depletion of the spermatogonial pool in immature human testis can be detected by histological methods soon after cytotoxic insult. The number of germ cells expressing spermatogonial markers significantly correlated with the leukemia therapy that patients had received prior testicular biopsy. Observations are well in accordance with existing knowledge that morphological damage to prepubertal human germ cells correlates with the cumulative dosage of cyclophosphamide (Uderzo *et al*. 1984, Muller *et al*. 1985, Ise *et al*. 1986). In the present study, this was also true of spermatogonial numbers at testicular relapse. The number of spermatogonia related to toxic therapy prior to relapse and not to the extent of leukemic infiltration in the interstitium as suggested earlier (Lendon *et al*. 1978).

We found that ALL therapy with cyclophosphamide depletes the number of CD9- and OCT4-positive spermatogonia almost entirely. In previous immunoprofiling studies on fetal human and immature rodent testicular tissue, CD9 and OCT4 have been considered to be putative surface markers for pluripotent germ-line stem cells (Gaskell *et al*. 2004, Kanatsu-Shinohara *et al*. 2004, Pauls *et al*. 2006). Our present observations indicate that both undifferentiated stem cell -like cells (CD9- and OCT4 -positive) and more highly differentiated progenitor-type spermatogonia (expressing MAGE only) are equally sensitive and primary targets for the cytotoxic effects of cyclophosphamide.

This raises the question of whether the immunohistochemically detected decrease in spermatogonial numbers during ALL therapy correlates to the fertility potential of the long-term survivors. Previous studies suggest that histologically documented germ cell damage can recover (Wallace *et al*. 1991). In another prospective study with consecutive testicular biopsies after ALL therapy, abnormal testicular morphology did not improve by one year after completion of chemotherapy (Muller *et al*. 1988). In our study, all patients with standard risk therapy had recovered normal testicular function with normal testicular size and endocrine function. Of the three survivors of high risk therapy, one patient showed total recovery of testicular function with a normal sperm count and serum hormonal levels whereas the other two showed testicular damage of a different degree. The recovered patient had received a cumulative cyclophosphamide dose of 10 g/m^2 intermittently by parenteral administration while one of the latter patients had received 9 g/m^2 of cyclophosphamide as maintenance treatment in low oral doses and another 5 g/m^2 of cyclophosfamide by parenteral administration. Earlier reports have shown that if cyclophosphamide is used in doses of < 7.5 g/m^2, normal sperm production is maintained (Aubier *et al*. 1989, Relander *et al*. 2000, Kenney *et al*. 2001b) and oligo- or azoospermia is likely if the patient is treated with > 7.5 mg/m^2 of cyclophosphamide (Relander *et al*. 2000, Kenney *et al*. 2001b). However, there are some reports of men having children even after

10-30 g/m^2 of cyclophosphamide (Shamberger *et al*. 1981, Jaffe *et al*. 1988), which indicates that individual variation is high. Our observations confirm the previous hypothesis that both cumulative dosage and the period of cyclophosphamide exposure as well as individual sensitivity affect the risk of the spermatogonial damage (Ise *et al*. 1986). Since significant recovery of spermatogenesis occurred during sexual maturation, immunohistochemical analysis of testicular spermatogonial cells at the end of cytotoxic therapy was shown to poorly predict the long term consequences of spermatogonial depletion and the future fertility potential of childhood leukemia survivors.

7 SUMMARY AND CONCLUSIONS

The present study was conducted to assess cancer therapy-related toxicity during the development of the testis. Special emphasis was placed on the studying mechanisms by which imatinib mesylate, doxorubicin and irradiation exert their toxicity during testicular development. In addition, the effect of the treatment of acute lymphoblastic leukemia on the child testis and on later fertility was investigated. The main conclusions of the present work are as follows:

1. Imatinib mesylate interferes with postnatal testicular development in rat. Imatinib inhibited the migration, proliferation and survival of germ cells and proliferation of peritubular myoid cells. However, most of the postnatal changes induced by imatinib were reversible and no transgenerational effects could be detected.

2. Germ cells were the primary, most sensitive targets for doxorubicin induced toxicity in the rat testis. Exposure to doxorubicin resulted in an increase in the number of apoptotic testicular germ cells and a reduction in the number of proliferating germ cells. At the same time, ultrastructural morphology, expression of cell-specific proteins and the number of Sertoli cells remained unaltered.

3. Spermatogonia in juvenile primate testis are a highly sensitive target of irradiation. Spermatogonial depletion and cessation of proliferation are acute responses that follow irradiation.

4. Testicular development proceeds normally in culture in the absence of any external factors for 48 hours. However, the short time-window for evaluation of the effects limits the applicability of organ culture, but the combination of organ culture and xenografting offers a unique opportunity to explore both the acute and long-term effects of cancer treatments.

5. The number of potential spermatogonial stem cells is decreased by leukemia therapy involving alkylating agent cyclophosphamide, but remains unaffected by standard-intensity ALL therapy or testicular leukemia. The spermatogonia that survive provide a good basis for the spontaneous recovery of spermatogenesis

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