CHARACTERIZATION OF HUMAN SEMINOMAS

APOPTOSIS, STEM CELL FACTOR AND MUTANT *RAS* AFFECT *IN VITRO* BEHAVIOR

KARAKTERISATIE VAN HUMANE SEMINOMEN

APOPTOSE, STAMCELFACTOR EN MUTANT *RAS* BEINVLOEDEN HET *IN VITRO* GEDRAG

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"De goede reiziger weet niet waarheen hij gaat"

Chuang Tzu

Voor Aad en Joke

CONTENTS

Abbreviations

AFP	α-fetoprotein
ASO	allele specific oligonucleotide hybridization
BAD	BCL-2 associated death promotor
BAG	BCL-2 associated athanogen
BAX	BCL-2 associated x protein
BCL-2	B-cell lymphoma gene-2
BCL-x	BCL-2 homolog-x
bFGF	basic fibroblast growth factor
BrdU	bromodeoxyuridine
CDH	carbohydrate dihexose
CH	choriocarcinoma
CIS	carcinoma *in situ*
CMH	carbohydrate monohexose
CT	combined tumor
CTH	carbohydrate trihexose
d.p.c.	days *post coitum*
EC	embryonal carcinoma (cell)
EG	embryonic germ (cell)
ES	embryonal stem (cell)
FCM	flow cytometry
FCS	fetal calf serum
Gb3	globotriosylceramide
Gb4	globoside
Gb5	galactosyl globoside
GCT	germ cell tumor
GL7	sialyl galactosyl globoside
hCG	human chorionic gonadotropin
IC	image cytometry
ICE	interleukin-1ß converting enzyme
IT	immature teratoma
Le^x	lacto-series glycolipid carrying SSEA-1
LIF	leukemia inhibitory factor
MEPCR	mutant-enriched PCR
MT	mature teratoma
NS	nonseminomatous TGCT
PCR	polymerase chain reaction
PGC	primordial germ cell
PLAP	placental alkaline phosphatase
SCF	stem cell factor
SE	seminoma
SLB	SCF, LIF, and bFGF
SS	spermatocytic seminoma
SSEA	stage-specific embryonic antigen
TGCT	testicular germ cell tumor of adolescents and adults
YS	yolk sac tumor

Note: genes will be referred to in *italics*, proteins in CAPITALS.

Chapter 1

SCOPE OF THIS THESIS

This thesis contains the results of a research project aimed at obtaining cell lines of seminomas, relatively rare human tumors. Seminoma cell lines, thus far lacking, would be important in the study of the pathobiology of human germ cell tumors.

Seminomas represent one of the two types of human testicular germ cell tumors of adolescents and adults (TGCTs), the other type being the nonseminomatous TGCTs. Seminoma cells are considered to represent the neoplastic counterpart of primordial germ cells, embryonic cells that give rise to the stem cells of gametogenesis. Nonseminomatous TGCTs are neoplastic caricatures of normal embryonic development. Because of this, TGCTs might be used as a model system to study aspects of human developmental biology.

Chapter 2 contains an overview of the present knowledge of TGCTs, addressing epidemiology and possible causes, tumor histology, and the pathobiological relation between the TGCT precursor (carcinoma *in situ*), seminoma and nonseminomatous TGCTs. The last part of this chapter will focus on the micro-environment of carcinoma *in situ* and seminoma cells. Knowledge of this environment could be essential for the development of an *in vitro* culture system for seminoma cells and the subsequent derivation of cell lines.

Recently, a growth factor-receptor system has been characterized that appeared very important for primordial germ cell development. In addition, this system of stem cell factor and c-KIT receptor has been shown to be involved in the development of various tumors. Chapter 3 will focus on the importance of the stem cell factor/c-kit system for primordial germ cells and their neoplastic derivatives, since the described findings might also be applicable to TGCTs, especially carcinoma *in situ* and seminoma cells.

Previous attempts to culture seminoma cells *in vitro* have only been partly successful, while cell lines have never been obtained. A reason for this failure could be the immediate onset of apoptosis or programmed cell death once the tumor cells are removed from their natural micro-environment. Chapter 4 gives a brief overview of the current knowledge of apoptosis (detection, genetic control, effects of growth factors, hormones and extracellular matrix) and serves as a basis to investigate whether the onset of apoptosis is indeed causing the difficulties encountered in attempts to culture seminoma cells.

Chapter 5 describes the aims of the various studies carried out in the course of the project. The results are presented and discussed in the Chapters 6 through 11. A discussion of the data and indications for future research are presented in Chapter 12.

Chapter 2

TESTICULAR GERM CELL TUMORS OF ADOLESCENTS AND ADULTS

2.1. Epidemiology and possible causes

Testicular germ cell tumors of adolescents and adults (TGCTs) constitute one third of all cancers in the age group of 15 to 45 years, being the most frequent cancer in this population. They account for 1-3% of all malignancies in caucasian males (1). Two entities of TGCTs can be distinguished: seminomas (SEs) and nonseminomatous TGCTs (NSs) (2). SEs preferentially occur in the fourth decade of life, while NSs become clinically manifest in the third decade (1,3,4). SEs and NSs account for 50% and 40% of the total of TGCTs, respectively (5,6). The remaining 10% comprizes tumors with both a SE and a NS component. These tumors occur around the age of 30 (3,4).

In about 5% of all patients both testicles are affected (bilateral TGCTs) (7,8). Familial occurrence of TGCTs has been reported (9). In blacks the epidemiology differs from that in whites. The incidence of TGCTs in blacks is lower than in whites and SEs become clinically manifest at a younger age. Intra-abdominal TGCTs are more frequent in blacks, most likely due to the unexplained higher incidence of undescended testis in this population (see below) (10,11).

The incidence of TGCTs has steadily increased, and now is three times as high as in 1940 (1,5,6,12). Thus far the factors causing the development of TGCTs and its rising incidence are unclarified (13). Established risk factors are undescended testis, prior TGCT, family history of TGCT and gonadal dysgenesis, among others associated with Klinefelter's syndrome (14,15). In addition, inguinal hernia, early puberty, sedentary lifestyle, testicular trauma and sexually transmitted disease have been documented as risk factors (16-18). An involvement of endogenous and exogenous oestrogens (19), as well as of androgens (especially during puberty) is suggested (20).

Besides in the testis, germ cell tumors (GCTs) of the adult testicular type occur at other anatomical localizations. The fact that these GCTs occur at specific sites, *i.e.* the gonads, mediastinum and midline of the brain (21-24), could indicate that a specific micro-environment is needed for their development. The extragonadal GCTs of the adult testicular type and the (immature) teratomas and yolk sac tumors found in infants and children (25), will not be addressed in this chapter, but the relation of the latter with TGCTs will be discussed in Chapter 12.

2.2. Histology

Skakkebæk showed for the first time that carcinoma *in situ* (CIS) cells are the precursors of both SEs and NSs (26,27). Like spermatogonia, CIS cells are in close contact with Sertoli cells, located between the basal membrane of seminiferous tubules and the tight junctions of Sertoli cells. Clear cytoplasm, distinct cell borders and hyperchromatic, enlarged nuclei morphologically characterize CIS cells (28-30).

SEs have a monotonous histology of solid fields of uniform tumor cells, surrounded by connective tissue septa, which contain varying numbers of inflammatory cells, in particular lymphocytes (Fig. 2.1A). SE cells are indistinguishable from CIS cells, except for their invasive behavior (13,26). Both have morphological, ultrastructural, and immunohistochemical similarities with primordial germ cels (PGCs) (26,28-30).

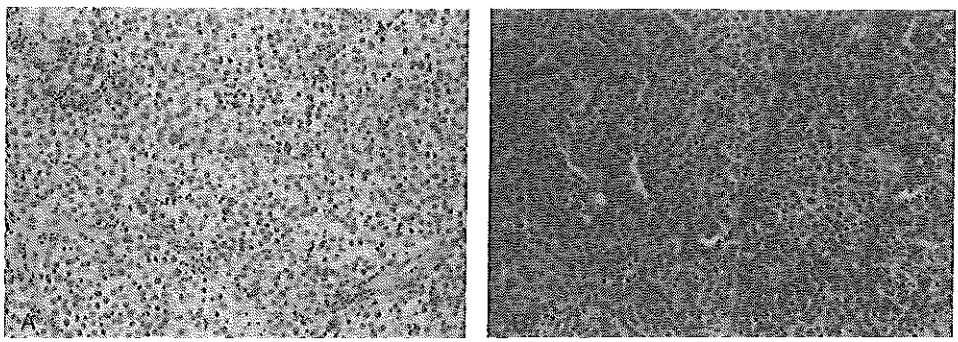

Figure 2.1. *Histology of a seminoma (A) and a spermatocytic seminoma (B), hematoxylin and eosin staining.*

In contrast to SEs, NSs can have a highly varied histology (2,5). They can be composed of one or more of the following histological types: embryonal carcinoma (EC), immature or mature teratoma, yolk sac tumor and choriocarcinoma. The different cell types, which form neoplastic caricatures of embryonic or extra-embryonic tissues, can be mixed or present in separate areas.

Besides TGCTs composed of only SE or NS, a group of tumors containing both cell types exists. This type of tumor is referred to as NS in the classification of the World Health Organization (2), and as combined tumor in the British classification (31).

In addition to SE and NS, another type of GCT can be distinguished in the testes of elderly men, namely spermatocytic seminoma. This tumor represents less than 5% of all GCTs. It is composed of sheets of small, medium and large cells with round nuclei (Fig. 2.1B). The chromatin is dense in the small, more open in the medium sized and often filamentous in the large nuclei. Thus for these morphological, as well as pathogenetic, clinical and histochemical reasons, spermatocytic seminoma is considered a separate GCT entity composed of neoplastic germ cells at a stage of maturation between spermatogonia and spermatocytes, that is not derived from CIS cells (32-35).

2.3. Pathobiological relation of seminomas and nonseminomas

Although it is known that both SE and NS develop from a CIS cell, the exact pathobiological relation between CIS, SE and NS is still unclarified. Two main hypotheses on the development of TGCTs exist. One hypothesis, the independent origin model, is based on the assumption that all SEs and NSs independently develop from their specific CIS cells (36-39). The other hypothesis, the linear progression model, assumes progression of a common CIS cell via a seminomatous stage, which is not necessarily clinically manifest, to NS (3,40-43) (Fig. 2.2).

Figure 2.2. *Representation of the two pathogenetic models for the development of testicular germ cell tumors of adolescents and adults: the independent origin model (A) and the linear progression model (B).*

The linear progression model is supported by findings in studies on human leukocyte antigens, ploidy, chromosomes, histochemistry, the reported borderline histology between SEs and NSs, and the presence of nonseminomatous components in metastases derived from testicular pure SEs (41,44-55). In spite of these supportive data, the linear progression model has not been proven.

2.4. Study of primary tumors and cell lines

The study of human NSs is facilitated by the existence of cell lines representing most nonseminomatous cell types (56-69) and the use of xenografts (61,67,70-73). Experiments can be performed using cell lines of pluripotent EC cells, which can be induced to differentiate by exposure to certain agents (*e.g.* retinoic acid, hexamethylene bisacetamide and bromodeoxyuridine), for example allowing analysis of changes in gene expression responsible for, or coinciding with, the process of differentiation (74,75). Some of these studies have focussed on the expression of cell surface glycolipids, *i.e.* molecules composed of a carbohydrate and a lipid moiety. Various groups of glycolipids can be distinguished according to their basic molecular structure (76). The three main groups are the so called globo-, lacto-, and ganglio-series (77). Among others, glycolipids are involved in early embryonic development and in mediation/modification of growth factor action (78-82). Therefore, glycolipids might be important in the development of TGCTs. The patterns of glycolipid expression in nonseminomatous cell lines correlate with their differentiation lineage. Andrews *et al.* (83,84) and Wenk *et al.* (85) have shown that undifferentiated EC cell lines are characterized by the expression of globo-series glycolipids. Upon induced or spontaneous differentiation of these cells into the various nonseminomatous cell types, the synthesis of globo-series glycolipids is down-regulated, while the synthesis of lacto- and ganglio-series glycolipids increases. Specific combinations of glycolipids are correlated with specific cell histological types and the way in which the various cell types are related can be studied using glycolipid analysis. No extensive data on the glycolipid pattern of primary SEs and NSs have been reported (86,87) and therefore the suggested close correlation between SEs and ECs (13) may be further studied using glycolipid assays.

Human SEs appear to form a unique tumor type, for which no animal model, no xenograft system (88), no cell lines and no sufficient *in vitro* culture system (89) are

available thus far. To gain more insight into the development of TGCTs and the relation between SEs and NSs one or more of the forementioned are needed. Optimal *in vivo* or *in vitro* culture conditions should result in survival and proliferation of SE cells, and eventually in the establishment of cell lines. Elucidation of the processes underlying the spontaneous or induced reprogramming of SE to NS, as suggested by the linear progression model, would then be possible.

2.5. The micro-environment of carcinoma *in situ* and seminoma cells

CIS and SE cells are assumed to be the malignant counterparts of PGCs, among others because of the presence on these cells of immunohistochemical markers for human fetal germ cells, including germ cell-specific alkaline phosphatase (detectable with antibodies to placental alkaline phosphatase, PLAP (3)) (90-93). This implicates that CIS is initiated at the stage of embryonal development when PGCs are present. PGCs first appear in a defined part of the yolk sac from where they migrate to the genital ridges (Fig. 2.3). Because of the forementioned relatively high incidence of bilateral TGCTs (7,8), it is assumed that CIS cells can already develop before colonization of the genital ridges takes place, *i.e.* before week 6 of development (94,95).

In seminiferous tubules (with spermatogenesis), a specific environment is created by the gonadal stroma cells, including Sertoli, Leydig and peritubular myoid cells. Sertoli cells nurse spermatogonia and maturing spermatogenic cells through the production of many factors (96), which might also be essential for CIS cells (Fig. 2.4). Among these

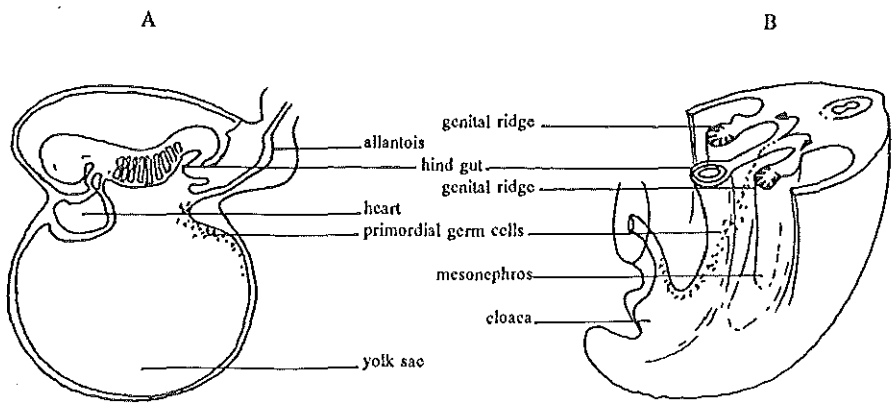

Figure 2.3. *Three weeks old embryo. The primordial germ cells are located in a part of the yolk sac, close to the allantois (A). The primordial germ cells migrate along the hindgut and the dorsal mesentery to the genital ridges (B).*

factors are transport/binding proteins, proteases, extracellular matrix components, cellular metabolites, and growth factors (96-98). One of these growth factors is stem cell factor (SCF) which has recently been shown to affect PGC survival and gametogenesis (99). Matsui *et al.* (100) reported that SCF is produced by the stromal cells lining the migration route of PGCs as well as by the cells of the gonadal ridges, while PGCs were found to express the SCF receptor c-*kit* (101). This receptor was also demonstrated on CIS and SE cells (102,103), suggesting that SCF supports these cells as well.

The Sertoli cell-germ cell interactions are not one-way events (104-110). A few germ cell-produced factors are known to affect Sertoli cell function: an as yet unidentified molecule increases Sertoli cell-transferrin mRNA levels (111), while nerve growth factor (112), for which receptors are present on Sertoli cells, is a candidate to explain some other effects, *e.g.* increased secretion of androgen-binding protein and inhibin, and an inhibition of aromatase activity in Sertoli cells (111,113-115). Interestingly, addition of nerve growth factor to *in vitro* cultures of seminiferous tubules containing CIS cells resulted in enhanced survival of these cells (116).

During fetal life, rat Sertoli cells normally express cytokeratins and vimentin. At the end of the fetal period and in the first days of postnatal life, cytokeratin expression is down regulated. In adults, only vimentin can be detected (117-119). The disappearance of cytokeratins coincides with the stop of rat Sertoli cell proliferation, the formation of the blood-testis barrier, the appearance of the first meiotic spermatocytes and the pubertal rise in androgens. Whether the intermediate filament expression is under hormonal control and/or related to the maturation status of the testis has not yet been elucidated. The fact

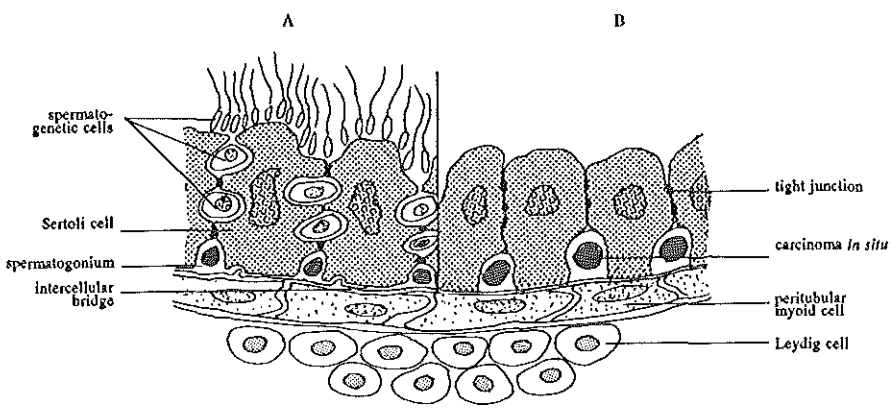

Figure 2.4. *Part of a seminiferous tubule containing spermatogenetic cells (A), or carcinoma* in situ *(B). The carcinoma* in situ *cells are at the site normally occupied by spermatogonia: in close contact with Sertoli cells and between the basal membrane and Sertoli cell tight junctions.*

that, in men, the ceased expression of cytokeratins can be "reversed" in situations of testicular disorders (120) could implicate a reversal to or mimicking of earlier stages of normal development (119). Recent immunohistochemical studies (our unpublished observations) showed that in CIS-containing and/or atrophic seminiferous tubules, the Sertoli cells express cytokeratins in combination with vimentin. This confirms previous observations (120) and suggests that atrophy and the development of CIS affect the intermediate filament expression of Sertoli cells by an unknown mechanism. The absence of meiotic spermatocytes from CIS-containing tubules and/or the early-embryonic nature of CIS cells could be related to this phenomenon.

The environment of CIS cells (and spermatogonia) is not influenced by Sertoli cells alone. The critical role of Leydig and peritubular myoid cells in the regulation of spermatogenesis and the androgenic status of the male has recently been recognized (96). As for Sertoli cell-CIS interactions, very little is known about the Leydig cell-CIS and peritubular myoid cell-CIS interactions. The interactions between the forementioned specialized gonadal cells and SE cells also remain unclarified. However, the presence of androgen receptors on CIS and SE cells (20) suggests an involvement of (Leydig cell-produced) androgens in the development of TGCTs, which could be related to an onset of TGCT development around puberty.

2.6. Animal models

In the 129J murine strain, which has been used in the study of human NSs (121-125), no seminoma-like tumors have been described. However, testicular seminomas have been reported to occur in dogs, stallions, bulls, rams and bucks (126,127). Canine seminomas macroscopically, histologically and ultrastructurally resemble human SEs (126,128). Like SEs, they are yellowish-white, lobulated and moderately soft, containing clear tumor cells and infiltrating lymphocytes. In spite of these similarities, it has been suggested but not proven that these tumors are the counterpart of human spermatocytic seminomas rather than of SEs (129,130). This suggestion is based on the presence in the canine tumors of giant cells resembling those in human spermatocytic seminomas. In addition, canine seminomas are never found in combination with nonseminomatous components, whereas these elements can be found together with human SEs. Further characterization of canine seminomas appears necessary to clarify whether they resemble human spermatocytic seminomas or can be used as an animal model representative of human SEs.

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Chapter 3

THE C-KIT/STEM CELL FACTOR SYSTEM IN THE DEVELOPMENT OF PRIMORDIAL GERM CELLS AND PATHOLOGY

3.1. C-KIT and stem cell factor

Identification of the feline sarcoma virus oncogene v-*kit* (1), led to the cloning and mapping to the White spotting (*W*) locus on human chromosome 4q11-q13 of the cellular proto-oncogene c-*kit* (2,3). This gene was shown to encode a type III transmembrane receptor kinase with extensive homology to the receptors for colony-stimulating factor-1 (encoded by c-*fms*) and the receptors for platelet-derived growth factor-α and -ß (1-3). Upon binding of the ligand, c-KIT autophosphorylates and associates with phosphatidylinositol 3'-kinase and phospholipase C-γ1 (4,5), while RAS is also activated (6). This intracellular cascade could be involved in the transduction of survival and/or proliferation signals.

Recently, a mouse fibroblast-derived factor was purified and characterized as the ligand for the c-KIT receptor (7). Subsequently, several groups simultaneously reported the cloning and sequencing of the gene encoding this ligand, which they named Stem Cell Factor (*scf*/SCF, the designation used here) (8-10), Kit Ligand (11), Mast Cell Growth Factor (12-14), or Steel Factor (5,15). In 1991, *scf* was mapped to the Steel (*Sl*) locus on human chromosome 12q22-q24 (16-18). From the cell-associated precursor of the ligand the soluble form is released upon proteolysis, similar to the secretion of colony-stimulating factor-1 and transforming growth factor-α from their membrane-associated precursors (8-11,14,19,20).

A variety of cellular programs is influenced by c-KIT and SCF: adherence, migration, survival, proliferation, differentiation/maturation, and secretion. The main cell types affected are those of the germ cell, mast cell, melanoblast, and hematopoietic lineages (21-23). Moreover, the c-KIT signaling pathway also appears to be involved in the development of placenta, nervous system, heart septa, lung, facial chondrogenic nuclei, and midgestational kidney. The diverse responses to SCF may reflect, among other factors, the density at the cell surface of the cell-associated form, the concentration of the soluble form, and its presentation in context with other cell-associated, soluble, or extracellular matrix-associated molecules (24).

3.2. Effects of c-KIT and stem cell factor on primordial germ cells

In mice homozygous for severe (lethal) *W* alleles, germ cells can be detected at 8 days *post coitum* (d.p.c.) but no further proliferation occurs (25). Several lines of evidence, including analyses of the effects of SCF on PGCs *in vitro* (26-28) and of *Sl* mutations on PGC development *in vivo* indicate that normal c-KIT/SCF function is required for the survival and/or proliferation of PGCs.

Murine PGCs are first distinguishable in the extraembryonic mesoderm proximal to the primitive streak around 7 d.p.c. (29). As early as 7.5 d.p.c., c-*kit* mRNA can be detected in PGCs (30). At 8 d.p.c. 10-100 PGCs can be found at the base of the allantois (31). During the migration from the hindgut to the genital ridges and the first 1-2 days upon reaching these sites, the PGC number increases to 2500-5000. At that time (12.5 d.p.c.), the gonadal ridge has undergone male or female differentiation (32). C-*kit* expression is detectable throughout the proliferation and migration period, and decreases as the female cells enter meiosis and the male cells become quiescent (30).

Genital ridges have been found to exert long-range effects on murine PGC numbers and direction of migration in culture (33). In addition, inverse gradients of *scf* and c-*kit* expression were detected along the murine PGC migratory route (24), with *scf* being highly expressed in the genital ridges and c-*kit* in the dorsal mesentery. *Scf* expression along the migratory route ceases when PGCs have reached the gonad, while expression in the genital ridges remains high. It has been suggested that the stromal cells along the migratory route produce cell-associated SCF, while soluble SCF is produced in the gonads (24). In analogy to the findings for mast cells (34), a gradient of soluble SCF would then elicit a chemotactic response in the PGCs, resulting in migration to the genital ridges. This migration might also be a haptotactic response to a concentration gradient of insoluble SCF, being cell- or extracellular matrix-associated.

The use of SCF-producing cell lines has resulted in the establishment of improved *in vitro* culture methods for murine PGCs (26-28). Cell-associated SCF, produced by feeder cell lines, was found to promote the adhesion of murine PGCs and sustain their survival, without induction of proliferation. The cell-associated form of SCF was superior to soluble SCF for survival of murine PGCs, although under some conditions the application of soluble SCF further improved the culture conditions. In analogy to the *in vitro* culture results for murine PGCs grown on SCF-producing feeders in the presence or absence of soluble SCF, it was found that cell-associated SCF supported long term hematopoiesis *in vitro*, while the soluble form could only support transient hematopoiesis. Interestingly, high concentrations of soluble SCF could partially overcome the lack of the cell-associated form (35).

SCF was shown to act in synergy with other growth factors and extracellular matrix proteins to support cells of the hematopoietic lineages (24). Synergy of SCF with leukemia inhibitory factor (LIF) and basic fibroblast growth factor (bFGF) resulted in the survival and extended proliferation of murine PGCs *in vitro*, allowing the subsequent derivation of pluripotent embryonic germ cells (36). This finding is interesting in view of the linear progression model, which assumes the reprogramming of SE cells to pluripotent stem cells, subsequently giving rise to embryonic and/or extraembryonic tissues in NSs (37).

3.3. Pathology related to the c-KIT/stem cell factor system

Evidence exists that the c-KIT/SCF system is involved in the development of a variety of tumors, including hematopoietic tumors, mast cell leukemia, melanoma, and brain, lung and breast cancer (24). High levels of c-*kit* transcripts were found in a high percentage of human SEs, but only in a few NSs examined in the same studies (38-41). Immunohistochemically, c-KIT was detected in SEs, but only rarely in NSs (39). SCF was found to be expressed in a low percentage of SEs and a high percentage of NSs in one study (40), while in another study a low expression was observed in all TGCTs examined (39). No evidence of amplification or gross rearrangements in the c-*kit* gene was found in any of the TGCTs examined so far. Although Murty *et al.* (40) identified sites of candidate tumor suppressor genes on the long arm of chromosome 12, one of which includes the *scf* locus, no relationship between expression of *scf* in some GCTs and the candidate tumor suppressor gene has been described.

It was recently confirmed that human piebaldism, a disorder of melanocyte development

associated with patches of unpigmented skin and hair, arises from aberrant c-KIT function (42-45). Defects in hematopoiesis and germ cell development have never been reported in heterozygous piebald individuals. The only reported human with homozygous piebaldism, a nine months old boy, had pigmentless skin and hair, blue irides, facial dysmorphia, deafness, developmental delay, but no anemia. Data on gonadal development were not presented (46).

In the murine system, many pathological germ-line *W* or *Sl* mutations have been reported, which in the homozygous state can result in (more or less severe) anemia, mast cell deficiency, white fur and sterility (16,47-50). In heterozygotes, these mutations cause a pattern of lacking coat pigmentation (spotting), while fertility, and red cell or mast cell numbers are normal (49-51). Similar mutations have been found in rats, but in homozygous mutants the anemia improves with age and the rats are fertile (52-54).

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Chapter 4

APOPTOSIS IN NORMAL DEVELOPMENT AND CARCINOGENESIS

4.1. Apoptosis

Rather recently, an important phenomenon controling cell fate during embryonal and adult life has been discovered and termed apoptosis (from the Greek word for "falling of leaves from a tree") or programmed cell death (1-5). Apoptosis is involved in elimination of specific cell subsets during embryogenesis and morphogenesis, thus affecting, among others, formation of the extremities, intestine, nervous system and degeneration of the female sexual organs in the male (6,7). Tissue homeostasis in adult organisms results from a balance of proliferation, differentiation and apoptosis, for example in the intestinal crypts (8). In the thymus, T-lymphocytes directed against self-antigens are apoptotically removed, while normal immune responses are modulated through apoptosis (9). In addition, the immune system can induce apoptosis throughout the organism to eliminate cells that might threaten homeostasis, *e.g.* virus-infected or damaged cells (9).

Tissue homeostasis can be disturbed in the sense that cell proliferation is no longer balanced by cell elimination through apoptosis. When the proliferation rate exceeds the apoptosis rate or when the apoptosis rate is decreased as compared to the homeostatic situation, tumor formation can occur.

4.2. Characteristics of apoptotic cells

A wide variety of events takes place in apoptotic cells, resulting in changes at the microscopic and molecular level. Regarding the apoptosis-inducing agents/situations, the features of apoptotic cells and the apoptosis-related genes that will be discussed in the following paragraphs, it has to be emphasized that these are all general aspects of apoptosis to which many exceptions and counter-examples have been found.

Microscopic features of apoptotic cells include condensed cytoplasm and chromatin, and cellular and nuclear buds (6,10,11). So called apoptotic bodies are formed upon breaking up of the nucleus into discrete fragments surrounded by a double-layered membrane, and by budding of the cell (Fig. 4.1). A major participation of the cytoskeletal elements ß-tubulin and actin in the formation of cellular buds and apoptotic bodies (12,13), associated with major deformation of the cell surface (14,15), is suggested. Changes of carbohydrates on the surface of apoptotic bodies allow recognition by neighbouring cells and macrophages (16,17). The macrophage vitronectin receptors have been implicated in this recognition process (18,19). Upon recognition, rapid phagocytosis ensures the elimination of apoptotic bodies before they can lyse (17,20).

In several cell types the activation of tissue transglutaminase during apoptosis is reported (5,21-24). This enzyme is involved in the cross-linking of intracellular proteins and is found at the highest concentrations in apoptotic bodies. It is suggested that transglutaminase activity leads to the formation of a rigid framework within apoptotic bodies, thus maintaining their integrity and preventing the leakage of intracellular substances to the extracellular space. This prevention of leakage, together with the forementioned phagocytosis, explains the absence of inflammatory reactions at the site of apoptosis (17,20).

At the molecular level, a main hallmark of apoptosis is internucleosomal DNA cleavage by endogenous endonuclease activity, resulting in the formation of oligonucleosome-sized

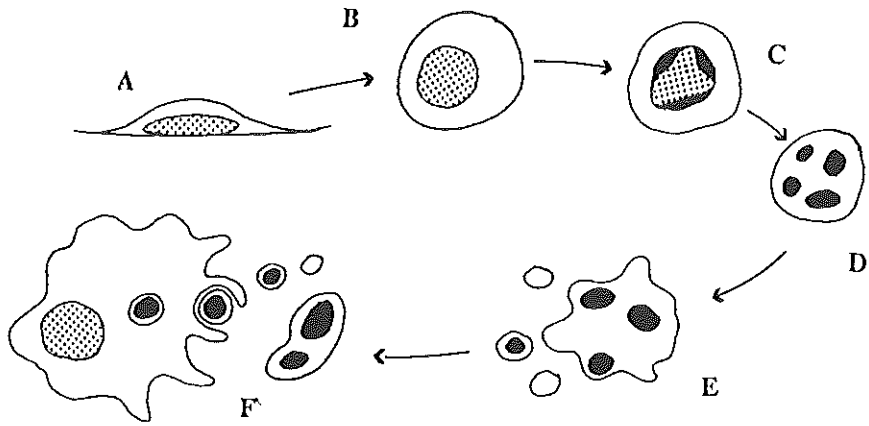

Figure 4.1. *Schematic representation of the apoptotic process. Upon receipt of an apoptosis-inducing signal, an adherent cell (A) rounds up (B) and its DNA marginates and condenses along the nuclear membrane (C), possibly a consequence of DNA fragmentation. This is rapidly followed by separation of the nucleus into discrete masses of condensed chromatin (D), and finally fragmentation of the cell into apoptotic bodies(E), which can be recognized and phagocytosed by macrophages or neighbouring cells (F).*

DNA fragments with the length of integer multiples of 180-200 base pairs (25). These fragments can be visualized as typical ladder patterns on electrophoresis gels. Preceding the formation of these ladders, large oligonucleosomes with lengths of 300,000 and/or 50,000 base pairs might be detected (26). These large fragments may also be formed without further digestion of the DNA. Therefore, internucleosomal fragments are important but not essential features of apoptosis. The presence of single or double strand DNA nicks can be visualized using DNA end labeling, either *in situ* on tissue sections (27) or in combination with flow cytometry (28).

Being important as intracellular messenger, calcium ions play a role in apoptosis (29). An influx of calcium ions coincides with the onset of some of the above mentioned events. Cytoskeletal components are sensitive to high levels of free calcium ions, while transglutaminase (5) and one of the candidate endonucleases, DNase I (30), are calcium-dependent. The fact that another candidate endonuclease, DNase II, is calcium-independent could explain the occurrence of DNA ladders under essentially calcium-free conditions (31,32).

4.3. Genetic control of apoptosis

An intricate and complicated interplay of many genes/proteins regulates apoptosis. In this section the effects of BCL-2, RAS and P53 will be briefly discussed. Table 4.1 provides an overview of other apoptosis-related genes described thus far.

The first gene identified as inhibitor of apoptosis was *bcl*-2 (57). This gene is overexpressed in non-Hodgkin's B-cell lymphoma as a result of a translocation (14,18) which couples the gene to an immunoglobulin-encoding sequence (58-60). Observations

in normal germinal center B-cells, gene transfer experiments using several cell types, and studies with transgenic mice confirmed that *bcl*-2 exerts its effect by inhibiting cell loss rather than by stimulation of proliferation (61). BCL-2 has been located to the inner mitochondrial membrane, the nuclear envelope and the endoplasmatic reticulum (62). The biochemical mode of action of BCL-2 remains unknown, although recent evidence suggests a role in the detoxification of reactive oxygen intermediates, among others generated as a result of aerobic life (63).

Besides the "specific" apoptosis gene *bcl*-2, well-known oncogenes and tumor suppressor genes can be involved in the control of apoptosis. The *ras* proto-oncogene, playing a role in the development of various tumors (64), may inhibit apoptosis (65) induced by certain chemotherapeutics (66) or by disruption of cell-matrix interactions (67). This effect of mutant *ras* is further discussed in the section on apoptosis and extracellular matrix. The tumor suppressor gene *p*53 also appears to be important in the control of apoptosis. P53 is involved in a cell cycle checkpoint for DNA integrity at the G1/S transition, which has to be passed before proliferation is allowed (68-70). The presence of chromosomal damage, *e.g.* radiation-induced breaks, results in the onset of P53-dependent apoptosis (71). Recently, P53 has also been implicated in apoptosis that is not induced by DNA damage (72,73). The presence of mutant *p*53 which is no longer able to induce apoptosis in response to DNA damage or subsequent mutations in other genes can result in tumor formation and will contribute to tumor progression (69).

4.4. Apoptosis upon growth factor or hormone withdrawal

Interleukin-2 was one of the first growth factors described to be involved in prevention of apoptosis. T-lymphocytes enter the apoptotic pathway upon withdrawal of this factor (74). Many other cell types are now known to depend upon growth factor or hormonal stimulation to survive (and proliferate): prostate or breast cells on steroids (75,76), vascular endothelial cells on fibroblast growth factor (77), mouse embryo cells on epidermal growth factor (78) and glial cells on platelet-derived growth factor (79). Stem cell factor suppresses apoptosis in various cell types, including primordial germ cells (80,81) and mast cells (82). These findings suggest that all cells in an organism depend on specific "survival factors" to escape apoptosis (1).

The cellular dependence on "survival factors" can be overcome by transfection and overexpression of *bcl*-2. Apoptosis of hematopoietic cell lines upon interleukin-3 withdrawal (57) and of neurons upon deprivation of nerve growth factor (83) is inhibited by BCL-2. It has been suggested that down-regulation of "survival gene" expression (including *bcl*-2) could follow growth factor deprivation (57). However, the loss of *bcl*-2 expression does not precede apoptosis when interleukin-3 is removed from certain interleukin-3-dependent cells (84). *P*53 is probably involved in stimulation of apoptosis upon removal of at least some growth factors. This is suggested by the finding that loss of wild-type P53 function allows interleukin-6-dependent cells to survive upon factor removal (73).

Table 4.1. Genes involved in the regulation and/or execution of apoptosis.

Name	Species	Apoptosis	Nature	Reference
a1	mouse	?		33
mcl-1	human	?		34
bcl-x_l	avian/mouse/human	-		35
bcl-x_s	avian/mouse/human	+	*bcl*-2 homolog	35
bax	mouse/human	+		36
bad	mammalian	+		37
*ced*9	nematode	-		38
bag-1	mammalian	-	BCL-2 binder	39
c-*myc*	rat/hamster	+		40
*cdc*2	human	+		41,42
*grb*3.3	human	+		43
irf-1	mouse	+		44
rb	human	-	cell cycle/	45
e2f	human	+	transcription	45
e1a	viral	+	regulator	45
*myd*118	mouse	+		46
*gadd*45	human/hamster	+		46
*myd*116	mouse	+		46
*gadd*34	hamster	+		46
ice	murine	+	cysteine protease	47
*ced*3	nematode	+		48
*cpp*32	human	+		50
ich-1_l	chick/human	+	*ice* homolog	52
ich-1_s	chick/human	-		52
nedd-2	mouse	+		53
*cpp*1	murine	+	serine protease	49
p*rice*	chick/human	+	protease	51
*crm*A	cowpox virus	-	*ice* inhibitor	54
tradd	human	+		55
fadd	human	+		55
rip	human	+	death domain gene	55
fas	human	+		9,55
*tnfr*1	human	+		55
rpr	fruit fly	+		55,56
fasl	human	+	ligand for FAS	9,55
tnf	human	+	ligand for TNFR1	55
*ced*4	nematode	+	?	48
?	hamster/rat	+	endonuclease	30-32

?, not known; +, enhances apoptosis; -, abrogates apoptosis.

4.5. Apoptosis upon disruption of cell-matrix interactions

Apoptosis is influenced by cell-matrix interactions (67,85). The interaction of cells with their surrounding extracellular matrix depends on the nature of the matrix components (*e.g.* fibronectin, vitronectin, collagen and laminin) and the matrix receptors (integrins) expressed on the cell surface. Frisch and Francis (67) have shown that the disturbance of epithelial cell-matrix interactions results in apoptosis. The term "anoikis" (derived from the Greek word for homelessness) was suggested for this form of apoptosis. An abrogation of anoikis by activated RAS was reported. These results corroborate the findings by Schlaepfer *et al.* (86) who have shown that interactions of cellular fibronectin receptors with the extracellular matrix component fibronectin supply the cells with a survival signal that is most likely mediated via the RAS pathway.

The fact that cells from certain tumor types can metastasize to various locations in the body indicates that these tumor cells do not die from anoikis once they are outside their normal micro-environment. In addition, they appear to be able to succesfully "adjust" to their new matrix and growth factor environment and give rise to new tumor nodules. The metastatic potential of tumor cells could thus correlate with the expression of apoptosis/anoikis blocking genes (67,85), for example through autocrine stimulation by growth and survival factors.

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Chapter 5

RESEARCH AIMS

Glycolipid characteristics of nonseminomatous cell lines are well defined. We analyzed the glycolipid patterns of primary seminomas (SEs) and nonseminomatous testicular germ cell tumors of adolescents and adults (TGCTs), of which no extensive glycolipid data are available, to investigate whether the data on nonseminomatous cell lines can be applied to primary tumors and to shed light on the relation between SEs and nonseminomatous TGCTs (Chapter 6).

No animal model for human SEs is currently available. With the aim of finding such a model, we investigated whether canine seminomas are comparable to human SEs, using a multidisciplinary approach (Chapter 7).

No optimal *in vivo* or *in vitro* culture system, allowing prolonged survival and proliferation of SE cells is available at present. We aimed at improving the *in vitro* culture conditions for SE cells, using a well-defined feeder layer and medium with fetal calf serum, stem cell factor, leukemia inhibitory factor and basic fibroblast growth factor (Chapter 8).

We analyzed the occurrence of apoptosis in intact SE tissue and SE cell suspensions to reveal whether the problems encountered thus far in the attempts to culture SE cells *in vitro* are due to the onset of apoptosis upon disruption of the cellular micro-environment, prior to culturing (Chapter 9).

To investigate the importance of *ras* mutations in the development of TGCTs, we screened a large series of primary SEs and nonseminomatous TGCTs for the presence of mutant N- or K-*ras*. Because of the ability of mutant *ras* to abrogate apoptosis induced by disruption of cell-matrix interactions, we also wanted to know whether the presence of a mutant N- or K-*ras* correlated with the results of our *in vitro* culture and apoptosis studies on SE cells (Chapter 10).

The data presented in Chapter 9 indicate that SE cells are susceptible to induction of apoptosis. To gain insight in the factors that possibly influence the onset of apoptosis in SE cells, as well as in the possible role in SE cells of regulators of apoptosis, we reviewed the literature. Findings possibly relevant for abrogation of apoptosis in SE cells, the application of which may allow the development of an *in vitro* culture system, are presented in Chapter 11. In this chapter, results from previous chapters and unpublished findings are discussed.

Chapter 6

GLYCOLIPIDS OF HUMAN PRIMARY TESTICULAR GERM CELL TUMORS

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Submitted

Background: The glycolipid content of germ cell tumor cell lines correlates with their differentiation lineage. Whether this reflects the situation in primary germ cell tumors is not known. Analysis of the glycolipid content of seminomas, which have not been extensively studied because of a lack of cell lines, might reveal their relationship with other germ cell tumor types.
Experimental design: The glycolipids of 38 human primary testicular germ cell tumors of adolescents and adults, comprising 19 seminomas, five embryonal carcinomas, five yolk sac tumors and nine (mixed) nonseminomas were analyzed, using thin-layer chromatography in combination with carbohydrate immunostaining. Samples of two testicular parenchyma containing abundant carcinoma *in situ*, two normal parenchyma with spermatogenesis, and one spermatocytic seminoma were also studied.
Results: Lactosylceramide (CDH) was detected in all embryonal carcinomas, but in less than half of the seminomas. Seminomas and embryonal carcinomas both contained globo-series glycolipids, including: globotriosylceramide (Gb3), globoside (Gb4), galactosyl globoside (Gb5) and sialyl galactosyl globoside (GL7). The lacto-series glycolipid Le^x was found in all embryonal carcinomas, but only in one seminoma. Gangliosides GD3 and GT3 were detected in many seminomas, but rarely in embryonal carcinomas. Yolk sac tumors displayed a heterogeneous glycolipid profile. Compared to seminomas and pure embryonal carcinomas, differentiated nonseminomas had reduced levels of globo-series glycolipids, especially Gb3 and Gb5, while CDH, Le^x, GD3 and GT3 were found in the majority of cases. Compared to normal testicular parenchyma, carcinoma *in situ*-containing parenchyma exhibited increased expression of globo-series glycolipids, most notably Gb3 and Gb5. The spermatocytic seminoma did not express GL7 and Le^x.
Conclusions: For the first time, we extensively studied the glycolipid content of seminomas. In addition, we show that the glycolipid content of nonseminomatous germ cell tumor cell lines reflects the situation in primary tumors. Globo-series glycolipids are similarly expressed in seminomas and embryonal carcinomas. The expression of Gb3 and Gb5 is reduced in nonseminomas upon differentiation. Le^x expression in nonseminomas including embryonal carcinomas, allows discrimination from seminomas. Expression of gangliosides in seminomas might indicate their maturation from gangliosides-negative precursor cells. Reprogramming of these precursors would result in the formation of Le^x-expressing embryonal carcinomas.

Introduction

The differentiation status of embryonal carcinoma (EC) and other nonseminoma (NS) cell lines derived from human testicular germ cell tumors of adolescents and adults (TGCTs) correlates with the expression of certain combinations of glycolipids in the plasma membrane (1-3). In EC cell lines, high levels of globo-series glycolipids, including globotriosylceramide (Gb3), globoside (Gb4), galactosylgloboside (Gb5) and sialyl galactosylgloboside (GL7) have been detected (3). Upon somatic differentiation, including the formation of neural cells, these glycolipids are down-regulated, while lacto- and ganglio-series glycolipids (including Le^x, and GD3/GT3, respectively)

Table 6.1. Glycolipid specificity of monoclonal antibodies used

Antibody	Glycolipid Structure*	Glycolipid Name
Globo-Series		
#	Glcß1→Cer	CMH
#	Galß1→4Glcß1→Cer	CDH
1A4-E10	Galα1→4Galß1→4Glcß1→Cer	Gb3 (CTH)
MC630	GalNAcß1→3Galα1→4Galß1→4Glcß1→Cer	Gb4 (globoside)
MC630	Galß1→3GalNAcß1→3Galα1→4Galß1→4Glcß1→Cer	Gb5 (SSEA-3)
MC813	NeuAcα2→3Galß1→3GalNAcß1→3Galα1→4Galß1→4Glcß1→Cer	GL7 (SSEA-3/4)
MC813	GalNAcß1→3Galß1→3GalNAcß1→3Galα1→4Galß1→4Glcß1→Cer 3 ↑ NeuAcα2→3Galß1	GL9 (SSEA-3/4)
Lacto-Series		
MC480	Galß1→4GlcNAcß1→3Galß1→4Glcß1→Cer 3 ↑ Fucα1	Le^x (SSEA-1)
MC480	Galß1→4GlcNAcß1→3Galß1→4GlcNAcß1→3Galß1→4Glcß1→Cer 3 3 ↑ ↑ Fucα1 $[Fuc\alpha1]_n$	extLe^x (SSEA-1)
Ganglio-Series		
R24	NeuAcα2→8NeuAcα2→3Galß1→4Glcß1→Cer	GD3
A2B5	NeuAcα2→8NeuAcα2→8NeuAcα2→3Galß1→4Glcß1→Cer	GT3

** Globo-series glycolipids contain Galα1→4Gal; lacto-series glycolipids contain GlcNAcβ1→3Gal; ganglio-series contain NeuAcα2→3Gal; gala-series contain Galβ1→Ceramide. # No antibodies to detect CMH and CDH are available; these molecules are detected using orcinol staining.*

become detectable in specific combinations and quantities depending on the lineage of differentiation, inducible by various agents, *e.g.* retinoic acid and hexamethylene bisacetamide (3-5). Cell lines derived from yolk sac tumors (YSs) can contain Gb3, Gb4 and gangliosides, while choriocarcinoma cell lines mainly express Gb3 and the stage-specific embryonic antigen 1 (SSEA-1)-carrying lacto-series glycolipid Le^x (3).

It is not known to which degree the data on cell lines are representative for primary tumors. In addition, data on seminoma (SE) cells are lacking, mainly since SE cell lines do not exist. Since glycolipids are expressed in a stage and lineage specific manner, data on the glycolipid profile of SEs may shed light on the relationship between SEs and NSs, which is still a matter of debate. Several investigators suggest that SE and NS are biologically independent (6-8), while others assume that NS develops through a, not necessarily clinically manifest, SE stage (9-12). According to this so-called linear progression model, SE cells become "reprogrammed" to EC cells, the undifferentiated stem cells of human NSs. This hypothesis is supported by morphological, ultrastructural, immunohistochemical, (interphase) chromosomal and

clinical analyses (11,13-19). We now studied the glycolipid content of primary TGCTs, including SEs and carcinoma *in situ* (CIS), the precursor of all TGCTs (20), to reveal the relationship between SEs and NSs, especially ECs.

Experimental design

A series of 49 orchidectomy specimens suspected of a GCT were used in this study. Tumor diagnosis was based on microscopic interpretation of a hematoxylin and eosin-stained 5 μm frozen tissue section and subsequently confirmed using immunohistochemical analyses on paraffin-embedded material. Representative fresh tumor parts were snap frozen in liquid nitrogen or dissociated in culture medium using crossed scalpel blades to obtain single cell suspensions that were cryopreserved. Since it is known that infiltrating lymphocytes can be present in SEs in variable amounts (7,21), we investigated whether these cells influenced the glycolipid analysis of SE cells. Therefore, the results of cryopreserved suspensions from five SEs, containing tumor cells and lymphocytes, were compared to those of the same suspensions after lymphocyte depletion. Upon lyophilization of all samples, glycolipids were extracted according to standard procedures (22,23) and analyzed using thin-layer chromatography in combination with carbohydrate immunostaining (4,5). The glycolipid specificity of the monoclonal antibodies used is depicted in Table 6.1.

Results and discussion

The glycolipid expression patterns of nonseminomatous cell lines has been shown to be related to the differentiation lineage of the cells (3-5). No extensive studies on primary NSs have been performed, nor has the glycolipid content of SEs been specifically studied (24-27), mainly due to a lack of cell lines. We analyzed the glycolipid expression in primary SEs and NSs to get more insight into the relationship between SE and EC. The results of our orcinol and immunostaining analyses are shown in Figure 6.1. All data concerning the glycolipid profiles of the analyzed samples are listed in Table 6.2 and summarized in Table 6.3.

Seminomas and carcinoma in situ

SEs are known to contain infiltrating lymphocytes (7,21), which could influence our tumor glycolipid analysis. Therefore, magnetic anti-CD2 coated beads were used to remove these inflammatory cells from SE cell suspensions. Thin-layer chromatography and subsequent orcinol or immunostaining for SSEA-1, SSEA-3 and SSEA-4, using pellets of either untreated or lymphocyte-depleted cell suspensions, revealed that lymphocyte depletion did not result in a marked change in glycolipid profile (Fig. 6.1). Orcinol staining revealed an additional band of unknown origin in the beads-treated samples which did not react with any of the monoclonal antibodies included in this study. Whether this band is specific for SEs needs further investigation. Gb3 and Gb4 were the major glycolipids in all five SE samples. Since lymphocytes did not interfere with our glycolipid analysis, we proceeded to use lyophilized tissue blocks from snap frozen samples for subsequent analyses.

Of 21 SEs analyzed, all tumors expressed the globo-series glycolipid GL7, while CDH was found in nine, Gb3 in 19, Gb4 in 20 and Gb5 in 10 SEs. None of the SEs (except one) expressed Le^x. The ganglio-series glycolipids GD3 and GT3 were present in 14 and 10 SEs, respectively. The expression level of the distinct glycolipids varied among the SEs. Especially concerning GL7, two groups of SEs could be distinguished: one with a low and one with a high level of expression. Since tumor cell enrichment by lymphocyte depletion did not result in a marked change in detection levels of the glycolipids and similar size tumor blocks were used for glycolipid extraction, the high and low glycolipid levels found in the tumor blocks apparently reflect differences in expression level and not a variation in the amount of tumor cells present in each sample.

The two CIS-containing parenchyma were characterized by the presence of Gb3 and Gb5 and the abundant expression of Gb4 and GL7. Le^x was absent, while trace amounts of gangliosides were detectable. These results attest to the phenotypic similarity of CIS and SE cells.

Two tumors, TL1049 and TL3544, were found to have high levels of glycolipid expression and contained an extended GL7 glycolipid, referred to as GL9, previously shown to be present in NT2/D1 cells (5). Interestingly, these tumors have previously been shown to contain a mutant *ras* gene (28) and exhibit an aberrant *in vitro* behavior (29). Sixteen other SEs, comprising 3 *ras* mutant and 13 wild-type tumors, did not show a correlation between the presence of a *ras* mutation and high glycolipid expression, while none of these *ras* mutant SEs expressed GL9.

Since expression of gangliosides is regarded a marker of differentiation (4), the finding of GD3 and GT3 in many SEs confirms the thought that SEs form a heterogeneous population. It can be speculated that the gangliosides-containing SE cells are derived from precursor cells which only express globo-series glycolipids. Whether primordial germ cells, the benign counterparts of SE cells, also show heterogeneity concerning glycolipid expression could be analyzed in future studies, using immunohistochemistry.

No SE cell lines are available today, although one cell line, designated S2, has been described to have some seminomatous characteristics (30). Analysis of the glycolipid profile revealed that S2 cells contain some Gb3, but mainly express CDH, Gb4, GL7 and Le^x, while Gb5 is not present (3). We confirmed the reported data on S2 in a blind test during this study, which allowed identification of the S2 origin of the sample (not shown). In combination with our findings of CDH and Le^x mainly in primary ECs (see below), and the absence of Gb5 in half of the SEs, the suggestion that S2 represents a tumor cell with an intermediate phenotype between SE and EC, but not a pure SE (3,30), is supported.

Embryonal carcinomas

The ECs expressed CDH, globo-series glycolipids and Le^x. All SEs (except one) lacked this latter marker, which can thus be used for the differential diagnosis between SE and EC. Two ECs were found to weakly express GD3, while only one tumor contained GT3.

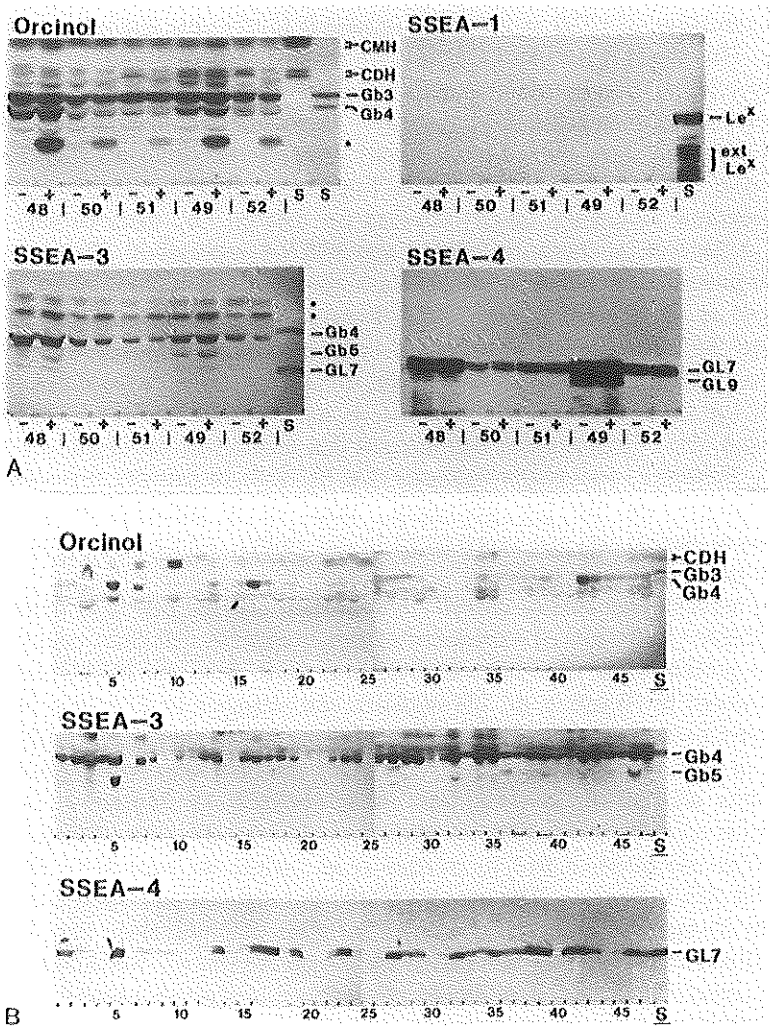

Figure 6.1. *Effect of lymphocyte depletion on glycolipid content of human seminomas. Upper and lower phase glycolipids were obtained from cell suspensions that were either untreated (-) or treated (+) with magnetic immunobeads to remove lymphocytes (A). Thin-layer chromatography immunostaining analysis of globo-series glycolipd expression in lower phase and upper phase extracts of human testicular germ cell tumors (B). Glycolipid standards are included on the right side of each plate (S). Plates were developed with chloroform/methanol/water (50:40:10) containing 0.05% calcium chloride and either stained for carbohydrate using orcinol/sulfuric acid spray (**Orcinol**) or labeled with monoclonal antibody directed to either **SSEA-1**, **SSEA-3** or **SSEA-4**. Lower phase glycolipids were included on Orcinol and SSEA-3 plates; upper phase glycolipids were included on SSEA-1 and SSEA-4 plates. Results represent bound antibody detected using alkaline phosphatase-conjugated second antibody. Asterisks (*) note a contaminant in lymphocyte depleted samples (Orcinol), and non-specific binding of second antibody to lipids present in lower phase extracts (SSEA-3). Samples are identified by number in Table 7.2.*

The expression of CDH is markedly enhanced in primary tumors, as compared to cell lines. This could mean that CDH is more rapidly converted into the derived globo-series glycolipids in cell lines cultured *in vitro*, especially since the expression of globo-series glycolipids is similar in primary tumors and cell lines. Results obtained in a NATO advanced study workshop (Andrews *et al.*, to be published elsewhere) on the expression of cell surface antigens by TGCT cell lines, applying immunohistochemistry and immunoflow cytometry, largely confirm our data on Le^x, detected with antibodies to SSEA-1 (as well as those on SSEA-3 and SSEA-4 expression). However, our data and those presented by Wenk *et al.* (3) show some differences with those obtained by Andrews *et al.*. The latter detected SSEA-1 antigen on all cells from the EC cell lines H12.1 and H12.2, whereas Wenk *et al.* could not detect this marker on these cell lines, using glycolipid analysis. Most likely, this is due to the fact that although SSEA-1 antigen can be carried on glycolipids, it is mainly presented at the cell surface as glycoprotein (31). The presented data suggest that this is true for the H12.1 and H12.2 cell lines. However, our results implicate that EC cells in primary tumors express the SSEA-1 antigen on the Le^x glycolipid, alone or in addition to expression on glycoproteins (which was not investigated in our study), whereas *in vitro*, this antigen is mainly carrried on glycoproteins.

Taken together, the studies on cell lines indicate that EC cell lines heterogeneously express SSEA-1 and reduced expression of this marker in cell lines could indicate its loss upon prolonged *in vitro* culture. Our data, those from the NATO workshop and those presented by Wenk *et al.* (3) implicate the presence in ECs of a large, globo-series glycolipids-expressing stem cell population, which (heterogeneously) expresses SSEA-1, *in vitro* mainly carried on glycoproteins and *in vivo* (also) on glycolipids.

Our data are not in keeping with those obtained by Motzer *et al.* (25) and Damjanov *et al.* (32), who could not immunohistochemically detect SSEA-1 expression in ECs. The use of MAb P12 by Motzer *et al.*, while we used MAb MC480 might account for this difference. The use of MC480 in combination with a 2-step detection method by Damjanov *et al.* might account for their findings, since they also failed to detect SSEA-3 expression in SEs (using the 2-step approach), which was detected by us in the present study and in an immunohistochemical analysis using the avidin-biotin method (not shown).

Yolk sac tumors

The glycolipid patterns of the two pure YSs, TL1013 and TL37R21, the latter derived from a xenografted NS with a YS component, are similar to those described for YS cell lines.

The four primary YSs with minor populations of other nonseminomatous cell types (as indicated in Table 6.2), did not display a clearly defined glycolipid profile. These heterogeneous glycolipid profiles could not be related to the types of tumor morphology distinguished by Pera *et al.* (33), *i.e.* solid and reticulated YS resembling rodent visceral and parietal endoderm, respectively. In contrast to four previously described YS cell lines (one lacking all detectable glycolipids) (3), these four primary YSs contain Le^x. Presence of this glycolipid could probably be attributed to EC or teratoma cells, that were immunohistochemically detected in these YSs as minor cell

Table 6.2. Glycolipids of human germ cell tumors

# Tumor	CDH	Gb3	Gb4	Gb5	GL7	Le^x	GD3	GT3
CIS and seminomas								
13. TL1804 (CIS/SE)	+	++	+++	+	+++			+
35. TL3724 (CIS/NS)	+	+	+++	+	++		+	
1. TL7573		++	+++	++	+++		+	
2. TL614		++	+++		+		+	+
14. TL3174				+	+		+	+
15. TL287	++	++	+++		+		++	+
16. TL8225	+	+++	+++	++	+++			
19. TL2207T3	+	+	+		++		+	
26. TL1487		++	+++		+			
27. TL229		++	+++	+++	+++		+	
29. TL2207T1		++	+++		++		++	++
37. TL8837		+	++		++		+	+
38. TL9089		+	+++	+	+++		++	+
39. TL212		++	+++	++	+++		++	+
41. TL8763			+	+	+++			+
42. TL74	+	+++	+++	++	+++			+
45. TL8888		++	++		+	+	+	
47. TL539		++	+++		++		+++	+
48. TL1049	+	+++	+++	++	+++		+++	
49. TL3544	+	+++	+++	++	+++			
50. TL8285	+	++	+		+			
51. TL9244	+	+++	+		+		+++	
52. TL4873	+	++	+		+			
Embryonal carcinomas								
5. TL5207	++	+++	+++	+++	+++	++		+
7. TL2207T2	+++	+++	++	++	+	+++		
17. TL3635	++	++	++	+++	+++	+		
28. TL524	+	++	+++	+	++	+	+	
43. TL269	+	++	+++	+	++	++	+	
46. TL87	+	++	+++	+++	+++	++		
Yolk sac tumors								
30. TL37R21							++	+
40. TL1013		+	++				++	+
4. TL7873 (MT)*	+	+	+++			++	+	
9. TL6322 (EC)*	+			+	+	+++	+	
25. TL1973 (EC,IT)*	++					+++		
36. TL7162 (EC)*		+	++	++	++	+		
Nonseminomas								
8. TL6745 (MT)		+	+		+		++	++
10. TL3819 (IT,MT,YS,CH)	+++	+		+		+++	+	+
11. TL3035 (MT,YS)	+		+		+		+++	+
22. TL37T1 (EC,IT,MT,YS)	++	+	+	+	+	++	+	
23. TL6936 (MT,IT,YS)	++	+	++	++	+++	++	++	+
31. TL189 (WT)			+		+		+	+
32. TL1348 (EC,IT,MT,YS)			+++	++	+++	+	+	+
33. TL37T2 (MT,EC)	+	+	++		++	+		+++
34. TL8007 (EC,IT,MT,YS)	+	++	+++	+	++	+	+	+
Spermatocytic seminoma								
44. TL8743	+	++	+++	+			+	
Non-germ cell tumors								
3. TL8558 (DC)		++	+++		+		+	+
18. TL4224 (L)	+		++		+		+	+
20. TL6661 (L)	+				+	+		
Testicular parenchyma								
12. TL1540	+		++		+			
24. TL1541			++		+		+	

Results represent a synthesis of thin-layer chromatography orcinol and immunostaining data. The scale is negative (no symbol) to strong positive (+++). Le^x antigen was carried on multiple glycolipid species. Abbreviations: CH, choriocarcinoma; CIS/SE, CIS/NS, carcinoma in situ *containing testicular parenchyma adjacent to a seminoma and nonseminoma, respectively; DC, dermoid cyst; EC, embryonal carcinoma; IT, immature teratoma; L, lymphoma of the testis; MT, mature teratoma; YS, yolk sac tumor. *Four YSs contained minor amounts of non-YS cells, as indicated; WT, testicular Wilms' tumor of germ cell origin.*

Table 6.3. Glycolipid expression in human germ cell tumors

Glycolipid	Cell type				
	N (2)	CIS/SE (23)	EC (6)	YS (6')	NS (8)
Globo-series					
CDH	1, +	11, +	6, ++	3, +	6, ++
Gb3		21, ++	6, ++	3, +	6, +
Gb4	2, ++	22, ++	6, +++	3, ++	7, ++
Gb5		12, ++	6, ++	2, ++	5, +
GL7	2, +	23, +++	6, ++	2, ++	7, ++
Lacto-series					
Le^x		1, +	6, ++	4, ++	6, ++
Ganglio-series					
GD3	1, +	15, ++	2, +	4, ++	7, ++
GT3		11, +	1, +	2, +	7, +

The number of samples (of the total number analyzed, indicated in brackets) expressing the indicated marker and the average immunostaining intensity are shown. Glycolipid structures were identified in this report by: i) co-migration on thin-layer chromatography plates with pure glycolipid standards and ii) by immunostaining using specific anti-glycolipid monoclonal antibodies. CIS, carcinoma in situ *containing testicular parenchyma; EC, embryonal carcinoma; N, normal testicular parenchyma; NS, nonseminomatous testicular germ cell tumor; SE, seminoma; YS, yolk sac tumor. 'Four YSs contained minor amounts of other nonseminomatous cell types, as indicated in Table 6.2. The results of a testicular Wilms' tumor were not included in the average staining intensity of nonseminomas. Expression is from absent (no symbol) to strong (+++).*

populations. Damjanov *et al.* (32) immunohistochemically detected Le^x in the YS cells of tumors containing at least EC and YS components, while pure YSs were not analyzed.

We conclude that pure YSs are characterized by at least lacking Gb5, GL7 and Le^x.

Other nonseminomatous TGCTs

Compared to SEs and ECs, the nine (mixed) NSs had reduced levels of globo-series glycolipids, especially Gb3 and Gb5, while CDH and Le^x were found in the majority of the samples. Eight NSs contained GD3 and GT3. The highest ganglioside levels were found in tumors with at least a mature teratoma component. One pure mature teratoma had trace amounts of Gb3, Gb4 and GL7, besides high levels of GD3 and GT3.

Our data on pure ECs and NSs with differentiated components, are in agreement with those reported for the cell line NT2 (3). EC cells express almost exclusively large amounts of globo-series glycolipids (apart from Le^x). The NSs with differentiated components are characterized by a lower expression of the globo-series glycolipids, especially Gb3 and Gb5, expression of the lacto-series glycolipid Le^x in the majority of

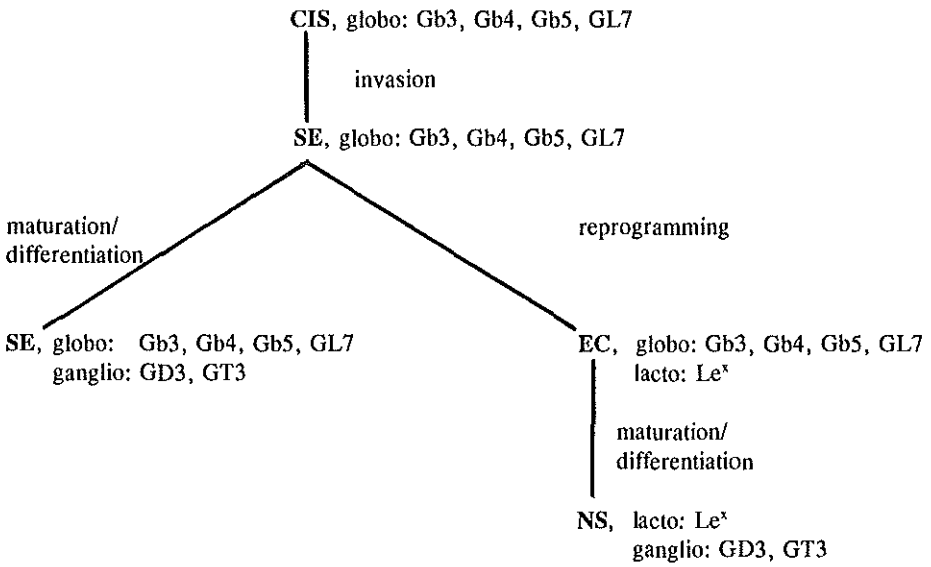

Figure 6.3. *Speculative model of the development of testicular germ cell tumors from carcinoma* in situ, *taking into consideration the glycolipid expression patterns of the various tumor types.*

the tumors and presence of the gangliosides GD3 and GT3, at the highest levels in tumors with at least a mature teratoma component. These data confirm the morphological observations of the presence of a minor stem cell population in differentiated NSs.

Spermatocytic seminoma, non-germ cell tumors and testicular parenchyma

The only spermatocytic seminoma in our series of primary testicular tumors was clearly distinguishable from all TGCTs in that it did not contain GL7 and Le^x. This supports the contention that spermatocytic seminoma is a separate GCT entity, not derived from CIS cells (34,35).

The dermoid cyst expressed Gb3, Gb4, GL7, GD3 and GT3. One B-cell lymphoma contained some CDH, while the other had low levels of CDH, Gb4, GL7 and Le^x.

In normal testicular parenchyma Gb3 and Gb5 could not be detected. However, Gb4 and GL7 were present, although at lower levels than in CIS-containing parenchyma.

Based on their glycolipid content, the spermatocytic seminoma, non-GCTs and the normal parenchyma of the testis could readily be discriminated from TGCTs and parenchyma containing CIS.

Concluding remarks

Our analysis of the glycolipid content of human primary TGCTs confirms the data obtained on nonseminomatous cell lines (3). Globo-series glycolipids are highly expressed in ECs, while the expression of especially Gb3 and Gb5 is reduced in differentiated nonseminomatous elements. In addition, we show that the globo-series glycolipids are expressed at comparable levels in CIS, SEs and ECs. The expression of

Le^x by ECs allows discrimination between this tumor type and SEs, which do not express this marker. Gangliosides are found in many SEs and almost all differentiated NSs, but are rare in ECs.

These results could be integrated in the speculative model shown in Figure 6.3. Primitive cells, *i.e.* CIS and SE cells, are characterized by globo-series glycolipids. These tumor cells could develop along two pathways. Either they mature (differentiate) in the germ cell lineage and start expressing gangliosides, or they are reprogrammed to become pluripotent EC cells and start expressing lacto-series Le^x. When these reprogrammed cells mature (differentiate) into various lineages they start expressing gangliosides as well.

The present data fit into the linear progression model, but do not prove it. Studies comparing the glycolipid profile of CIS and adjacent tumor, either SE or NS, should be performed to further investigate this model.

Methods

Tumor handling

Fourty-nine orchidectomy specimens, suspected of a TGCT were collected at the operation theater or pathology department of collaborating hospitals. Representative parts of tumor and adjacent normal parenchyma were snap-frozen using liquid nitrogen. The remaining parts were put in culture medium (DMEM/F12, with 103 kU/L penicillin, 103 mg/L streptomycin, 43 mg/L gentamycin, 365 mg/L-glutamin, Gibco, Paisley, UK) and taken to the laboratory for further processing. Tumor diagnosis was based on microscopic interpretation of a hematoxylin and eosin-stained 5 μm frozen tissue section. Fresh representative samples of all tissue components were fixed in 4% (v/v) formalin for paraffin embedding, or snap frozen in liquid nitrogen. Remaining tumor parts were dissociated in culture medium at room temperature, using two crossed scalpel blades. Tissue fragments were allowed to settle in a 50 ml tube in 30 ml culture medium. The supernatant, containing mostly single cells (as analyzed by phase contrast microscopy using a Zeiss Axiovert microscope), was washed twice with culture medium. To the cell suspension 10% (final volume) dimethylsulfoxide was added slowly. The suspension was aliquotted, frozen in a Kryo 10 Series 2 automated freezer (Planer Biomed, Sunbury-on-Thames, UK), and stored in liquid nitrogen.

Tumor characterization

Typing according to the classification of the World Health Organization (7,21) was based on histology and immunohistochemical analysis of expression of germ cell specific alkaline phosphatase (detected with antibodies to placental alkaline phosphatase), α-feto protein, human chorionic gonadotropin (Dako, Glostrup, DK) and cytokeratins 8 and 18 (Beckton Dickinson, San Jose, USA) using representative paraffin and frozen tissue sections (11).

Classification revealed 19 SEs and 19 NSs, the latter comprising five pure ECs, one mature teratoma (MT), five YSs (one pure and four with minor amounts of other nonseminomatous cell types, as indicated in Table 6.2), one testicular Wilms' tumor of germ cell origin (36), and seven mixed tumors. The mixed NSs comprised two tumors

with EC, immature teratoma (IT), MT and YS, one with IT, MT, YS and choriocarcinoma, one with MT and YS, and one with IT, MT and YS. Separate tumor nodules were used from two other mixed tumors; one with EC, IT, MT and YS besides EC with MT, the other with two SE nodules besides an EC component. The separate samples from these two tumors are referred to as T_1, T_2 and T_3 (for the latter), and are regarded as individual tumors. Besides the above mentioned tumors, samples of two normal parenchyma with spermatogenesis and of two parenchyma with abundant CIS were analyzed, as were a spermatocytic seminoma, one pure YS (TL37R21) derived from a xenografted mixed NS (TL37), one dermoid cyst and two testicular B-cell lymphomas.

Lymphocyte depletion

Cryopreserved single cell suspensions from five SEs, containing SE cells and lymphocytes, were rapidly thawed at 37^{0}C, washed in 10 ml culture medium, and counted. The suspensions were treated with a 2.5 fold excess (relative to the total cell number) of magnetic beads coated with anti-CD2 monoclonal antibody (Dynal, Skoyen, N) to deplete lymphocytes. After 15-20 min incubation at room temperature with gentle shaking, 4 ml of culture medium was added, and the beads were removed using a magnetic particle collector (Dynal). The supernatant, containing enriched SE cells was removed. The beads were washed twice with culture medium and all supernatants were pooled. Removal of the lymphocytes was verified by microscopic examination of a cytospin preparation with hematoxylin and eosin staining. After treatment with magnetic beads, all suspensions contained less than 15% lymphocytes. Similar volumes of packed cells from untreated or beads-treated samples were used for glycolipid extraction.

Lyophilization and glycolipid extraction

The SE cell suspensions (either treated with magnetic beads or not) and the frozen tumor blocks were lyophilized overnight in a Freeze Mobile 12SL (Virtis Sentry, Gardiner, USA). Upon lyophilization, samples were sent to Philadelphia in numbered tubes, without information on tumor histology to assure an objective assay. Glycolipids were extracted from an approximately equal volume of packed tumor cells using isopropyl alcohol:hexane:water (55:25:20, v/v/v), as previously described (22). Total lipid extracts were partitioned into an upper and lower phase according to the method of Folch-Pi (23). The upper phase was desalted using C18 reverse phase columns (Analytichem, Harbor City, USA).

Glycolipid analysis

Major glycolipids were detected using orcinol staining. Specific glycolipids were identified by co-migration with pure glycolipid standards and by immunostaining with specific monoclonal anti-carbohydrate antibodies (4,5). Briefly, 5 μl of each glycolipid sample was streaked onto Whatman HP-FK silica gel plates and subjected to ascending chromatography using a solvent system of chloroform:methanol:water (50:40:10 v/v/v) containing 0.05% (w/v) calcium chloride. After drying, the thin-layer chromatography plates were coated with 0.5% (w/v) polyisobutyl-methacrylate (Aldrich, Milwaukee,

USA) in diethyl ether for 1 min, blocked for 2 hr with 5% bovine serum albumin (Sigma, St. Louis, USA) in phosphate-buffered saline, and then reacted with primary antibody overnight at 4°C. Bound antibody was detected using a 2 hr incubation at 4°C with alkaline phosphatase-conjugated goat anti-mouse antibody (HyClone, Logan, USA) diluted 1:1000. Color reaction was obtained through incubation with bromochloroindolyl phosphate (Fisher Biotech, New Jersey, USA) and nitroblue tetrazolium (Sigma) for 1 hr at room temperature (37).

Monoclonal antibodies

Anti-carbohydrate monoclonal antibodies (MAbs) were obtained and used as described previously (4). Gb3 was detected using MAb 1A4-E10 (4); Gb4 and Gb5 were detected using MAb MC630 to SSEA-3 (38); GL7 was detected using MAb MC813 to SSEA-4 (39); Le^x was detected using MAb MC480 to SSEA-1 (40,41); GD3 was detected using MAb R-24 (42); GT3 was detected using MAb A2B5 (43); The glycolipid carbohydrate structures recognized by these reagents are listed in Table 6.1. Gangliosides are designated according to the nomenclature of Svennerholm (44). Glycolipids are designated according to the recommandations of the IUPAC Nomenclature Committee (45).

Acknowledgements

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Chapter 7

SEMINOMAS OF THE CANINE TESTIS; COUNTERPART OF SPERMATOCYTIC SEMINOMA OF MEN?

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Background: Dogs develop germ cell tumors of the testis at a relatively high rate. It is not known to what degree these tumors resemble various human testicular neoplasms.
Experimental design: The epidemiology and morphology of a series of spontaneous canine testicular tumors, collected between 1985 and 1991, was analyzed, and compared with human testicular germ cell tumors. DNA content analysis of representative samples was performed using flow cytometry and image cytometry. Eight human spermatocytic seminomas were studied in parallel.
Results: All canine tumors had the histopathologic features reported as typical for dog testicular seminomas. These tumors could show both an intratubular and an invasive component. Most of them were pure (78%), while they could be combined with a Leydig cell tumor, a Sertoli cell tumor, or both. No somatic, placental or yolk sac cells were identified, and there was no carcinoma *in situ*. A bimodal age distribution, with a peak around 1 year of age and between 4 and 16 years of age, was found for all pure and mixed testicular tumors, except for those composed of a Leydig cell and a seminoma component. These tumors were all present in dogs older than 7 years, being significantly older ($p<0.01$) than dogs with a pure tumor of either type. All Sertoli cell and Leydig cell tumors were diploid. No consistent peritriploid DNA content, characteristic of human testicular germ cell tumors, was found for canine seminomas, which most often had a diploid DNA content. Human spermatocytic seminomas always contained diploid tumor cells, and showed a relatively low number of high ploidy cells, comparable to canine seminomas of the testis.
Conclusions: The so-called seminomas of the canine testis are tumors of old age. Histologically, these tumors are composed of a single cell type with some variation without evidence of differentiation. It is proposed that canine seminomas correspond to human spermatocytic seminomas. It is thought that the Leydig elements in these tumors represent a reactive change rather than biphasic differentiation of a single stem cell capable of germinal and sex-cord cell development.

Introduction

The tumors of the human adult testis, comprising approximately 95% germ cell tumors (GCTs) and 5% gonadal stromal tumors, are in general rare tumors (1-3% of all cancers in males). In spite of this, these testicular germ cell tumors of adolescents and adults (TGCTs) are the most common malignancy in young men, and a still increasing incidence is reported (1).

Multidisciplinary research resulted in increasing understanding of the biology of these tumors. Clinico-pathologically they can be divided into seminomas (SEs) (50%) and nonseminomatous TGCTs (NSs) (40%), and those composed of both histologies, the combined tumors (CTs) (10%) (2,3). They all originate from carcinoma *in situ* (CIS) (4), which can frequently be found in the adjacent parenchyma of an invasive tumor (5 for review). Polyploidization is suggested as an early event in the pathogenesis because of the consistent peritriploidy of these tumors as well as of CIS (6-9). Evolution of these tumors is most probably associated with net loss of (parts of) chromosomes (6,10 for review). The majority of human TGCTs are clinically manifest at postpubertal age, with a median age for NSs of 25 year, for SEs of 40 year, and for

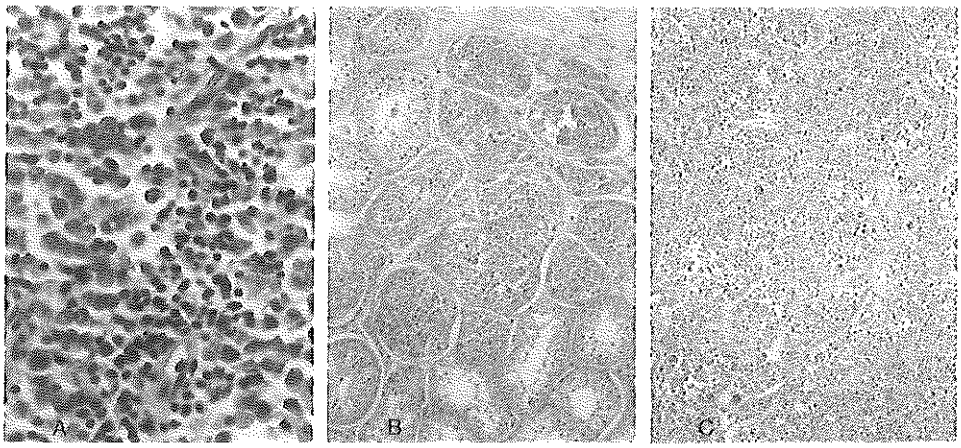


Figure 7.1. *Representative hematoxylin and eosin-stained tissue sections showing the morphology of canine seminoma (A, x640), an intratubular (B) and an invasive component (C) of the same case (x160). Note the lymphocytic infiltrates in the invasive tumor.*

CTs in between the two (1,6,10).

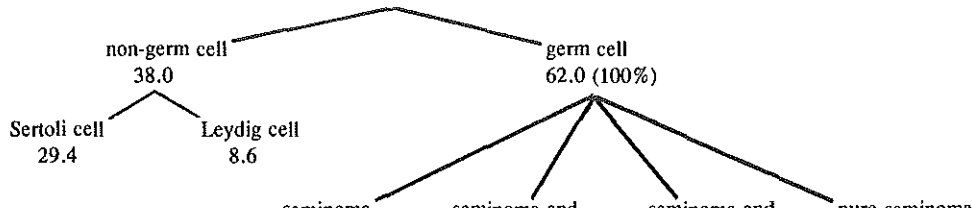
To study the factor(s) involved in the initiation, promotion, and progression of human TGCTs, a proper animal model would be of great value. The only available and intensely studied model, the murine teratocarcinomas (spontaneous or induced), is not satisfactory because it lacks SE and CIS (11). In addition, the spontaneous tumors in this model develop prepubertally. In general, domestic animals show a low incidence of TGCT-like tumors (11), with the exception of dogs, who show an incidence even higher than that in humans (1,3,12,13). To address the question whether canine testis tumors are a proper model for human TGCTs, we studied the morphology, epidemiology, and DNA content of a large series of spontaneous canine testicular tumors.

Experimental design

A series of 187 testicular tumors of dogs collected at the Veterinary Faculty of the University of Utrecht between 1985 and 1991 was included in this study. Re-examination of the representative hematoxylin and eosin-stained paraffin sections of 106 tumors, supposed to contain at least one seminoma component, showed no discrepancies with the original diagnosis. Therefore, the tumors not re-examined (n=10), and reported to contain a seminoma according to the original diagnosis, were also included in this study. All samples were thoroughly histologically screened for the presence of NS-like as well as CIS-like components. In addition, the presence of nongerm cell tumor components, *e.g.* Sertoli cell and/or Leydig cell tumors, was scored. The age of the affected dogs (when available) was studied in correlation with the histology of the tumor.

Because of the possible pitfalls of DNA flow cytometry (FCM) for DNA content

Table 7.1. Distribution of the histology of spontaneous canine testicular tumors in total (n=187=100%), and separately for those with at least one seminoma component (n=116=100%, indicated in brackets).



no slides available:		1.3 (1.9)		4.3 (6.9)
intratubular:	0.4 (0.8)	2.2 (3.4)	2.1 (3.4)	9.6 (15.5)
invasive:		0.0 (0.0)	3.2 (5.2)	20.2 (32.7)
intratubular/invasive:		2.2 (3.4)	2.6 (4.3)	13.9 (22.5)

measurements, *e.g.* inability to distinguish diploid and tetraploid tumor cells from normal host cells, and especially in paraffin-embedded tissue the risk of missing minor aneuploid cell populations (14,15), we also used image cytometry (IC) in this study. This technique solves the latter problems, but results in a lower stem line resolution due to the lower number of nuclei analyzed. Using both approaches, DNA content analysis was performed on representative samples of different canine testicular tumors. The percentage of hyperpentaploid cells, determined by IC, was compared with the FCM data. In addition, eight human spermatocytic seminomas (SSs), tumors composed of more mature germ cells with a different pathogenesis than TGCTs (16 for review), were analyzed.

Results and discussion

For morphological and epidemiological reasons, the spontaneous or induced murine teratocarcinomas are less suitable as models for human TGCTs (1,3,4,10,11,17,18). We suggest that they are the counterparts of GCTs of the human infantile testis (19 for review). In addition to mice, other animals show a low incidence of germ cell tumors, except dogs (11 for review). We studied the morphology (n=116), epidemiology (n=187), and ploidy (n=33) of spontaneous testicular germ cell tumors of dogs to analyse whether these tumors are a proper model for human TGCTs.

Morphology

The tumors of our series, as well as the nonincluded tumors available at the Pathology Department of the Veterinary Faculty of the University of Utrecht, the Netherlands, showed no nonseminomatous components, in agreement with earlier

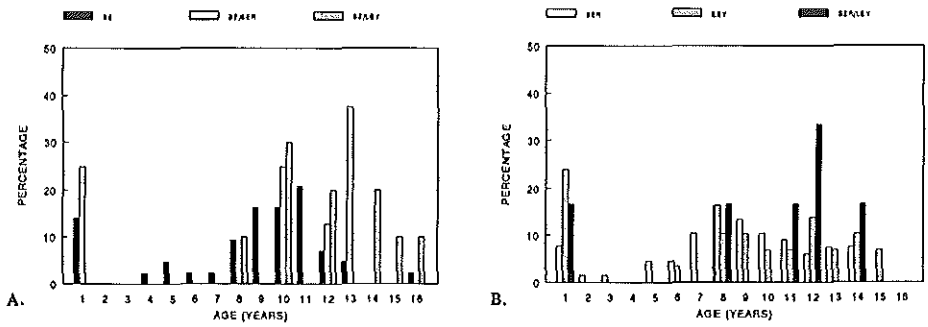


Figure 7.2. *Schematic representation of the age (years) at time of presentation of the different histologic categories of canine testicular tumors; containing at least a seminoma component (A); without a seminoma component (B). (SE, seminoma; SER, Sertoli cell tumor; LEY, Leydig cell tumor).*

publications (20 for review), but in contrast to the reported intracranial canine germ cell tumors (21-23). Neither was carcinoma *in situ* identified in the parenchyma adjacent to the canine testicular tumors. All testicular germ cell tumors in dogs were morphologically identical seminomas, showing solid sheets of moderately pleomorphic mononuclear cells, with round to ovoid nuclei, a dense chromatine structure and a single large nucleolus. The cells showed a moderate amount of basophilic cytoplasm and usually distinct cell borders (Figure 7.1A). Identical seminoma cells could grow within the seminiferous tubules (Figure 7.1B) (intratubular variant) or as invasive tumor (Figure 7.1C). Lymphocytic infiltrates, localized in the stromal compartment of the tumor were distributed throughout the invasive tumors, but also frequently in the surroundings of an intratubular seminoma component. In 95% of the specimens containing invasive seminomas (n=79), of which interpretable adjacent parenchyma was available, an intratubular component could be detected, which was most often multifocal. Typically, the intratubular seminoma cells occupied the whole cross section of the tubules. Multifocal origin for seminomas was earlier described (24).

Epidemiology

In contrast to the rather low overall incidence of human TGCTs (1 to 3%), a 10 times higher incidence of testicular germ cell tumors in dogs is reported (1,12,13,25). Testicular germ cell tumors in dogs and humans share certain epidemiological features, in particular, the risk factor cryptorchidism (26-28). The incidence of seminomas and gonadal stromal tumors in our series is summarized in Table 7.1, showing that 38% (n=71) were Sertoli cell and/or Leydig cell tumors, while the remaining (n=116, 62%) contained at least one seminoma component. Most seminomas were pure (77.6%), whereas some were combined with gonadal stromal tumors. In contrast to the

Table 7.2. Summary of the ploidy analysis of the canine testicular tumors and human spermatocytic seminomas using flow cytometry (FCM) and the presence of hyperpentaploid cells as measured by image cytometry (IC).

Ploidy	Histology																	
	inv se			intrat se			inv/intrat se			Leydig			Sertoli			SS		
	FCM	IC		FCM	IC		FCM	IC		FCM	IC		FCM	IC		FCM	IC	
		-	+		-	+		-	+		-	+		-	+		-	+
diploid	7	5	2	9	5	0	14	0	0	4	2	0	6	2	0	5	4	1
peridiploid	4	0	2	3	1	0	2	0	0	0	0	0	0	0	0	0	0	0
total	11	5	4	12	6	0	16	0	0	4	2	0	6	2	0	5	4	1
hyperdiploid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
hypotetraploid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	2

Abbreviations: inv se, invasive seminoma; intrat se, intratubular seminoma; inv/intrat se, mixed invasive and intratubular seminoma; Leydig, Leydig cell tumor; Sertoli, Sertoli cell tumor; SS, human spermatocytic seminoma; FCM, DNA flow cytometry; IC, image cytometry; -, less than 0.5% hyperpentaploid cells; +, more than 0.5% hyperpentaploid cells.

rarity of the combination of Sertoli and/or Leydig cell tumors with human TGCTs (29), this combination was found in 22.4% in this series of canine tumors, in agreement with earlier reports (13,27). Our results (not shown) confirm the reported histologic similarities between gonadal stromal tumors of the canine and human testis (11,20).

The distribution of tumor histologies in our series with at least a seminoma is similar to that in the subgroup of which the age at presentation is known (not shown). Within this latter group (Figure 7.2A), two peaks of incidence for tumors composed of only a seminoma or a seminoma mixed with a Sertoli cell tumor were identified, the first at 1 year of age (n=8), and the second (n=44) after 3 years of age. A bimodal age distribution was also suggested by the data of Innes (25). The peak of incidence for seminomas, around the 10th year of age is in agreement with other studies (27,30-32). Pure Sertoli cell and pure Leydig cell tumors, or the combinations (collected within the same time period between 1985 and 1991), also show a bimodal age distribution (Figure 7.2B). The dogs with a mixed seminoma and Leydig cell tumor (mean 12.1 years $\pm$ 2.6, n=10) were significantly older than the dogs with the purecounterparts (seminoma: 8.5 years $\pm$ 3.7, n=43; Leydig cell tumors: 8.7 years $\pm$ 4.9, n=29) (Mann-Whitney U test; $p<0.01$). This suggests a secondary development of those Leydig cell tumors which are mixed with a seminoma, possibly due to a prolonged gonadotropin drive resulting from atrophy induced by the seminoma component (33). Therefore, it is questionable whether the Leydig cell components are neoplastic or hyperplastic, a theme already discussed some 50 years ago (25 for review).

DNA content

Because of the complementary value of FCM and IC for DNA content analysis (14,15), we used both techniques on representative samples of canine testicular tumors. All controls (six normal parenchyma and five epididymis samples) showed similar

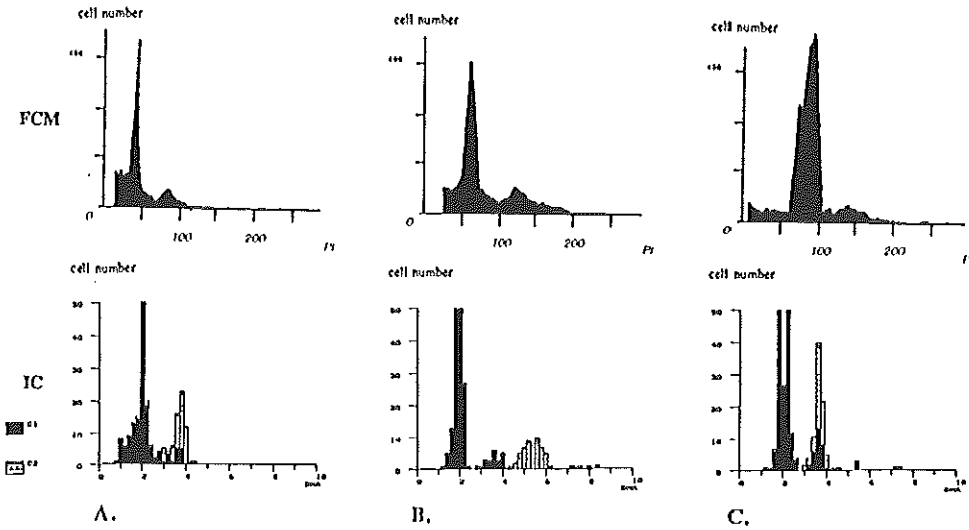


Figure 7.3. *Representative examples of a DNA content analysis using flow cytometry (FCM) and image cytometry (IC) of canine testicular seminoma cells, showing a diploid tumor without hyperpentaploid cells (A); a diploid tumor with hyperpentaploid cells (B); and a hyperdiploid tumor with hyperpentaploid cells (C).*

results using both approaches (not shown). Hyperpentaploid cells were found in less than 0.5%, in the same range as published (34 for review). The results obtained by FCM (DNA-index) and IC (ratio of hyperpentaploid cells) are summarized in Table 7.2. A high G2M population was found in all samples studied by FCM. The stem line data of the tumors derived from FCM are in agreement with the IC DNA-index measurements when obvious tumor populations could be identified (not shown). Three representative examples are shown in Figure 7.3; a diploid seminoma without hyperpentaploid cells (A), a diploid seminoma with hyperpentaploid cells (B), and a hyperdiploid seminoma with hyperpentaploid cells (C). Ploidy analysis showed that Sertoli and Leydig cell tumors, either pure or combined with other components, were always diploid. Less than 0.5% of hyperpentaploid cells was present.

FCM showed hyperdiploidy in 4 out of 11 invasive and 3 out of 12 intratubular seminomas. In addition, 2 out of 16 samples with both intratubular and invasive seminoma components were hyperdiploid. Both hyperdiploid invasive seminomas studied by IC contained hyperpentaploid cells. In addition, IC revealed a heterogeneous DNA content in two diploid invasive seminomas by FCM, with the presence of hyperpentaploid cells. The diploidy of five intratubular seminomas was confirmed using IC, and no hyperpentaploid cells were detected. The presence of hyperpentaploid cells was already suggested by the highly pleomorphic cells detected by morphological screening (illustrated in Figures 7.1B and C). No correlation between ploidy and age

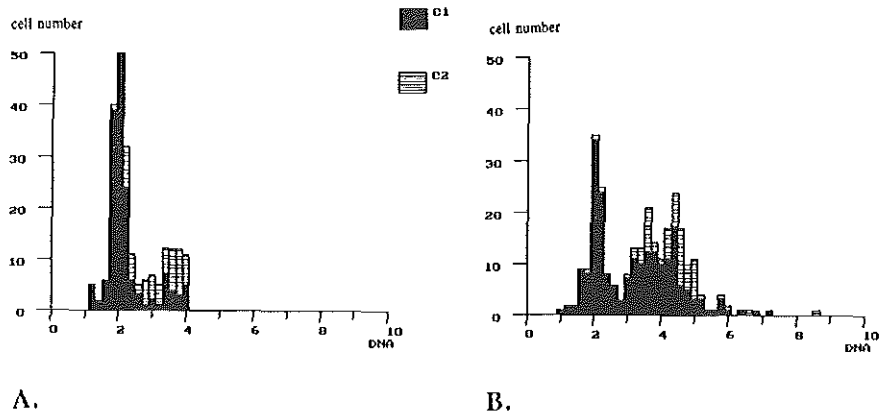


Figure 7.4. *Two representative examples of a diploid (A) and an aneuploid (B) human spermatocytic seminoma, analyzed by image cytometry.*

of the dog or histology of the tumor was found. After correction for background (0.5% hyperpentaploid cells), four invasive seminomas showed the presence of hyperpentaploid cells (range 0.5-5.9%, mean 2.9) (not shown). This is in contrast with data on human SEs and NSs (unpublished data), showing a relatively high frequency of hyperpentaploid cells, and a consistently near triploid DNA content (6-8).

Human spermatocytic seminomas

Because of the higher incidence of testicular seminomas in older dogs, as well as the absence of carcinoma *in situ* and nonseminomas, we hypothesize that these tumors are similar to human SSs. This tumor shows the histology of more mature spermatocytic cells, and has a different pathogenesis than TGCTs (16,35). Cryptorchidism is not reported as a risk factor (36). Ploidy may be an informative parameter to support our hypothesis. Thus far, published data on ploidy of SSs are discordant. One IC study reported five diploid, three tetraploid and three aneuploid SSs (36). While using the same technique, a consistent aneuploidy is found in another study (n=7) (37). One FCM study reported 33% diploid and 67% aneuploid SSs (n=9) (38). In addition, 57% were also found to be aneuploid (two near diploid, one tetraploid and one aneuploid) using FCM (35). In addition to our study (this chapter), a comparative DNA content analysis using both IA and FCM of SSs was recently published (39). All three SSs included were aneuploid, but diploid tumor cells were present. We performed IC on eight SSs (see Table 7.2), of which three were aneuploid (one hyperdiploid and two hypotetraploid) and five were diploid using FCM (35,38). Two

representative examples are illustrated in Figure 7.4, showing a diploid sample without hyperpentaploid cells (A), and an aneuploid sample (B), with hyperpentaploid cells. No discrepancies in ploidy were present between the FCM and IC data (not shown). Four out of eight SSs samples contained more that 0.5% hyperpentaploid cells (mean 2.1%, range 0.5 to 5.6%). The presence of hyperpentaploid cells is concordant with the histology of this tumor (40). The specific keratin expression pattern in human SSs (cytoplasmatic punctate) (40) might be an additional marker.

Concluding remarks

Histologically, no carcinoma *in situ* and nonseminomatous components have been found in this large series of canine testicular germ cell tumors. The seminomas clinically present at a relative high age, and are combined with sex cord elements in approximately 25% of the cases. DNA content analysis pointed to a similarity of canine seminomas and human SSs. This is supported by the benign behavior of human SSs (16 for review) and canine seminomas, showing metastases in 12.5% according to data of the Department of Pathology of the Veterinary Faculty of the University of Utrecht, and 6 to 11% according to the literature (41). In contrast, human SEs present with metastases in 20 to 30% (42).

Our findings are in favor of the hypothesis, already put forward in the early 60s (30), that canine seminomas are more similar to human SSs than to human SEs. Therefore, we propose to reclassify canine testicular seminomas as spermatocytic seminoma.

Methods

Histology

One hundred and eighty seven spontaneous canine testicular tumors collected at the Veterinary Faculty, University of Utrecht (The Netherlands) from 1985 through 1991, were classified according to the World Health Organization classification (3,13) on the basis of representative paraffin hematoxylin and eosin-stained slides. Histologically, the following pure or combined tumors (n=187) were distinguished: seminomas (intratubular or invasive), Sertoli cell tumor and Leydig cell tumor. The slides of 106 tumors containing a seminoma component were reviewed (10 were not available, *i.e.* eight seminomas and two seminomas/Sertoli cell tumors according to the original diagnosis). The age of 62 seminoma-bearing dogs of which the histologic diagnosis was checked was known.

Single parameter DNA flow cytometry

Representative samples (n=49) of all histological tumor types of 33 cases were analyzed by DNA flow cytometry (FCM); *i.e.* 12 intratubular, 11 invasive and 16 combined intratubular and invasive seminoma components, four Leydig cell tumors and six Sertoli cell tumors. In addition, six normal and five epididymis samples were measured. A 5-μm thick hematoxylin and eosin-stained slide was used for a histologic check for the tumor components processed for FCM. Depending on the amount of material, 2 or 3 50-μm thick sections were used for single cell isolation. Either

complete tissue sections were used, or parts of tissue sections in which a single component was isolated. Flow cytometry was carried out as described (43) with some minor modifications (based on 44). Briefly, tissue sections were deparaffinated, rehydrated by sequential washes in 100, 95, 70, and 50% (v/v) ethanol, and stored in distilled water overnight at room temperature. Dissociation was performed for 30 min at 37°C using 1 ml 1% (w/v) pepsine (Sigma Chemical Company, St. Louis Missouri) in 0.9% NaCl solution, pH 1.5, while vortexing every 5 min. Isolated cells were washed in buffered saline solution, centrifugated at 1,000xg for 5 min, and passed through a 100-μm filter. After another washing step, the pellet was resuspended while vortexing in 250 μl solution A (low salt stain: containing 3% PEG 8000, 0.05 mg/ml of propidium iodide, 180 units/ml of RNAse A, 0.1% Triton X-100, 4 μmol/ml sodium citrate) and incubated for 20 min at 37°C, after which 250 μl solution B (containing 3% PEG 8000, 0.05 mg/ml of propidium iodide, 0.1% Triton X-100, 400 μmol/ml sodium citrate) was added, and stored in the dark at 4°C, overnight. Before analysis, the suspensions were passed through a 50-μm pore filter (Stokvis & Smits B.V., Haarlem, The Netherlands). Cell analysis was performed on a FACSCAN flow cytometer (Becton Dickinson, Mountain View, California), with interface, and "Consort 30" data acquisition (Becton Dickinson).

Analysis of the FCM data was only performed when sufficient numbers of normal host cells were present, as judged from the hematoxylin and eosin-stained slide, to assure the position of the diploid stem line. When different histologic components were present within one specimen, the ploidy of these different cell populations was analyzed by carving the paraffin sections before processing in such a manner that one component was enriched as compared with the others. This allowed the assignment of an enlarged peak in the flow histogram to the enriched cell population. DNA content is expressed as the DNA index, *i.e.* the ratio of propidium iodide intensity of the modal G0/G1 peak of the aneuploid population and the modal G0/G1 peak of the diploid normal cells in the suspension. A diploid cell population has, by definition, a DNA index of 1.0.

Image cytometry

In addition to FCM, IC was done on 16 total and 17 purified seminoma samples of 15 cases; *i.e.* 6 intratubular, 9 invasive, as well as 2 Leydig cell and 2 Sertoli cell tumors. A further 11 testis samples (6 normal parenchyma and 5 epididymis samples) without tumor were studied, as well as eight human SSs.

One 50-μm section, adjacent to the sections used for FCM, was prepared for IC. Similar to FCM preparation, the sections were deparaffinated and rehydrated using decreasing ethanol concentrations. Subsequently, the sections were enzyme-treated with 0.05% pronase (Sigma) during 30 min at 37°C. The reaction was stopped with cold phosphate-buffered saline. The cell suspension was then filtrated and centrifuged. The supernatant was replaced by 2% (g/w) polyethylene glycol in 50% ethanol until a concentration of about 20,000 cells/ml was reached. Monolayer preparations were then made by cytocentrifugation of 1 ml of the cell suspension. These preparations were air-dried and stored at 4°C until staining (45). For the DNA measurements, Feulgen AzurA (Chroma, Germany) staining was used. Image cytometry was carried out with

LEYTAS, as previously described (46). Briefly, LEYTAS integrates a microscope with automated functions (Autoplan, Leica, Germany) and an image analysis computer (MIAC; Leica). The image cytometer is fully automated and enables cell selection at low magnification and measurement of selected objects at high magnification. In addition, the high magnification images are stored and displayed on a television monitor. The procedure in the present investigation consisted of selection and measurement of all epithelial cells. Subsequently, the preparation was screened for cells with higher density and size. The procedure was finished when either the whole preparation had been screened or when the maximum number of objects (n=320) had been selected. LEYTAS cytometry includes meticulous artefact rejection to remove falsely selected objects (*e.g.* overlapping nuclei). In addition, the few remaining artefacts were removed by visual evaluation of the stored images. Thus the obtained histograms were based on single cells only. Integrated optical density histograms were converted into DNA histograms by using the integrated optical density of the normal cell population as a reference value. For the present investigation, the ratio of the number of hyperpentaploid cells and the total number of cells was compared with the stem line determination by FCM.

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Chapter 8

HETEROGENEITY IN THE *IN VITRO* SURVIVAL AND PROLIFERATION OF HUMAN SEMINOMA CELLS

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Abstract

The *in vitro* culture conditions allowing survival and initial proliferation of murine primordial germ cells from 10.5 days *post coitum* embryos, which include the use of a murine embryonal fibroblast (STO) feeder, were applied to 21 human seminomas, composed of tumor cells which are considered as the malignant counterparts of human primordial germ cells. Cells from 18 seminomas attached poorly to STO, and only few survived through day 10. In contrast, three seminomas showed a higher degree of attachment. Two of them showed initial proliferation and enhanced survival: 30 days for tumor SE1 and 25 days for tumor SE3. Tumor SE1 was more extensively studied, using the culture conditions allowing the derivation of pluripotent embryonic germ cells from 8.5 days *post coitum* murine primordial germ cells, which include the use of STO feeder, stem cell factor, leukemia inhibitory factor and basic fibroblast growth factor. The presence of stem cell factor was necessary and sufficient for colonies of tumor cells to form during the first 3 days of culture. While the cell number decreased after day 3 in medium without fetal calf serum, it increased till day 9 in medium containing fetal calf serum. No reprogramming of SE1 cells to pluripotent cells was observed. Our data indicate that seminomas form a tumor population with a heterogeneous *in vitro* behavior, not equivalent to that of 8.5-10.5 days *post coitum* murine primordial germ cells.

Introduction

In humans, a unique histological entity of testicular germ cell tumors of adolescents and adults (TGCTs) exists, namely seminomas (SEs), which are composed of tumor cells that are considered to be the malignant counterpart of human primordial germ cells (PGCs) (1). No animal model for these tumors is known, and they cannot be cultured *in vitro* for a prolonged period. To develop an *in vitro* culture system, we studied the use of Sertoli cell feeders and observed an enhanced survival (2). Because of the number of animals repeatedly needed as Sertoli cell donors, as well as of the heterogeneity of the feeder preparations, we looked for alternatives.

SE cells are indistinguishable from the cells of carcinoma *in situ* (CIS), the precursor of all TGCTs (3), except for their invasive behavior (3,4). Both have morphological and immunohistochemical similarities to PGCs (3,5), and like PGCs most SEs express the stem cell factor (SCF) receptor c-*kit* (6,7). Therefore, we assumed that SE cells share micro-environmental requirements with PGCs. Using a murine embryonal fibroblast (STO) feeder, initial proliferation and survival up to day 6 has been described for murine PGCs isolated from 10.5 days *post coitum* (d.p.c.) embryos (8). The use of soluble or membrane bound SCF, which has an important role in gametogenesis (9), and/or leukemia inhibitory factor (LIF), which maintains the pluripotent phenotype of murine embryonal stem (ES) cells (10,11) and embryonal carcinoma (EC) cells (12), allowed enhanced survival and proliferation (13-16). Further addition of basic fibroblast growth factor (bFGF), probably involved in the regulation of germ cell proliferation (17,18), resulted in long-term proliferation of murine PGCs (19,20). Under the latter conditions, pluripotent embryonal germ (EG)

cells can be derived from 8.5 d.p.c. PGCs (19,20). This is interesting in view of the linear progression model, which assumes the reprogramming of SE cells to pluripotent stem cells, subsequently giving rise to embryonic and/or extra-embryonic tissues in nonseminomatous TGCTs (NSs) (4).

Therefore, we have now studied the survival of cells from 21 primary SEs in co-culture with STO cells. The effect of SCF, LIF and bFGF on the cells from the SE with the longest survival on STO feeder was analyzed.

Materials and methods

Tumor handling

Twenty-one orchidectomy specimens from patients suspected of having a germ cell tumor were collected during surgery in collaborating hospitals. Macroscopically representative parts of the tumor and the adjacent normal parenchyma were partly snap frozen using liquid nitrogen, partly put in medium A (DMEM/F12, with 100 kU/L penicillin, 100 mg/L streptomycin, 40 mg/L gentamycin, 365 mg/L-glutamin, Gibco, Paisley, UK) and taken to the laboratory for further processing. Fresh representative samples of all components were used for imprints and subsequently fixed in 4% formalin (J.T.Baker, Deventer, NL) for paraffin embedding. After imprint and frozen section diagnosis of SE (using a hematoxylin and eosin-stained slide), the tumor was mechanically disaggregated at room temperature, using two crossed scalpel blades. Tissue fragments were allowed to settle in a 50 ml tube in 30 ml of medium A. The supernatant, containing almost only single cells (as analyzed by microscopy), was washed twice with medium A and either directly cultured or cryopreserved. To the cell suspension 10% (final concentration) dimethylsulphoxide (Merck, Darmstadt, FRG), was added slowly. The suspension was aliquotted, automatically frozen in a Kryo 10 Series 2 (Planer Biomed, Sunbury-on-Thames, UK) (-2°C/min to -5°C, -1°C/min to -40°C, -5°C/min to -160°C) and stored under liquid nitrogen.

Tumor characterization

Histological typing of the tumors was performed according to the World Health Organization classification (1,21), aided by immunohistochemistry for the expression of germ cell specific alkaline phosphatase (detected with antibodies to placental alkaline phosphatase (PLAP)), α-fetoprotein (AFP), human chorionic gonadotropin (hCG) (Dako, Glostrup, DK) and cytokeratins 8 and 18 (Becton Dickinson, San José, USA) on representative paraffin sections, while c-*kit* expression was immunohistochemically detected on frozen sections. All stainings were carried out using an immunoperoxidase technique at room temperature with 3,3' diaminobenzidine tetrahydrochloride (Fluka Chemie, Buchs, CH) visualization, as described previously (22).

Feeders

STO cells were cultured in T25 flasks (Costar, Cambridge, USA) at 37°C in a humid atmosphere with 5% carbon dioxide in air, using 5 ml of medium A containing 10% fetal calf serum (FCS) (Gibco) and subcultured once a week. The feeders were grown in 0.1% gelatin (Sigma, St. Louis, USA) coated six or 12-well tissue culture

plastic plates (Costar) or T25 flasks and at confluence treated with 10 μg/ml mitomycin C (Sigma) for 3 h. After a triple wash with phosphate-buffered saline (PBS) the feeders were kept in medium A containing 10% FCS and SE cells were inoculated on the next day.

Seminoma-STO co-cultures

Single cell suspensions from 21 SEs (coded SE1 to SE21) were seeded onto STO feeder. Two tumors were cultured using the cryopreserved suspension. Nineteen tumors were cultured using fresh cell suspensions, while from nine of these tumors cryopreserved suspensions were also used. Seminoma cells were seeded in either a T25 flask (10^7 cells per flask) or a 6-well plate ($5x10^6$ cells per well), in respectively 5 and 2 ml of medium A with 10% FCS. After overnight incubation at 34°C in a humid atmosphere with 5% carbon dioxide in air, medium was taken off and half a volume of fresh medium was given to the culture. The old medium was spun down at 1000 r.p.m. for 5 min and half the volume was returned to the culture. Subsequently, half of the medium was changed every other day.

Seminoma proliferation in the presence of growth factors

Feeder-containing six-well plates were seeded with $5x10^6$ cryopreserved SE cells per well after rapid thawing in a 37°C water bath (experiments 1, 2 and 3). In experiments 1 and 2 four wells (referred to as wells 1-4) were inoculated for each condition, while in experiment 3 wells were inoculated in duplicate (wells 3 and 4). The wells contained 2 ml of medium A, with or without 10% FCS, with or without 60 ng/ml human recombinant SCF (provided by Amgen, Thousand Oaks, USA) (experiment 2 and 3) or a combination of 60 ng/ml SCF, 10 ng/ml human recombinant LIF (provided by Dr. J.K. Heath, Dept. of Biochemistry, University of Oxford, UK) and 1 ng/ml human recombinant bFGF (Gibco) (experiment 1, 2 and 3) (this combination of growth factors is referred to as SLB). Seminoma cell number and colony size in wells 3 and 4 were counted on days 3 and 9 in experiment 1, on days 1, 3 and 9 in experiment 2 and on days 1, 3 and 6 in experiment 3. Cultures were kept at 34°C in a humid atmosphere with 5% carbon dioxide in air, while half of the medium was changed every day.

On day 1 of culture 10 μM bromodeoxyuridine (BrdU) (Sigma) was added to wells 1 and 2 in experiments 1 and 2. After overnight incubation, cultures were fixed at room temperature in 70% ethanol for 15 min. The bottoms of the wells were cut into two sections using a hot scalpel blade. The sections were immunohistochemically stained either for cytokeratin, using 3,3' diaminobenzidine tetrahydrochloride visualization (22), or for germ cell-specific alkaline phosphatase (using antibodies to PLAP) and BrdU (Organon Teknika, Boxtel, NL), using a double-staining technique according to Hardonk and Harms (23). PLAP was stained using an immunoperoxidase with 3-amino-9-ethyl carbazole (Sigma) visualization, while BrdU was stained with an immuno alkaline phosphatase with fast blue BB salt (Sigma) visualization. This procedure was repeated for wells 3 and 4 (in all three experiments) after incubation with BrdU from day 8 to 9.

For all conditions, the cells present in three visual fields at a 320 x magnification

were counted, using a Zeiss Axiovert phase contrast microscope (Zeiss, Germany), equipped with a Sony charge-coupled device camera and screen (Sony, Japan), to allow evaluation of the observed cells by two individuals.

Statistical analysis

Welch's *t*-statistics (24,25) was used to analyse the influence of serum and growth factors on SE colony number and size. Analysis was done for each culture condition separately, comparing the counts of the fixed time points, *i.e.* days 3 and 9 in experiment 1, days 1, 3 and 9 in experiment 2 and days 1, 3 and 6 in experiment 3. All calculations were performed using Stata software (26).

Results

Tumor characterization

Immunohistochemically, all 21 tumors, which were located in the testis, were negative for AFP, while they showed consistent membranous staining for PLAP and c-KIT. Six tumors were negative for cytokeratin and hCG. In five tumors cytokeratin-expressing cells and in three tumors hCG-positive cells were detected. Seven tumors showed cytokeratin as well as hCG expression.

Seminoma-STO co-cultures

The cells of all tumors, except three (SE1, SE2 and SE3), showed poor attachment to STO feeder, and only few cells survived through day 10 (not shown). While fresh and cryopreserved SE2 cells only showed enhanced attachment, fresh SE1 and SE3 cells also survived for over 24 days. Cryopreserved SE1 cells survived for up to 15 days, while no cryopreserved suspensions of SE3 were available. In all SE1 and SE3 cultures initial proliferation was observed (not shown).

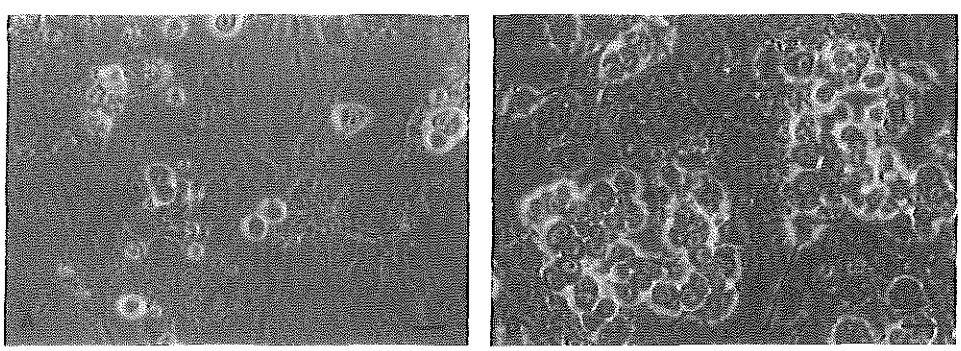

Figure 8.1. *Morphology of SE1 cells on STO feeder on day 9 of culture in medium without fetal calf serum (FCS) and growth factors (A) and in medium with FCS and the combination of stem cell factor, leukemia inhibitory factor and basic fibroblast growth factor (SLB) (B). Only in the presence of FCS and SLB colonies of up to 40 cells were detectable. Scale bar = 30 μm.*

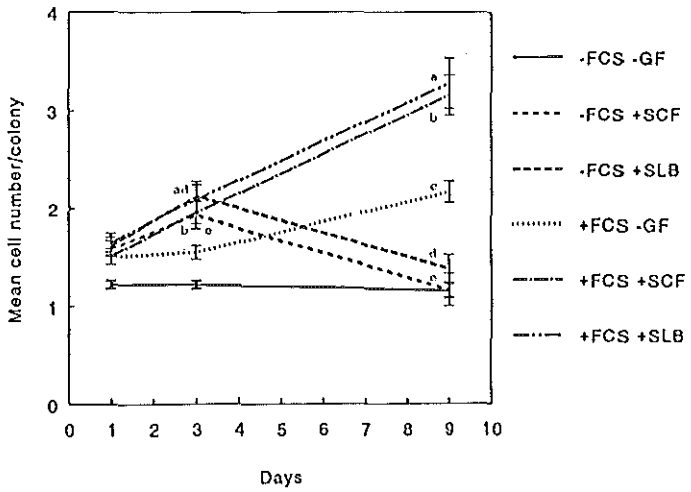

Figure 8.2. *Mean size of SE1 colonies over time under the various culture conditions. Results are from experiment 2 as a representative example. Vertical bars indicate standard errors. The letters a-e indicate a significant ($p<0.01$ in all cases, except for a and e on day 3 with $p<0.05$) increase or decrease in mean colony size as compared to the previous count of the colony size. (FCS, fetal calf serum; GF, growth factor(s); SCF, stem cell factor; SLB, the combination of stem cell factor, leukemia inhibitory factor and basic fibroblast growth factor).*

Seminoma proliferation in the presence of growth factors

Because of the availability of cryopreserved cell suspensions and the better performance in culture of SE1, these cells were used to study the effect of growth factors. Therefore, SE1 cells were cultured on STO feeder in DMEM/F12 with or without FCS, SCF or SLB. All data shown in the figures and presented in the text are from experiment 2; experiments 1 and 3 yielded essentially similar results.

The morphology of SE1 cells on STO feeder, in medium without FCS and growth factors or medium with FCS and SLB at day 9 of culture, is shown in Figure 8.1. In medium without FCS, feeder quality had morphologically declined and colonies were absent, while large colonies were present in medium with FCS. Under FCS-free conditions, SE cells were found on top of the STO cells, while they seemed to sink into the feeder layer when exposed to FCS-containing medium.

The mean colony size of SE1 over time, under the various conditions, is shown in Figure 8.2. The mean increases in the colony number and of the mean colony size for the days 1-3 (first period) and days 3-9 (second) period were calculated. Under FCS-free conditions, without growth factors, no change in total cell number was observed during the first period, while this number decreased during the second period (data not shown). The colony number significantly ($p<0.01$) decreased after day 3 (data not shown). During the entire experiment the mean colony size was constant (1.2 cells per colony). The use of SCF or SLB with FCS-free medium resulted in an increase in total cell number during the first period and a rapid decrease in this number after day 3(data not shown). The colony number was constant until day 3, but decreased significantly ($p<0.01$) during the second period. The mean colony size increased significantly

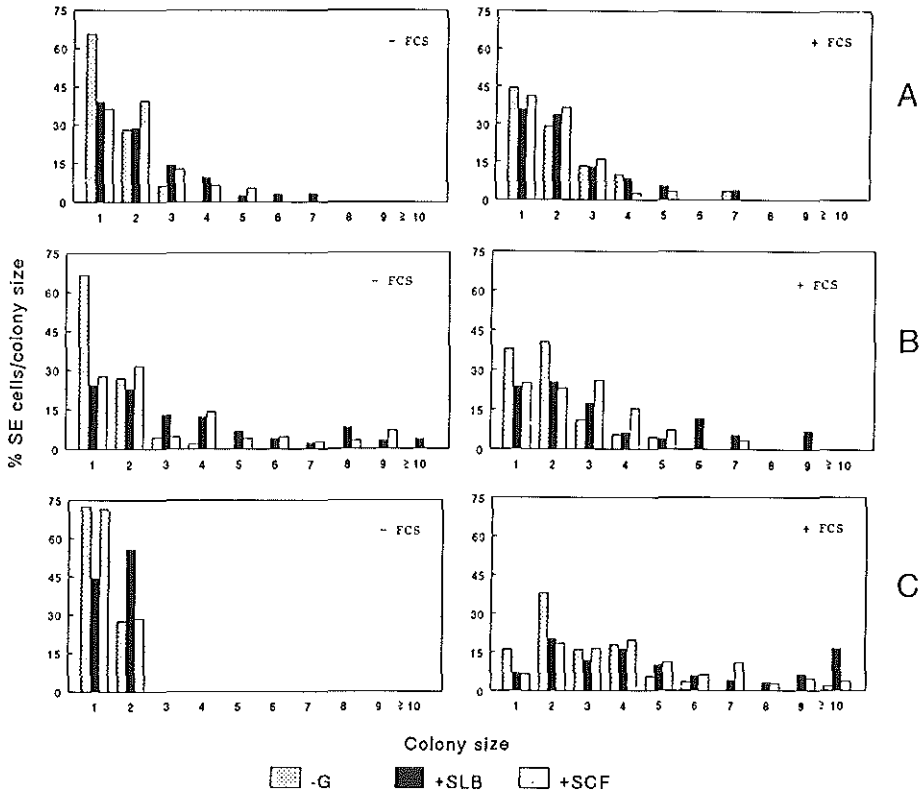

Figure 8.3. *Distribution of SE1 cells over colonies of various sizes at days 1 (A), 3 (B) and 9 (C). (FCS, fetal calf serum; GF, growth factor(s); SCF, stem cell factor; SLB, the combination of stem cell factor, leukemia inhibitory factor and basic fibroblast growth factor).*

($p<0.01$) until day 3 and decreased significantly ($p<0.01$) afterwards. In FCS-free medium no colonies with more than two cells were detected on day 9, irrespective of the presence of growth factors. In FCS-containing medium the colony number was constant throughout the experiment, irrespective of the presence of growth factors. In the absence of growth factors, total cell number (not shown) and mean colony size were constant until day 3 (mean size 1.6 cells per colony). Both increased during the second period. This increase was only significant ($p<0.01$) for the mean colony size (mean size 2.2 cells per colony at day 9). In the presence of SCF or SLB, total cell number (not shown) and colony size significantly ($p<0.05$) increased during the whole culture period. In the presence of SCF, the mean colony size on days 3 and 9 was 2.0 and 3.2 cells per colony, respectively, while in the presence of SLB the mean size was 2.1 and 3.3 cells per colony, respectively. Figure 8.3 illustrates the range of the colony sizes under the various conditions on days 1, 3 and 9. In the absence of FCS and growth factors only colonies of 1 and 2 cells were present at day 9, whereas the use of these additives resulted in the formation of much larger colonies (up to >10 cells per colony) at this time point. Only in the cultures with SLB a few colonies of up

to 40 cells were detected outside the counted fields.

No obvious morphological and immunohistochemical (PLAP and cytokeratin expression) changes were identified during the *in vitro* culture (not shown).

PLAP staining and BrdU incorporation

The counts of PLAP-positive cells confirmed the data obtained with morphological phase-contrast detection of SE1 cells in culture. SE1 cell cultures were incubated with BrdU from day 1 to 2 or from day 8 to 9 to detect DNA synthesis. The percentage of PLAP-positive cells showing BrdU incorporation ranged from 10 to 24% on day 2, while 0.4 to 17% of the cells were labeled at day 9. No differences were found for the various culture conditions. On days 2 and 9, cells with incorporated BrdU were detected in colonies of all sizes.

Discussion

We have previously shown (2) and confirmed in the present study that SE cells cultured without feeder layer (on tissue culture plastic, in DMEM/F12 containing 10% FCS) die within 3 days (data not shown). This was also found for the cells (SE1) with the longest survival on STO feeder, even in the presence of SCF or SLB (not shown). Therefore, we conclude that SE cells need contact with a specific matrix, which might be provided by feeder cells, possibly through interactions of the membrane-bound form of SCF and the receptor c-KIT, in order to survive and proliferate. This is supported by the finding of the same survival of the SE1 cells on STO with or without additives during the first 3 days of culture. Since the results from the present study, are similar to those reported previously on the use of Sertoli cell feeders for SE culture (2), we conclude that the homogeneous STO feeders seem to form a good alternative for the use of Sertoli cell layers.

The more extensively studied tumor SE1 showed an increase in colony size during the first 3 days of culture on STO feeder, using both SCF and SLB, irrespective of the presence of FCS. From days 3 to 9, colony size increased in the presence of FCS alone, or with added SCF or SLB. Probably because of quality decline (morphological changes) of STO in FCS-free medium, the number and colony size of SE1 decreased from day 3 onwards. Therefore, FCS seems to be necessary to directly support the STO cells, while its effect on SE cells seems indirect and through the feeder layer.

During the first 3 days of culture, the colony number of SE1 cells was constant for all conditions. This indicates that proliferation of SE1 cells, for which the presence of SCF was necessary and sufficient, caused the growth of the colonies, instead of clustering of the cells owing to (enhanced) cell motility. From day 3 onwards all cultures containing FCS showed an increase in colony size.

Three SEs (SE1, SE2 and SE3) had a plating efficiency on STO similar to that found for 8.5 d.p.c. murine PGCs (30%) (19), while two of them (SE1 and SE3) initially proliferated, just like 10.5 d.p.c. murine PGCs (8). However, 18 of the 21 SEs studied had a plating efficiency on STO of less than 1%. In spite of survival of the attached cells from these SEs to about day 10, no proliferation was found. Apparently, SEs form a tumor population with a heterogeneous *in vitro* behavior,

differing in attachment to STO feeder cells and subsequent survival and proliferation. The SE cells with an attachment and initial proliferation similar to that of 8.5 d.p.c. murine PGCs showed no reprogramming to pluripotent stem cells under any of the applied conditions, as judged by their unchanged morphology and continued expression of membrane-bound germ cell-specific alkaline phosphatase (detected with antibodies to PLAP). Therefore, we conclude that the differentiation state of SE cells is not similar to that of 8.5-10.5 d.p.c. PGCs. However, this does not exclude a linear progression model for CIS, SE and NS. The differences in the *in vitro* behavior of SEs and murine PGCs might be related to the crucial role of the age (d.p.c.) of the latter in the ability to respond to growth factors; 11.5-12.5 d.p.c. murine PGCs do not proliferate when co-cultured with feeder cells in the presence of SCF or LIF (13,16).

The heterogeneity in the *in vitro* behavior did not correlate with the expression of the markers hCG and/or cytokeratins 8 and 18. The differentiation status of human TGCT cell lines has recently been described to correlate with the expression of distinct glycolipids, among others carrying the stage-specific embryonic antigens 1, 3 and 4 (27). Our preliminary results from an extensive study of the glycolipid profile of primary human TGCTs, especially SEs (28), revealed no distinct differentiation status of SE1, SE2 and SE3 as compared with the other SEs described here. The SCF receptor c-KIT was detected on all SEs.

Recently, we found a possible explanation for the aberrant *in vitro* behavior of SE1, SE2 and SE3. From 40 SEs analyzed, including 17 of the 21 tumors described in this paper, these tumors were the only three containing mutant N- or K-*ras* (29). Interestingly, suppression of apoptosis by mutant *ras* has been reported (30), and we have indications that mechanical dissociation of SE tissue results in apoptosis of the tumor cells (31). These indications are in agreement with the findings by Frisch and Francis (32), who recently reported on the induction, and abrogation by mutant *ras*, of apoptosis by disruption of cell-matrix interactions. Therefore, we conclude that a higher degree of attachment, alone or in combination with enhanced survival and initial proliferation of SE cells *in vitro*, might be related to the presence of mutant *ras*, which possibly interferes with the apoptotic pathway. *Ras* mutations indicate an unfavorable prognosis in childhood acute lymphocytic leukemia (33) and non-small cell lung cancer (34,35), while an enhanced *in vitro* proliferative capacity is reported for adult acute myeloid leukemia with a poor prognosis (36). In view of these and our findings we are currently investigating the prognostic relevance of the *in vitro* behavior and presence of *ras* mutations in SEs.

Acknowledgements

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Chapter 9

APOPTOSIS OF HUMAN SEMINOMA CELLS UPON DISRUPTION OF THEIR MICRO-ENVIRONMENT

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Submitted

Abstract

One of the main obstacles encountered when trying to culture human seminoma cells *in vitro* is massive degeneration of the tumor cells. We investigated whether dissociation of tumor tissue, to obtain single cell suspensions for *in vitro* culture, results in the onset of apoptosis. Using morphological analysis and *in situ* end labeling, less than 4% apoptotic tumor cells were detected in intact tissue from 11 out of 14 seminomas. In these 11 tumors, apoptosis-specific DNA ladders indicative of internucleosomal double strand DNA cleavage, were not detected on electrophoresis gels. In contrast, three seminomas with over 12% apoptotic tumor cells in the intact tissue and all analyzed (pure) seminoma cell suspensions, obtained after mechanical dissociation of intact tumor tissue, showed DNA ladders. Flow cytometric analysis of *in situ* end labeled seminoma suspensions showed DNA breaks in up to 85% of the tumor cells. Indicated by cell morphology and DNA degradation, seminoma cells appear to rapidly enter the apoptotic pathway upon mechanical disruption of their micro-environment. No expression of *p*53 and of the apoptosis-inhibitor *bcl*-2 was detectable in intact seminoma tissue or cell suspensions. Our data suggest that abrogation of apoptosis might be crucial to succeed in culturing human seminoma cells *in vitro*.

Introduction

Besides proliferation and differentiation, apoptosis (programmed cell death) is a main mechanism controling cell fate during embryogenesis, morphogenesis and tissue homeostasis (1-5). Recently, Frisch and Francis (6) reported that epithelial cells undergo apoptosis upon disruption of their interactions with the extracellular matrix, in a process they named anoikis. Apparently, interactions between cells and their matrix, mediated by integrins (the matrix receptors), provide the cells with a survival and/or proliferation signal, which blocks anoikis. Pesce *et al.* (7,8) reported that murine primordial germ cells (PGCs) die apoptotically at extragonadal sites during embryogenesis and during *in vitro* handling upon isolation from the embryo. This *in vitro* apoptosis could be blocked by the presence of specific growth factors, *i.e.* stem cell factor (SCF) or leukemia inhibitory factor (LIF) (7). Thus, besides specific cell-matrix interactions, growth factor-receptor interactions are involved in prevention of apoptosis.

Not only extracellular factors are correlated with induction of apoptosis. Several intracellular factors have also been implicated. BCL-2 (located in the membrane of mitochondria, nucleus and endoplasmatic reticulum (9)), was the first oncogene product reported to interfere with apoptosis, sustaining cell survival without increasing proliferation rates in non-Hodgkin's lymphoma (10-14). BCL-2 has been reported to block apoptosis upon growth factor withdrawal or disruption of cell-matrix interactions (11,15). Nuclear P53, which constitutes a checkpoint for DNA integrity during the cell cycle (16), has recently been implicated in the induction of apoptosis (16-18). Upon DNA damage, *p*53 expression is enhanced and the damaged cell enters a P53-dependent apoptotic pathway. Removal of certain growth factors can also result in the onset of P53-dependent apoptosis (19,20). In several cell types and upon induction by

various stimuli, apoptosis can also proceed in a P53-independent way (21,22).

Primary seminomas (SEs), tumors considered to be the malignant counterparts of PGCs (23-26), occur at specific localizations, *i.e.* in the gonads (27,28), mediastinum (29) and midline of the brain (30). Like PGCs, SE cells express the SCF receptor c-*kit* (31-33). Thus, SE cell survival and proliferation appear to depend upon a very specific micro-environment and growth factor supply. These findings suggest that a lack of apoptosis and a differentiation block could have contributed to tumor formation.

Recently we reported that attempts to culture human SE cells *in vitro* were hampered by massive degeneration of the tumor cells within the first three days of culture (33). We now investigated whether SE cells die apoptotically upon disruption of their micro-environment, prior to *in vitro* culturing. Furthermore, we immunohistochemically analyzed whether SE cells express *bcl*-2, and whether death of the SE cells coincides with enhanced *p*53 expression.

Materials and methods

Tumor handling and characterization

Fourteen fresh orchidectomy specimens, macroscopically identified as SEs, were collected at the operation theater or pathology department of collaborating hospitals. Representative parts of the tumors were snap frozen using liquid nitrogen. The remaining was put into medium A (DMEM/F12, with 103 kU/L penicillin, 103 mg/L streptomycin, 43 mg/L gentamycin, 365 mg/L-glutamin, Gibco, Paisley, UK) and taken to the laboratory for further processing. The SE histology of the tumor was confirmed through microscopic examination of a hematoxylin and eosin stained 5 μm frozen tissue section. Representative samples were fixed in 4% formalin (J.T.Baker, Deventer, NL) for paraffin embedding. Subsequently, the tumors were conclusively diagnosed according to the classification system of the World Health Organization (34-36), using immunohistochemistry for germ cell-specific alkaline phosphatase (detected with antibodies to placental alkaline phosphatase), α-feto protein, human chorionic gonadotropin (Dako, Glostrup, DK) and cytokeratins 8 and 18 (Becton Dickinson, San José, USA) on representative paraffin sections, as described (37). In addition, frozen sections from all SEs were aceton fixed for 10 min and screened for *bcl*-2 expression, using the 100α antibody (1:20) (provided by Drs. F. Pezzella and D.Y. Mason) and the streptavidin-biotin detection method. Expression of *p*53 in aceton-fixed frozen tissue sections and in cytospins of 1% formalin-fixed cell suspensions (see below) was examined using the DO-7 antibody (1:75) (Dako) and a two-step detection method (37).

Fresh tumor tissue was mechanically dissociated in a suitable volume of medium at room temperature using two crossed scalpel blades. Tissue fragments were allowed to settle in a 15 or 50 ml tube containing 10 or 30 ml medium, respectively. The supernatant contained almost only single cells, as analyzed by phase contrast microscopy using a Zeiss Axiovert microscope. From the supernatant a volume containing at least 2 x 10^6 cells was fixed at 0°C in 5 ml 1% formalin (in phospate buffered saline, PBS) for 30 min. Cells were spun down at 1500 rpm for 5 min and resuspended in 200 μl ice-cold PBS. Subsequently, the suspension was mixed with

400 µl ice-cold 96% ethanol. Samples were stored at -20°C until further processing for flow cytometry (FCM). To the remaining fresh single cell suspension 10% (final concentration) demethylsulfoxide (Merck, Darmstadt, FRG), was added slowly. The suspension was aliquotted, automatically frozen in a Kryo 10 Series 2 (Planer Biomed, Sunbury-on-Thames, UK) (-2°C/min to -5°C, -1°C/min to -40°C, -5°C/min to -160°C) and stored under liquid nitrogen.

Lymphocyte depletion

Cryopreserved single cell suspensions from 12 SEs, containing SE cells and lymphocytes, were rapidly thawed at 37°C, washed in 10 ml culture medium, and counted. The suspensions were treated with a 2.5 fold excess (relative to the total number of SE cells and lymphocytes) of magnetic beads coated with anti-CD2 monoclonal antibody (Dynal, Skoyen, N) to deplete lymphocytes. After 15-20 min incubation at room temperature with gentle shaking, 4 ml of culture medium was added, and the beads were removed using a magnetic particle collector (Dynal). The supernatant, containing enriched SE cells was removed. The beads were washed twice with culture medium and all supernatants were pooled. Removal of the lymphocytes was verified by microscopic examination of a cytospin preparation with hematoxylin and eosin staining. After treatment with magnetic beads, all suspensions contained less than 15% lymphocytes. Similar packed cell volumes from untreated or beads-treated samples were used for DNA extraction.

Detection of DNA breaks

One of the early events during apoptosis is single or double strand DNA cleavage by endogenous endonuclease activity (38). DNA breaks in tissue sections can be visualized using *in situ* end labeling (ISEL) (39), while end labeling in combination with FCM can be applied to analyse cell suspensions (40). The occurrence of characteristic internucleosomal breaks is confirmed by the detection in cell lysates of 200 bp DNA fragments, and multimers of that, on electrophoresis gels (38). Morphological hallmarks of apoptosis are condensation of chromatin and cytoplasm, and cell fragmentation (41-43).

In situ end labeling

For ISEL (39), 2 µm paraffine sections were deparaffinated, rehydrated and incubated at 80°C, in 2xSSC (0.3 M NaCl, 30 mM sodium citrate) for 20 min. After a triple aqua bidest wash, the slides were treated with 20 mg/L pronase E (Sigma, St. Louis, USA) in PBS at room temperature for 30 min, rinsed with running tapwater, incubated in buffer B (50 mM Tris, 5 mM $MgCl_2$, 10 mM ß-mercapto-ethanol, 0.005% BSA, pH7.5), dehydrated using 50, 70 and 100% ethanol, and airdried. Positive controls were incubated with 200 µg/L DNase I (Boehringer, Mannheim, Germany) in buffer C (10 mM Tris pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 25 mM KCl) at 37°C for 15 min, and washed with buffer B. Subsequently, all slides were incubated at 15°C for 1 hr in buffer B containing dATP, dCTP, dGTP, biotin-16-dUTP (0.01 mM each) (Boehringer) and 20 kU/L DNA polymerase I (Promega, Madison, USA). Polymerase was not added to negativecontrols. After PBS washes, endogenous

peroxidase activity was blocked using 0.1% H_2O_2/PBS and, after PBS washes, slides were incubated with avidine labeled horseradish peroxidase (1:1000) (Sigma) in 1% BSA/0.5% Tween 20/PBS. Subsequently, slides were PBS washed and the immunoreaction was visualized using 3,3'-diaminobenzidine tetrahydrocloride (Fluka Chemie, Buchs, Switzerland)/H_2O_2. After rinsing with tapwater, slides were counterstained for 5 sec with 1% methyl green (Merck, Darmstadt, Germany). Slides were rinsed with aqua bidest and, after removal of excess bidest using filterpaper, with aceton. These washes were repeated once. Slides were dipped in two batches of aceton/xylol (1:1), for 2 sec/batch, cleared in xylene and embedded in Pertex (Histolab Products AB, Västra Frölunda, Sweden). The percentage of apoptotic cells was scored by counting a total of 150-580 viable, or morphologically apoptotic and labeled SE cells in five representative low power microscopic fields at a 400x magnification.

Flow cytometry

For FCM (40), fixed cells were washed in PBS and resuspended in buffer D (50 mM Tris pH 7.8, 5 mM $MgCl_2$, 10 mM ß-mercapto-ethanol, 1kU/L DNA polymerase I, 0.2 mM dATP, dCTP, dGTP and biotin-11-dUTP). After incubation at 15°C for 90 min the cells were washed with 0.1% Triton-X-100/PBS, and resuspended in 4xSSC (0.6 M NaCl, 60 mM sodium citrate) containing 2.5 mg/L avidin-fluorescein isotiocyanate (FITC) (Vector Laboratories, Burlingame, USA), 0.1% Triton-X-100 and 5% (w/v) nonfat dry milk. Staining was performed at 37°C for 30 min. Subsequently, the cells were washed in PBS. DNA was counterstained with 5 mg/L propidium iodide (PI) (Calbiochem, La Jolla, USA) or 1 mg/L 4',6-diamidine-2'-phenylindole (DAPI) (Calbiochem) in PBS at 4°C for 30 min. Flow cytometry was performed on a FACSCAN (PI stained samples) or VANTAGE (DAPI stained samples) flow cytometer (Becton Dickinson) with excitation at 488 nm or 351/364 nm, respectively. The following parameters were measured: forward light scatter, perpendicular light scatter, FITC fluorescence (515-545 nm), and fluorescence of the DNA-PI complex (563-607 nm) or DNA-DAPI complex (488 nm). Cell debris was excluded from analysis by appropriate forward light scatter treshold setting.

Detection of DNA ladders

DNA was isolated (44) from: 1) three 10 μm slides from snap frozen SE blocks; 2) snap frozen pellets from fresh SE cell suspensions; 3) snap frozen pellets from either lymphocytes-containing or -depleted SE cell suspensions, that had previously been cryopreserved. Cells were lysed in 400 μl buffer (10 mM Tris, 400 mM NaCl, 2 mM EDTA, 100 μg/ml proteinase K (Boehringer)), overnight at 37°C. The lysate was extracted with 500 μl phenol/chloroform (1:1) and subsequently with chloroform/isoamylalcohol (24:1). DNA was precipitated by addition of 50 μl 3M sodium acetate and 800 μl 100% ethanol, and overnight incubation at -20°C. The pellet was spun down, washed with 70% ethanol, vacuum dried, dissolved in 100 μl TE (10 mM Tris, 0.1 mM EDTA) with 50 mg/L RNase A (Sigma) and incubated at 37°C for 30 min. Subsequently, 20 μl solution was subjected to electrophoresis in a 1.8% agarose gel at 60 V for 2-3 hr.

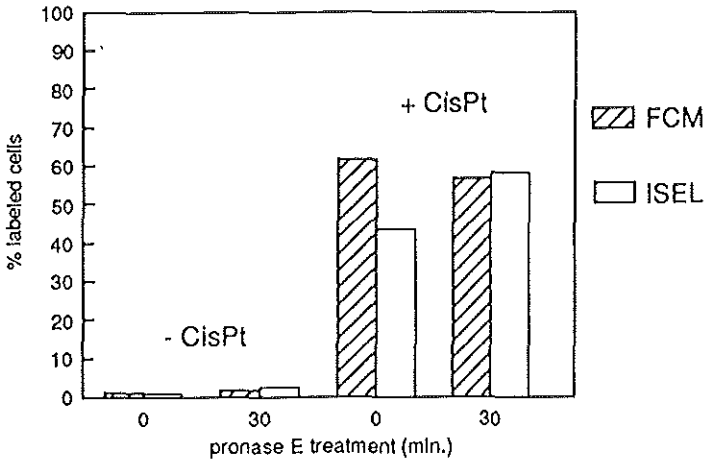

Figure 9.1. *Detection of apoptotic CHO cells after incubation without or with cisplatin, indicated as - CisPt and + CisPt, repectively. Labeled cells were detected using flow cytometry (FCM) or* in situ *end labeling (ISEL), without (0 min) or with (30 min) pronase E treatment.*

Results

Prior to analysis of the tumor samples, it was ensured that results obtained with ISEL and FCM were comparable. Therefore, apoptosis was induced in chinese hamster ovary (CHO) cell cultures by cisplatin treatment. In a DMEM + 10% FCS (Gibco) containing T75 flask, $2x10^6$ CHO cells were seeded. Upon attachment, cells were incubated with 21 μM cisplatin (Bristol-Myers Squibb, Woerden, the Netherlands) for two hours. After washes with medium, the cells were incubated for 48 hr. Floating cells were harvested by centrifugation of the culture medium at 1000 rpm for 5 min. After addition of 10 ml fresh culture medium, attached cells were harvested from the flasks using cell scrapers and spun down. Cells from untreated cultures, harvested by scraping, were used as negative control. All samples were split into two fractions; one was fixed in 4% formalin at room temperature for 1 hr and paraffin embedded (for ISEL), the other was fixed in 1% formalin and stored under ethanol (for FCM). Prior to ISEL or FCM, the samples were pronase E treated for 0 or 30 min at room temperature. Upon ISEL, the percentage of morphologically apoptotic and labeled cells was determined by counting a total of at least 100 cells. In the negative controls a low percentage of labeled cells was detected (Fig. 9.1). Without pronase treatment, ISEL and FCM detected 0.5% and 0.8% labeled cells, respectively. These percentages increased to 2% for ISEL and 1.3% for FCM when pronase was used prior to labeling. In the samples of floating cells from treated cultures, application of pronase did not markedly affect the percentage of labeled cells detected with FCM (61% without, 57% with pronase treatment, respectively). However, pronase treatment of paraffin sections of these cells was necessary to avoid underestimation of the number of apoptotic cells. With pronase treatment, 58% of the CHO cells was found to be apoptotic, *i.e.* comparable to the FCM results, while this number was only 43%

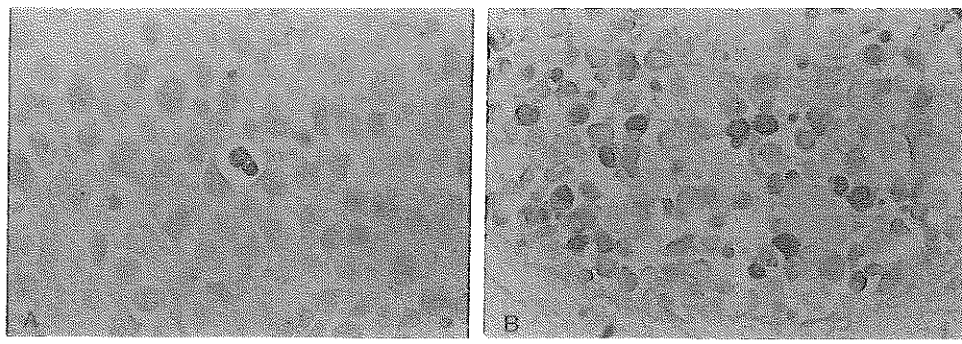

Figure 9.2. *A representative example of the* in situ *end labeling of a seminoma with less than 4% apoptotic cells (TL229) (A) and of a seminoma with 20% apoptotic tumor cells (TL1049) (B).*

without pronase (Fig. 9.1). Probably, pronase treatment is necessary to provide full access of DNA polymerase to paraffin embedded cells. The ISEL and FCM results were confirmed by the presence of DNA ladders only in the cisplatin-treated cultures (not shown).

Based on the above results, paraffin embedded SE tissue blocks were analyzed using 30 min of pronase treatment at room temperature and ISEL, while SE cell suspensions were not pronase treated and analyzed by FCM. In paraffin embedded intact tissue from 11 out of 14 SEs less than 4% of the tumor cells had morphological characteristics of apoptosis and DNA strand breaks (Fig. 9.2A). In three SEs this number was higher, *i.e.* 20%, 13%, and 15%, respectively (Fig. 9.2B). Apoptosis-specific DNA ladders, indicating internucleosomal double strand DNA cleavage, were not detected in intact tissue of the SEs with less than 4% apoptotic cells (Fig. 9.3A). In contrast, these ladders were present in the three SEs with upto 20% apoptotic cells (not shown). All lymphocytes-depleted SE cell suspensions obtained after mechanical dissociation showed ladder patterns; for tumors TL614 and TL4942 the ladders had an increased intensity as compared to the ladders of not-depleted suspensions (Fig. 9.3B). In not-lymphocyte-depleted cell suspensions up to 85% of the SE cells contained nicked DNA, as detected with FCM (Fig. 9.4). In the intact tissues of tumors TL614 and TL4942 a relatively high amount of lymphocytes was present. The cell suspensions of these tumors contained very few SE cells, either viable or apoptotic. In Table 9.1 the results on all SE tissue blocks and cell suspensions are summarized.

Two tumors (TL6209 and TL6329) underwent additional analysis. From each tumor, three pairs of tissue blocks of approx. 0.125 cm^3 were incubated at 4°C and three pairs at 34°C, in medium A. At both temperatures, the pairs were incubated for 1, 4, or 16 hr, respectively. From each pair, one block was fixed in 4% formalin for paraffin embedding, while the other was snap frozen in liquid nitrogen for DNA

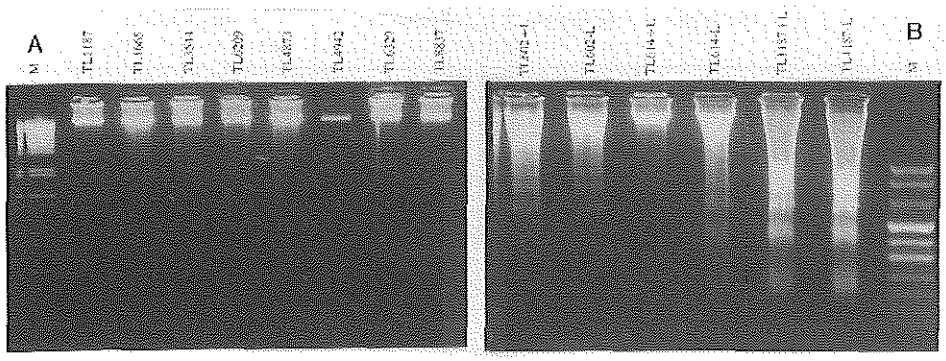

Figure 9.3. *In intact tissue from 11 out of 14 seminomas no DNA ladders were detectable upon electrophoresis, as shown here for eight tumors (A). In single cell suspensions, obtained upon mechanical dissociation of tumor tissue, DNA ladders were detectable, as shown here for three tumors. The ladders were sometimes more intense when lymphocytes were depleted from the suspensions (B). -L, depleted of lymphocytes; +L, not depleted of lymphocytes; M, marker, in A:* PstI*-digested phage lambda DNA, in B: marker 8 (Boehringer).*

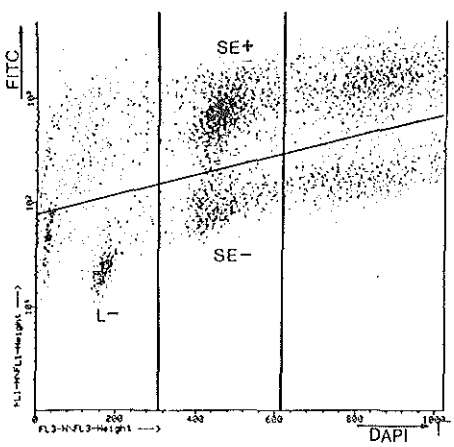

Figure 9.4. *Representative example of the flow cytometric analysis of end labeled seminoma cell suspensions without lymphocyte depletion, obtained upon mechanical dissociation of tumor tissue (TL3544). SE+, apoptotic seminoma cells; SE-, intact seminoma cells; L-, intact lymphocytes; FITC, fluorescence signal indicating labeling of DNA strand breaks; DAPI, fluorescence signal indicating cellular DNA content.*

Table 9.1. Apoptosis in seminoma tissues and cell suspensions.

Tumor	ISEL % apopt	FCM % apopt	DNA Ladder		
			Tissue	Suspension SE+L	SE-L
TL229	1	68	-	+/-	+/-
TL602	2	71	-	+	+
TL614	2	17	-	-	+
TL1049	20	85	+/-	+	+
TL1187	0	79	-	+	+
TL1665	0	80	-	+/-	+/-
TL2207	13	68	+/-	+	+
TL3544	4	45	-	+	+
TL4873	2	75	-	+	+
TL4942	2	5	-	-	+/-
TL6209	1	82	-	NA	NA
TL6329	2	85	-	NA	NA
TL8114	15	NA	+	+	+
TL8837	0	NA	-	-	NA

Intact seminoma tissue blocks were analyzed by in situ *end labeling (ISEL) of single strand DNA breaks. Flow cytometry (FCM) was applied to analyse the percentage of apoptotic seminoma cells in cell suspensions. Lysates from frozen sections of intact tissue and cell suspensions, either depleted of lymphocytes (SE-L) or not (SE+L), were analyzed for the presence of DNA ladders. -, absent; +, present; +/-, weakly present; NA, no sample available for analysis.*

analysis. Figure 9.5 and Table 9.2 show that in both tumors the apoptotic process was slowed down by keeping the micro-environment intact, while incubation of the cells at 4°C resulted in a further delay in the onset of apoptosis. Whereas mechanical dissociation of tumor tissue resulted in immediate apoptosis of up to 85% of the cells, incubation of tissue blocks at 4°C for 4 hr resulted in apoptosis of up to 16% of the SE cells.

Expression of *bcl*-2 could be immunohistochemically detected in infiltrating lymphocytes but was absent from the SE cells in all of the analyzed tumors (not

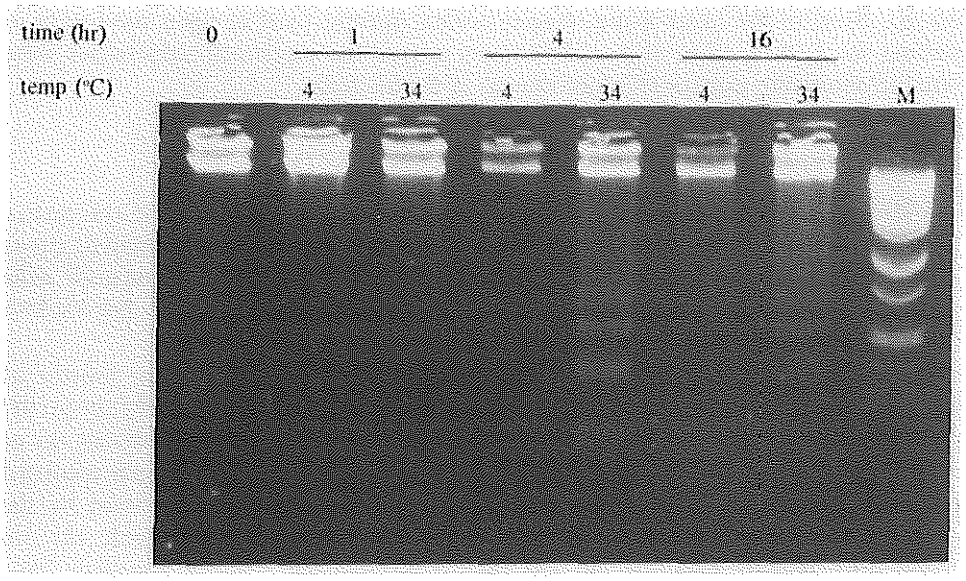

Figure 9.5. *DNA ladders in cultured intact tissue of seminoma TL6209. Tissue was incubated for 1, 4, or 16 hr at 4 or 34°C in medium A. M, marker,* PstI*-digested phage lambda DNA.*

Table 9.2. Percentage of morphologically apoptotic and *in situ* end labeled seminoma cells in cultured intact tissue.

Incubation Time (hr)	TL 6209 4°C	TL 6209 34°C	TL 6329 4°C	TL 6329 34°C
0	1	1	2	2
1	23	10	8	8
4	16	18	5	30
16	17	41	5	100

Intact seminoma tissue blocks (0.125 cm^3) were incubated in vitro, for 1, 4, or 16 hr at 4 or 34°C in medium A. The percentage of apoptotic cells was determined by counting a total of at least 250 viable or morphologically apoptotic, labeled seminoma cells.

shown). None of the intact tissue samples (either directly fixed or upon incubation in medium for 1, 4 or 16 hr at 4 or 34°C), nor any of the single cell suspensions were found to detectably express *p*53 (not shown) in the SE cells.

Discussion

Apoptosis can be induced by various agents, including disruption of cell-matrix interactions (6), growth factor withdrawal (1), oxidative stress (45) and cytotoxic drugs (46). We have now shown that SE cell suspensions contain up to 85% apoptotic cells immediately after disruption of the cellular micro-environment, while very few (<3%) apoptotic cells are present in intact SE tissues.

Several lines of evidence suggest that the *ras* oncogene can inhibit the process of apoptosis (6,46,47). Frisch and Francis (6) reported that cells are (partially) protected against anoikis (apoptosis upon disruption of cell-matrix interactions) by mutant *ras* or overexpression of *bcl*-2. Schlaepfer *et al.* (48) have shown that the integrin-mediated anoikis-suppressing activity of the extracellular matrix is most likely passed by the RAS pathway. Using another approach, we recently found that oncogenic *ras* can inhibit drug-induced apoptosis, in cells transfected with the c-Ha-*ras* oncogene (46). Our observation that SEs bearing a mutant *ras* showed enhanced survival and proliferation in co-cultures with embryonal fibroblast feeder layers, as compared to SEs with wild type *ras* (33,47), could be based on the apoptosis/anoikis-abrogating activity of mutant *ras*.. However, the four *ras* mutant tumors among the SEs analyzed here (TL614, TL1049, TL3544, TL8837) (33,47), were indistinguishable from the non-mutant tumors in the performed assays. The exact relation between the onset of apoptosis upon tumor dissociation, the presence of a mutant *ras* gene and *in vitro* behavior is subject of further study.

Apoptosis of cultured murine PGCs, which appear to need a specific micro-environment for both *in vivo* and *in vitro* survival and proliferation, can be suppressed by SCF (7). We previously reported that the addition of SCF to cultures of SE cells (with an activated *ras* gene) resulted in colony formation (33). In analogy with the findings on murine PGCs (49,50) this was probably due to abrogation of apoptosis (*i.e.* extension of cell survival) and prolonged proliferation, without an increase in proliferation rate.

We suggest that in our experiments, SE cells rapidly entered the apoptotic pathway upon mechanical disruption of their micro-environment and/or deprivation of cell-matrix interactions and growth factors. Preliminary tissue culture results indicate that in an environment with intact cell-matrix interactions apoptosis of SE cells is delayed.

The analyzed apoptotic process in SE cells appears to be independent of (enhanced) *p*53 expression, which could not be immunohistochemically detected. In addition, the absence of *bcl*-2 expression is in concordance with the (high) susceptibility of SE cells to apoptosis. Future analysis of the expression of *bcl*-2 family members in SE cells should yield more information on the control of apoptosis in these cells.

Blocking the onset of apoptosis appears crucial for succesfull *in vitro* culture of SE cells. Once apoptosis can be abrogated, the pathobiological relation between the two histological types of human primary testicular germ cell tumors of adolescents adults, namely SEs and nonseminomatous testicular germ cell tumors (for which *in vitro*

culture conditions and cell lines are available) might be studied *in vitro*.

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Chapter 10

N- AND K-*RAS* MUTATIONS IN PRIMARY TESTICULAR GERM CELL TUMORS: INCIDENCE AND POSSIBLE BIOLOGICAL IMPLICATIONS

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Abstract

Recently, conflicting results have been reported on the incidence of *ras* mutations in primary testicular germ cell tumors of adolescents and adults (TGCTs). In four studies a low incidence of mutations (less than 15%) in a variety of TGCTs or derived cell lines was found, whereas in two other studies a high incidence of N- or K-*ras* mutations (over 40%) was shown. A total of 62 testicular seminomas and 34 nonseminomatous TGCTs were studied thus far. The largest series consisted of 42 TGCTs, studied on paraffin embedded tissue. We present the results of analysis for the 2presence of N- and K-*ras* mutations, in codons 12, 13 and 61, in snap frozen samples of 100 primary TGCTs, comprising 40 seminomas and 60 nonseminomatous TGCTs.

Using the polymerase chain reaction and allele specific oligonucleotide hybridization, mutations were found in five SEs (three in N-*ras* and two in K-*ras*, all codon 12), and in one NS (K-*ras*, codon 12). To exclude underestimation of the incidence of *ras* mutations in TGCTs due to the presence of an excess of wild type alleles in the analyzed sample, a polymerase chain reaction technique preferentially amplifying K-*ras* alleles with a mutation in codon 12 was applied to all seminomas. This approach, allowing a 250 times more sensitive assay, resulted in the detection of only one additional seminoma with a mutation.

Based on critical analysis of published data and on our results from the largest series of frozen samples investigated thus far, we conclude that N- or K-*ras* mutations are rare and apparently not essential for initiation or progression of TGCTs.

Introduction

The p21 proteins encoded by N-, H- and K-*ras* are involved in signal transduction from activated receptors in the cellular membrane to the nucleus. Mutations in these genes, particularly those in codons 12, 13 and 61, cause a decrease in intrinsic guanosine triphosphatase activity, leading to constitutive activation of the encoded protein (1,2). *Ras* mutations have been detected in a wide variety of human neoplasms, although with varying incidence (2), and are considered to represent only one of many events in the multistep process of transformation (3).

A low incidence of K-*ras* codon 12 mutations (less than 15%) has been found in all studies on human primary testicular germ cell tumors of adolescents and adults (TGCTs) (4-7). In contrast, conflicting data have been reported for N-*ras*. One study showed mutations at codon 12 or 61 in 13 of 20 seminomas (SEs) and two of three nonseminomatous TGCTs (NSs) (5). Another study, comprising only SEs, revealed N-*ras* codon 61 mutations in four of 14 tumors (4). No mutations were found in N-*ras* codons 12 or 61 in two studies, comprising 28 SEs and 31 NSs (6,7). A consistent finding is the absence of mutations in N-*ras* codon 13; H-*ras* codon 12, 13, and 61; and K-*ras* codons 13 and 61 in TGCTs and derived cell lines (4-9).

Several factors may explain the varying findings on the incidence of *ras* mutations in TGCTs. Besides possible geographical influences (2), technical factors related to the method of fixation of the tissue samples, frozen (4,5,7) versus paraffin embedded (6), might be involved. In addition, the applied polymerase chain reaction and allele

specific oligonucleotide hybridization (PCR/ASO) might underestimate the incidence of mutations when an excess of wild type alleles is present, since it allows detection of one mutant among 40 wild type alleles (10). This could especially be important for SEs, which may contain a high percentage (over 80%) of nonmalignant cells (4,11). To overcome this problem, Mulder *et al.* (4) used suspensions of SE nuclei sorted on the basis of their aneuploid DNA content. An alternative is the use of a PCR technique which preferentially amplifies mutant alleles (mutant-enriched PCR, MEPCR). It has been shown by Kahn *et al.* (12) that this approach allows detection of one mutant among 10,000 wild type alleles using ASO (MEPCR/ASO).

We have studied snap frozen samples of 100 primary TGCTs, comprising 40 SEs and 60 NSs, using PCR/ASO for detection of N- and K-*ras* codons 12, 13, and 61 mutations. In addition, MEPCR/ASO for K-*ras* codon 12 was used on all SEs.

Materials and methods

Tumor samples

One hundred primary TGCTs and adjacent parenchyma (when present) were collected at the operation theatres of collaborating hospitals. After frozen section diagnosis (using a hematoxylin and eosin-stained tissue section), representative parts of tumor and adjacent parenchyma were snap frozen in liquid nitrogen. Typing according to the World Health Organization classification (11) was based on histology and immunohistochemical analysis of expression of germ cell specific alkaline phosphatase (detected with antibodies to placental alkaline phosphatase, PLAP), α-fetoprotein (AFP), human chorionic gonadotropin (hCG) (Dako, Glostrup, DK) and cytokeratins 8 and 18 (Beckton Dickinson, San Jose, USA) using representative paraffin and frozen tissue sections, as described (13).

DNA extraction

DNA was extracted from the snap frozen tumor samples, as described (14). From each sample three 10 μm thick sections were collected in 100 μl lysis buffer (10 mM Tris, 400 mM NaCl, 2 mM EDTA, 1% SDS, 10 μg/ml proteinase K (Sigma, St. Louis, MO), pH 8.2) and lysed overnight at 37°C. Subsequently the samples were extracted twice with an equal volume of phenol/chloroform. After precipitation in ethanol/sodium acetic acid, the DNA pellet was dissolved in 10 mM Tris, 0.1 mM EDTA to a final concentration of approximately 0.5 μg/μl. From each sample 0.5 μl (approx. 250 ng DNA) was used for PCR. The agarose gel electrophoresis performed to confirm the presence of PCR products, as described in the next section, was also used to confirm the presence of comparable amounts of DNA in each PCR. Matched, carcinoma *in situ*-containing parenchyma of tumors with a *ras* mutation was also studied, when available (n = 4).

Polymerase chain reaction

Amplification was performed in a reaction mixture which consisted of buffer (50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 0.01 mM EDTA, 0.5 mM DTT, 0.1% Triton X-100) (Promega, Madison, WI), 0.2 mM of each

nucleotide (dATP, dTTP, dCTP, dGTP) (Pharmacia, Woerden, the Netherlands), 1 U Taq polymerase (Promega), 0.5 μM of both sense and anti-sense primer in a total volume of 50 μl. Four different primer sets, described by Verlaan-de Vries *et al.* (15), were used to amplify the K-*ras* 12 and 13, K-*ras* 61, N-*ras* 12 and 13, and N-*ras* 61 regions. The reaction mixture was covered with paraffin oil. Samples were placed in a Thermal Cycler (Perkin-Elmer, Norwalk, VA) and heated to 94°C for 1 min. The PCR included 35 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec, and extension at 72°C for 1 min. Finally, the samples were kept at 72°C for 2 min and subsequently stored at 4°C till further analysis. All samples were tested for the presence (or absence, in the negative control, containing H_2O instead of DNA) of the desired fragment by agarose gel electrophoresis and ethidium-bromide staining, using 4 μl of the PCR mixture.

*Mutant-enriched polymerase chain reaction for K-*ras *codon 12*

Amplification was performed in a reaction mixture which consisted of buffer (3.5 mM $MgCl_2$, 15 mM $(NH_4)_2SO_4$, 60 mM Tris-HCl pH8.4), 0.2 mM of each nucleotide (dATP, dTTP, dCTP, dGTP), 1.5 U Taq polymerase, 1 μM of both sense and anti-sense primer in a final volume of 50 μl. Using a mismatched sense primer (introducing a *Bst*N1 restriction site in the wild type allele) together with an antisense primer (12), K-*ras* 12 regions were amplified. The samples were heated to 96°C for 5 min, followed by one cycle at 96°C for 2 min, 55°C for 1 min, and 73°C for 1 min. During 24 cycles, the samples were brought to 96°C for 30 sec, 55°C for 30 sec, and to 73°C for 1 min. Each sample (12.5 μl) was then digested overnight at 37°C to completion with 5 U endonuclease *Mva*1, an isoschizomer for *Bst*N1 (Boehringer, Mannheim, Germany) in 26 μl buffer H (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithioerythritol) (Boehringer). In a second round of PCR, under the same conditions as described above, except for the use of the mismatched sense primer and a nested antisense primer, only the uncleaved (mutated) fragments were amplified. To show the effect of mutant-enrichment the second PCR was also applied to undigested amplification product of the first PCR.

Allele specific oligonucleotide hybridization

Paraffin oil was removed from the PCR-samples by extraction with diethyl ether. DNA was denatured at 96°C for 3 min and 1 μl of PCR mixture from each sample was dotted on a dry Nytran-N membrane (Schleicher & Schuell, Dassel, Germany), which had previously been soaked in H_2O and 10xSSC, and air-dried. DNA from previously characterized mutation-carrying samples was used as positive control. These controls were available for all K-*ras* 12 positions (16) and for K-*ras* 13 position 1 and K-*ras* 61 position 3 (17). As negative control a sample of the PCR mixture without DNA was used. DNA was crosslinked to the membrane by exposure to UV light for 2 min.

The N- and K-*ras* 12 and 13 wild type alleles were detected by specific wild type probes, and identification of K-*ras* 12 mutations was performed using 6 specific probes after conventional PCR, and 6 after mutant-enriched PCR (15,18). Two mixtures of three probes (codon positions 1 and 2) were used for K-*ras* 13 and N-*ras* 12 and 13, while for K-*ras* 61 and N-*ras* 61 three mixtures of three mutation specific probes (for

positions 1, 2, or 3 of the codons) were applied (15). Five picomoles (1 μl) of probe was endlabeled using 2.5 U T4 polynucleotide kinase (Gibco BRL, Breda, the Netherlands) in 4 μl reaction mix (50 mM Tris.HCl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM EDTA, 0.1 mM spermidine, 10 μCi (1 μl) ^{32}P γ-ATP (Amersham, Den Bosch, the Netherlands)). After incubation at 37°C for 30 min, 70 μl of 10 mM Tris/0.1 mM EDTA and 1 μl tRNA were added and the mix was centrifuged through a Clontech-100 column (Clontech Laboratories, Palo Alto, CA).

All blots were soaked in aqua dest in hybridization tubes. The aqua dest was replaced by 2 ml hybridization mix (3 M tetramethylammonium chloride, 50 mM Tris pH 7.5, 5 mM EDTA, 1 % SDS, 1 % (w/v) milk protein (Protifar) (Nutricia, Zoetermeer, the Netherlands). After prehybridization for 60 min at 56°C, 20 μl labeled probe was added and hybridization was done for 1 hr at 56°C. Blots were washed in hybridization mix without milk protein at 56°C for 30 min, taken from the tubes, rinsed with 2xSSC (0.3 M NaCl, 0.03 M sodiumcitrate, pH 7.0), dried, and exposed overnight to an Xomat^{tm} film (Eastman Kodak, Rochester, NY) at -70°C. Subsequently, blots were washed at higher stringency in 5xSSPE (50 mM sodium phosphate, 0.9 M NaCl, 5 mM EDTA)/0.1 % SDS, for 30 min, to remove any probe mismatched to the PCR product. In the PCR/ASO, temperature was kept at 57°C for N-*ras* 61, 59°C for K-*ras* 61, 61°C for N-*ras* 12 and 13, and 63°C for K-*ras* 12 and 13, while the temperature was 61°C in the MEPCR/ASO for K-*ras* 12. The blots were again rinsed with 2xSSC, dried, and exposed for 1 to 4 days, as described. Mutations could be detected comparing the dot signals obtained before (to verify the presence of PCR products) and after the stringent wash.

Identification of mutations

The exact nature of the N-*ras* 12 mutations was revealed by cycle sequencing (Cycle Sequencing Kit, Perkin-Elmer Cetus, Norwalk, CT) using a ^{32}P-labeled sense primer. In brief, the tumor samples were amplified in triplicate using conventional PCR. The PCR products were pooled and subjected to electrophoresis on a 2.5% preparative agarose gel. The fragments were excised from the gel and incubated with 6 U agarase (Boehringer) for 1 hr. at 45°C. The purified DNA fragments were ethanol precipitated at -70°C for 15 min and dissolved in 25 μl of sterile water. Twenty cycles of denaturation at 95°C (1 min), annealing at 63°C (1.5 min), and extension at 72°C (2 min) were performed in a thermal cycler (Hybaid^{TM}, Teddington, United Kingdom). The sequencing reactions were analyzed on a denaturing 6% polyacrylamide sequencing gel at 60W for 2.5 hr. The gel was exposed at -70°C for 16 hr, as described above, with an intensifying screen.

Results

Classification of the obtained tumors revealed 40 SEs and 60 NSs. The group of NSs comprised 22 pure tumors (12 embryonal carcinomas (ECs), four yolk sac tumors (YSs), five teratomas (T) (both mature and immature) and one choriocarcinoma (CH)), 35 tumors containing EC mixed with one or more other components (CH, SE, T, YS), two tumors containing CH and T, and one tumor containing T and YS.

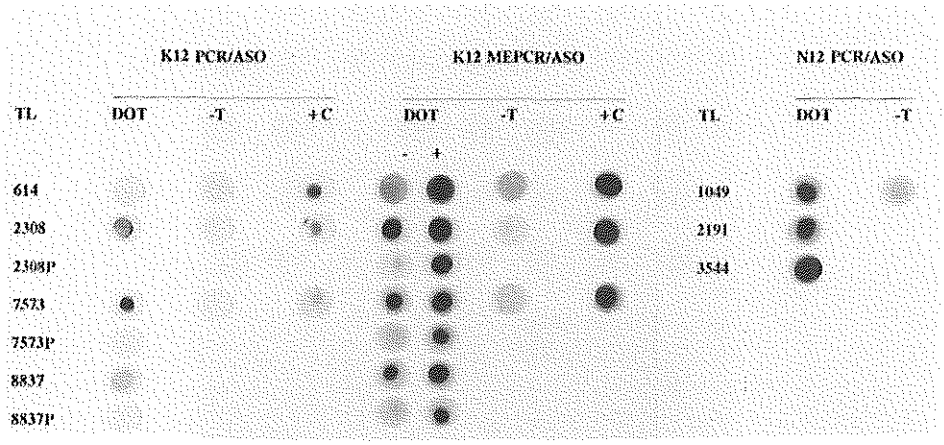

Figure 10.1. *Dot blot signals (after high stringency wash) of all mutant tumors after amplification of the N-or K-*ras *12 region, using PCR/ASO or MEPCR/ASO. TL, tumor; P, parenchyma adjacent to the tumor; DOT, signal of the mutant tumor; -T, signal of a representative mutation-negative tumor; +C, signal of the corresponding positive control containing a GGT to GCT transition (for TL614), a GGT to TGT transition (for TL2308), or a GGT to CGT transition (for TL7573 and TL8837); - and + indicate MEPCR/ASO without, respectively with* MvaI *digestion of the product from the first PCR, resulting in mutant enrichment for the + condition.*

A low incidence of codon 12 mutations was found in both N- and K-*ras* whereas no mutations in N- and K-*ras* 13 or 61 were detected in any of the analyzed TGCTs. Three SEs (TL1049, TL2191, TL3544) were found with a mutation in N-*ras* 12, while two SEs (TL7573, TL8837) and one NS (TL2308, containing EC, T, and YS) with a K-*ras* 12 mutation were detected (Fig. 10.1).

Using the PCR/ASO approach, mutations in some tumor samples might have remained undetected due to the presence of an excess of wild type alleles. Therefore suspensions of cell nuclei were prepared from 25 frozen NS samples, stained with propidium iodide, and analyzed by flow cytometry to determine the fraction of aneuploid (tumor) and diploid (nontumor) cells. The fraction of aneuploid cells varied between 22 and 93%, while 18 tumors contained more than 50% aneuploid cells.

It has previously been shown that in SE samples the fraction of nontumor cells can be greater than 90% (4). We therefore decided to repeat the mutation analysis in our series of SEs using MEPCR/ASO for K-*ras* 12. In this procedure mutant alleles are preferentially amplified, hereby allowing the detection of one mutant among 10,000 wild type alleles (12). The analysis revealed one extra mutant SE (TL614). The previously detected mutations were confirmed, showing an intensified signal after MEPCR/ASO, compared to PCR/ASO and MEPCR/ASO without *Mva*1 digestion (Figs. 10.1 and 10.2).

Parenchyma, adjacent to a mutant tumor was available for TL614, TL2308, TL3544, TL7573, and TL8837. Only the parenchyma of TL614 did not contain carcinoma *in situ* (CIS) and therefore this sample was not studied further. In the

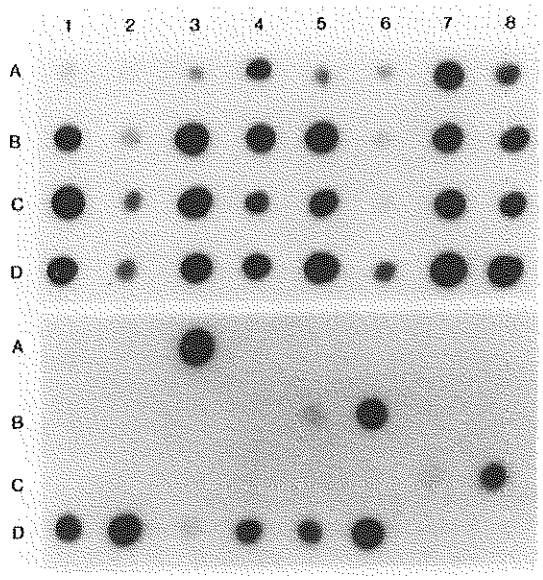

Figure 10.2. *Example of dot blot analyses of mutant-enriched polymerase chain reaction products for detection of K-*ras *12 mutants. The upper A-D panel represents results using the wild-type specific probe, which detects GGT. The lower A-D panel represents results after hybridization with the probe specific for the CGT sequence. Both blots were exposed after washing at high stringency. The first six positions (A1-A6) represent the positive controls for all possible mutations of the first or second base of codon 12 (A3 correspoding with CGT). The remaining positions (A7, D8) are examples of testicular germ cell tumors, with paired signals from each tumor: without (left dot) and with* MvaI *digestion (right dot) after the first amplification. For example: C7 (without digestion) and C8 (with digestion), parenchyma of tumor TL7573, note the enhancement in signal intensity after* MvaI *digestion using the mutant specific probe; B5 and B6, tumor HT85; D1 and D2, tumor TL7573; D3 and D4, parenchyma of tumor TL8837; D5 and D6, tumor TL8837.*

parenchyma of TL7573 and TL8837 a weak signal was found using MEPCR/ASO without *Mva*1 digestion, while no signal was found for TL2308 (Figs. 10.1 and 10.2). Using MEPCR/ASO the signals for the parenchyma of TL7573 and TL8837 were intensified while the mutation in TL2308 was now detectable. Since the parenchyma of TL7573 and TL8837 contained micro-invasive SE cells we cannot determine whether the *ras* mutation was only present in these SE cells or also in the CIS component. However, the findings for the parenchyma of TL2308, containing only CIS, indicate that *ras* mutations can occur in a noninvasive precursor lesion. No N-*ras* mutation was found in the parenchyma of TL3544 (not shown). Since only PCR/ASO was used to detect N-*ras* mutations, the presence of a mutation in CIS cells cannot be excluded, in analogy to the findings for the parenchyma of TL2308.

The nature of the detected mutations is summarized in Table 10.1, showing identical base conversions in both tumor and the adjacent parenchyma.

Table 10.1. Summary of the nature of the N- and K-*ras* codon 12 mutations, in testicular seminomas (SE) and testicular nonseminomatous germ cell tumors (NS), and their adjacent carcinoma *in situ* containing parenchyma (P).

Sample	Histology	N-*ras* 12 mutation	K-*ras* 12 mutation
TL614	SE		GGT → GCT gly → ala
TL1049	SE	GGT → GAT gly → asp	
TL2191	SE	GGT → GAT gly → asp	
TL2308	NS		GGT → TGT gly → cys
TL2308	P		GGT → TGT gly → cys
TL3544	SE	GGT → GCT gly → ala	
TL7573	SE		GGT → CGT gly → arg
TL7573	P		GGT → CGT gly → arg
TL8837	SE		GGT → CGT gly → arg
TL8837	P		GGT → CGT gly → arg

Discussion

Since no H-*ras* mutations have been detected in TGCTs or derived cell lines (4-9), we did not screen our samples for mutations in this gene. To analyse the incidence of N- and K-*ras* codon 12, 13, and 61 mutations, the PCR/ASO was applied to 100 primary TGCTs, while all 40 SEs in this series were also subjected to MEPCR/ASO to detect mutations in K-*ras* codon 12. Only six SEs and one NS were found to contain a *ras* mutation. Six of these mutations (five in SEs and one in NS) were already detected using PCR/ASO. Our data, showing an incidence of K-*ras* mutations of 4%, are concordant with those reported (4-7). Although using the same approach (frozen samples and conventional PCR/ASO), we did not find a high percentage of mutations in N-*ras* 12 and 61, as reported by Ganguly *et al.* (5). This could be due to difficulties in their interpretation of the mutation-specific hybridization signals.

Taking into account the sensitivities of the two detection methods used (one mutant among 40 or one mutant among 10,000 wild type alleles for PCR/ASO and

MEPCR/ASO, respectively), the minimal percentage of mutant alleles that needs to be present for detection can be calculated. A consistent overrepresentation of (parts of) 12p, on which K-*ras* is located, is found in TGCTs (19-22), with a copy number of about six. Assuming the presence of one mutant (codon 12) and five wild type K-*ras* alleles per mutant tumor cell, at least 5.5% of the cells in a sample has to carry the mutant allele to allow detection with PCR/ASO, while 0.02% is needed using MEPCR/ASO. Our comparison of the conventional and mutant-enriched PCR-based methods indicates that the low incidence of K-*ras* 12 mutations in TGCTs must be due to the absence of mutations in the samples studied, and not to the treshold of the PCR/ASO.

The study of flow-sorted SE nuclei by Mulder *et al.* (4) showed N-*ras* mutations in four out of 14 cases. Three of these mutant tumors were studied for heterogeneity for the presence of mutant alleles between separate areas within one tumor. Two tumors were shown to be heterogeneous. Of the two K-*ras* mutant tumors, coded HT31 and HT85 in the study of Mulder *et al.* (4), HT31 was also found to be heterogeneous. We confirmed the data on these K-*ras* mutant SEs, which were analyzed in addition to the 40 SEs in our series, using MEPCR/ASO (Fig. 2, only shown for HT85). Therefore, it cannot be excluded that tumor heterogeneity for *ras* mutations leads to underestimation of the incidence of these mutations.

A pathogenetic event can be considered of importance for tumor formation when it can be demonstrated in a high percentage of malignant cells within one tumor and in most independent tumors of the same histology. Taking together the results described here and those of Mulder *et al.* (4), Moul *et al.* (6) and Ridanpää *et al.* (7), a total incidence of *ras* mutations in primary TGCTs of 11% is found, with 18% in SEs and 4% in NSs. Together with the heterogeneity for *ras* mutations within a single tumor (4), and the detection of a *ras* mutation in parenchyma containing CIS and not in the adjacent TGCT (6), this supports the contention that *ras* mutations are not essential in the initiation or progression of TGCTs. However, a role of K-*ras* in the development of TGCTs by relative overrepresentation of (parts of) the short arm of chromosome 12 cannot be excluded. The difference between SEs and NSs concerning the incidence of mutations needs further investigation. The question whether mutations can already be present in noninvasive CIS, or only in the (micro-)invasive tumor, can be answered using microdissection of tissue sections of parenchyma under histological control to specifically isolate CIS cells.

Recently, Arends *et al.* (23) reported on the suppression of apoptosis in rat fibroblast cell lines by mutant H-*ras*. In this context, it is interesting that *in vitro* culture analysis revealed a consistent difference between 14 SEs without and three with a *ras* mutation (24). Using a co-culture system with an embryonal fibroblast feeder, tumors TL1049, TL3544 and TL7573 (all associated with a strong hybridization signal in PCR/ASO) showed enhanced survival and proliferation. TL8837 and TL2191 were not available for *in vitro* culture. Frisch and Francis (25) recently reported on the induction, and abrogation by mutant H-*ras*, of apoptosis by disruption of epithelial cell-matrix interactions. Since we have indications that SE cells also enter the apoptotic pathway immediately after isolation from their micro-environment (26), suppression of apoptosis by mutant *ras* could explain the enhanced survival and proliferation *in vitro*

of the mutant tumors. It is unlikely that dysfunction of other proteins in the RAS pathway, mimicking the effect of *ras* mutations, are involved in the development of TGCTs, because of the absence of SEs without *ras* mutations which could be cultured *in vitro*. The possible tumor heterogeneity of the K-*ras* mutation in TL614, suggested by the presence of less than 5.5% mutant cells, and its relation to *in vitro* behavior will be studied.

N- and K-*ras* mutations indicate an unfavorable prognosis in childhood acute lymphocytic leukemia (27) and nonsmall cell lung cancer, especially adenocarcinoma (16,28), respectively. In addition, an enhanced *in vitro* proliferative capacity is reported for adult acute myeloid leukemia with a poor prognosis (29). In view of these and our findings we will investigate the prognostic relevance of *ras* mutations in TGCTs.

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Chapter 11

APOPTOSIS AND SEMINOMA CELLS

11.1 Introduction

In this chapter, unpublished experiments mainly aimed at abrogation of seminoma (SE) cell apoptosis are presented. Our observations are discussed in the light of a literature review.

11.2. Factors possibly influencing the onset of apoptosis in seminoma cells

Our study of human SE cells indicates that disruption of the cellular micro-environment could be an important apoptosis-inducing factor. Our results are in agreement with those obtained by Frisch and Francis (1) and Boudreau *et al.* (2). The latter reported on the induction of interleukin-1ß converting enzyme (ICE) and apoptosis in mammary epithelial cells when cultured on matrices that do not meet (apparent) cellular requirements. The apoptosis abrogating effect of the association of insulin receptor substrate-1 with the αvß3 integrin in response to insulin stimulation, reported by Vuori and Ruoslahti (3), further stresses the importance of the interactions between growth factors, integrins and intracellular signalling for cell survival. In the case of GCTs, specific cellular requirements could explain the fact that these tumors mainly occur at specific anatomical localizations (4-7).

Schlaepfer *et al.* (8) reported that integrin-mediated signal transduction in murine fibroblasts is linked to the RAS pathway by the growth factor receptor-bound protein GRB2, while Fath *et al.* (9) described the apoptosis-inducing effect of a GRB2 isoform in rat thymuses which inhibits transactivation of a RAS-responsive element. Moreover, mutant *ras*-expressing rat and hamster fibroblasts do not (or at a lower level) express certain matrix receptors (10). Apparently, mutant *ras* overcomes the need of cells for interactions with the extracellular matrix to survive and proliferate. In view of these findings, our observation that SE cells with a mutant *ras* show increased attachment to feeder cells and prolonged survival and initial proliferation during *in vitro* culture is interesting.

To investigate whether a relation exists between the expression of mutant *ras* and integrins, we studied the presence of the αv integrin subunit in SEs containing either a wild type or mutant *ras*. We detected the αv subunit on SE cells from 8/24 tumors, but its absence was not correlated with the presence of mutant *ras* (unpublished observation). Our data on the expression of the αv integrin subunit on SE cells corroborate those obtained by Timmer *et al.* (11) who described a heterogeneity between SEs in the expression of other integrin subunits.

Knowledge of the composition of the seminomatous extracellular matrix and the presence of integrins on SE cells might help to optimize *in vitro* culture conditions by providing the cells with the required substratum. However, keeping cell-matrix interactions intact does not appear to provide the cells with all the survival and proliferation signals needed, since we found intact tissue blocks to undergo apoptosis *in vitro* within a few days (unpublished observation). In addition, transplantation of SE tissue blocks to orthotopic or ectopic sites in 25 nude rats and 11 SCID (severe combined immunodeficient) mice never resulted in tumor formation. To confirm the apoptotic death of the tumor cells, tissue blocks were removed from three rats and

formalin fixed, within four days upon transplantation. Morphologically apoptotic tumor cells were subsequently detected in slides from the paraffin-embedded material. The other rats were kept under observation for six months to two years. The SCID mice were observed for one to five months upon transplantation. All animals were euthanized, without any signs of tumor formation, when their physical condition had declined from old age. At autopsy, fibrotic tissue parts (as analyzed using morphological interpretation of formalin fixed material) could be retrieved in two mice, one or three months upon transplantation, respectively. In the other mice, no transplanted tissue blocks could be retrieved (unpublished observations).

Not only the disruption of cell-matrix interactions, but also oxidants or stimulators of cellular oxidative metabolism were recently shown to be capable of apoptosis induction (12). Under physiological conditions, the ability of a cell to maintain an appropriate oxidant-antioxidant balance allows cell survival. Cells and organisms have "developed" an intricate system of antioxidants, including superoxide dismutase, catalase, tocopherol and ascorbic acid, to detoxify reactive oxygen intermediates, formed as a result of metabolic processes, and survive the oxidative siege that is associated with aerobic life. However, the oxidative agents and the detoxification arsenal also endow cells and organisms with some sort of selection system to be used for specific cell elimination through apoptosis upon oxidant-antioxidant disbalance (12). An example of specific cell elimination through oxidative-stress is found in the blastocyst (13). Extracellular H_2O_2 was identified as a direct inducer of apoptosis, specifically killing cells with the developmental potential to make trophectoderm that might remain in the embryonic tissues, thus protecting the embryo from ectopic trophectoderm. The surviving embryonic cells exploit glutathione-dependent protection mechanisms against oxidation. Interestingly, Strohmeyer *et al.* (14) reported an altered glutathione S-transferase isoenzyme pattern and a decrease of glutathione S-transferase activity in TGCTs as compared to normal testicular parenchyma. This finding could explain the high susceptibility of TGCTs to oxidative stress, among others induced by antitumor drugs and possibly by the *in vitro* manipulations performed by us (Chapters 8 and 9).

Reactive oxygen intermediates can rapidly react with cellular macromolecules (15), either directly or ultimately resulting in damage to cellular structures such as membranes (16). The fact that BCL-2 has been found to be located in the cellular membranes of mitochondria, endoplasmatic reticulum and nucleus, points to a role in protecting the membranes from reactive oxygen intermediates-induced damage. Recently, this protective function was indeed reported (17). Non-physiological agents, such as the anti-oxidant Trolox (Hoffmann-La Roche), have also been found able of blocking oxidative stress-induced apoptosis (18). Preliminary results, using medium supplemented with Trolox in which SE cells were collected right after disruption of their micro-environment, indicate that this agent can block the immediate onset of apoptosis in these cells, as monitored by the absence of DNA ladders on electrophoresis gels and of apoptotic SE cell populations upon fluorescent DNA nick labeling and flowcytometry (unpublished observation).

During normal spermatogenesis, apoptotic spermatogonia can be detected in the seminiferous tubules (19-21). The exact function of the apoptotic death of these cells

remains unclarified but it has been speculated that it serves to eliminate spermatogonia with genetic abnormalities (22,23). Since apoptosis occurs in groups of spermatogonia linked by intercellular cytoplasmic bridges (20), it is presumed that these bridges are involved in mediation of apoptotic signals. Interestingly, intercellular bridges have not been found between CIS or SE cells (24). This might explain the survival and proliferation of these harmful cells, which will only apoptotically die when more stringent conditions are applied (*e.g. in vitro* manipulations or the use of cytotoxic drugs or irradiation), in spite of their genetic abnormalities.

11.3. The possible role in seminoma cells of genetic regulators of apoptosis

We studied the expression of *bcl*-2 in intact SEs and single cell suspensions and found that BCL-2 is not immunohistochemically detectable in either of these. In contrast to findings by others (25-30), we could not detect *p*53 expression in intact SEs, using freshly frozen and paraffin-embedded samples. This could be due to the use of different antibodies in the various laboratories, as well as to the use of paraffin-embedded samples in some studies and freshly frozen samples in others. Since we could not immunohistochemically detect (enhanced) *p*53 expression in SE cells upon disruption of their micro-environment, we consider the possibility that their apoptotic death under these circumstances is independent of (enhanced) *p*53 expression.

Besides *bcl*-2 and *p*53, many other genes are known to regulate the apoptotic process. Recently, genes belonging to the so-called *bcl*-2 gene family have been found to encode BCL-2 homologues with either death promoting or inhibiting activity. Oltvai *et al.* (31) reported the finding of a BCL-2 associated protein which they called BAX (BCL-2 associated X protein). This protein has extensive amino acid homology with BCL-2, especially in the so called BCL-2 homology regions 1 and 2 (BH1 and BH2), which are involved in dimerization. BAX can heterodimerize with BCL-2 or homodimerize to counter BCL-2 activity (*i.e.* accelerate cell death) when overexpressed, however only in the presence of a death signal. BCL-2 mutants that fail to interact with BAX can no longer inhibit cell death (32). Yang *et al.* (33) reported the existence of BAD (BCL-x_l/BCL-2-associated death promoter homolog), a heterodimeric partner for BCL-x_l and BCL-2 containing the BH1 and BH2 domains. BAD was shown to be able of displacing BAX in the heterodimers formed with either BCL-x_l or BCL-2 and reverse the death repressor activity of BCL-x_l but not of BCL-2. No evidence for the existence of BAD/BCL-2/BAX or BAD/BCL-x_l/BAX heterotrimers was found. The association between BCL-x_l and BAD was stronger than that between BCL-2 and BAD. The detected protein interactions give rise to an apoptosis regulation model in which BCL-x_l and BCL-2 must heterodimerize with BAX to inhibit death and BAD can bind to BCL-x_l, displacing BAX into homodimers, to induce death. Susceptibility to cell death is then best correlated with the distribution of BAX over hetero- and homodimers (Fig 11.1). Interestingly, Miyashita and Reed (34) reported that P53 is a direct transcriptional activator of *bax*, while it was shown by Miyashita *et al.* (35,36) and Selvakumaran *et al.* (37) that P53 downregulates expression of *bcl*-2.

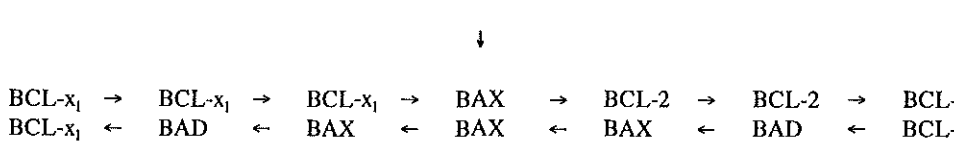

Figure 11.1. *Schematic representation of homo- and heterodimer formation between proteins of the BCL-2 family. The susceptibility of a cell to apoptosis-inducing stimuli is best correlated with the distribution of BAX over homo- and heterodimers, with BAX homodimer formation resulting in high susceptibility.*

Genes not homologous to *bcl*-2 have been found to code for proteins that interact with BCL-2. Takayama *et al.* (38) described BAG-1 (BCL-2-associated athanogene-1 (from the Greek word athanos, referring to anti-death)) to be a novel BCL-2-binding protein, without homology to the BCL-2 family. Co-expression of *bag*-1 and *bcl*-2 provided markedly increased protection from cell death. BAG-1 was shown to have a certain homology to ubiquitin and ubiquitin-like proteins, normally involved in targeting proteins for degradation. Thus, a possible role for the ubiquitin-like domain in BAG-1 may be to serve as a site for attachment of ubiquitin and subsequent proteolytic degradation. Ubiquitin conjugation has been shown to regulate the turnover of other proteins that are thought or known to play a role in cell death regulation, including tumor suppressor P53 (39,40). In addition, a requirement for ubiquitin for radiation-induced apoptosis in thymocytes has been reported (41).

A novel group of genes has recently been described to be involved in the execution of the apoptotic process. This group comprises the genes encoding ICE (42) and the ICE-like proteases CED-3 (43), ICH-1 (44), NEDD2 (45), CPP32 (46) and TX (47), which are thought to act at the point in the apoptotic pathway at which the various routes of inducing stimuli finally converge, *i.e.* at the beginning of the final common pathway. Interestingly, the cowpox virus gene *crm*A has been reported to encode a powerful inhibitor of ICE (48). Transfection or injection of this gene into certain cells renders them resistent to apoptosis (49). Because ICE family members are thought to act at the start of the final common pathway, the use of inhibitors like *crm*A should result in blocking of apoptosis in response to a wide variety of stimuli.

As mentioned, we could not immunohistochemically detect BCL-2 in intact SEs. This finding could offer an explanation for the high susceptibility of these cells to apoptosis-inducing stimuli, like disruption of cell-matrix interactions and radiation (50) (which results in the formation of reactive oxygen intermediates (16)). Since specific cell types have been shown to respond to specific apoptosis inducing stimuli in a specific way, data obtained on the regulation of apoptosis in various cell types can not be directly applied to SE cells. It appears necessary to fully elucidate the control of apoptosis in SE cells in reponse to various stimuli, before progress can be made in blocking these processes. Further information on the genes involved in regulation of the apoptotic processes in SE cells might be obtained from additional immunohistochemical studies. These could first focus on the expression of *bcl*-2 and *ice* family members. Western blot analysis of immunoprecipitates from extracts of pure

SE cell suspensions, obtained after lymphocyte removal through negative selection using anti-lymphocyte antibodies or through positive selection using anti-placental alkaline phosphatase (detecting germ-cell specific alkaline phosphatase) coated magnetic beads, might reveal which molecules are involved in the regulation of SE cell apoptosis and interact prior to and upon the induction of apoptosis. These studies could lead to a specific intervention with the endogenously important regulators of SE cell apoptosis.

Transfection with apoptosis-abrogating genes, especially those controling the final common pathway, could be useful to improve SE cell survival *in vitro*. We have already tried to transfect SE cells with the gene encoding SV40 large T antigen, using the calciumphosphate precipitation technique and a micro-injection approach. In addition, we applied electroporation in an attempt to transfect SE cells with *bcl*-2. All these attempts were unsuccessful and results are unpublished. Since only proliferating cells can be transfected successfully and since only cells from the few *ras*-mutant SEs initially proliferate *in vitro*, it might be better to directly bring anti-apoptosis proteins into SE cells, in stead of genes. The use of micro-injection methods or fusion strategies using liposomes containing the required factors could be considered.

Regarding the induced overexpression of proliferation-inducing genes in SE cells, attention has to be paid to the fact that these genes might also induce apoptosis. This observation was first reported for c-*myc* (51-55). Under favorable cell culture conditions, *e.g.* the presence of sufficiently high growth factor and/or serum concentrations, c-*myc* (overexpression) promotes cell proliferation. However, in the (virtual) absence of these factors, high c-*myc* expression results in the onset of apoptosis. Apparently, when an intracellular proliferation signal is not accompanied by suitable external stimuli the apoptotic pathway is switched on. This phenomenon could occur upon transfection of SE cells kept under suboptimal *in vitro* conditions and could already be involved in the onset of apoptosis in SE cells as described in Chapter 9. A cell cycle/apoptosis controling gene that offers a growth advantage *in vivo* could induce the onset of apoptosis once the tumor cells are transferred to a suboptimal growth condition lacking vital factors, *e.g.* growth factors and/or extracellular matrix.

11.4. Possible involvement of ligand-receptor interactions in tumor development and prevention of seminoma cell apoptosis

Primary SEs appear to depend upon a specific micro-environment and growth factor supply since they occur at specific localizations and express the stem cell factor (SCF) receptor c-*kit*. This suggests that a lack of apoptosis could have contributed to tumor formation, in keeping with the finding that SCF can abrogate apoptosis of murine primordial germ cells (56). Immunohistochemically, we (unpublished observation) and others (57) could not detect *scf* expression in SEs (except for rare individual cells), which indicates the absence of autocrine c-KIT stimulation. In human serum the concentration of SCF is in the 3 μ/L range (58). This is high, as compared to for example erythropoietin which circulates in the 30 ng/L range (59,60). Whether circulating SCF can support SE cells remains to be investigated.

Interestingly, Stevens and Mackensen (61) reported that introduction of mutant *scf*

into 129J mice resulted in an increased incidence of testicular teratoma (from 2.5 to 6.9%). Since tumors were not formed in all animals, it appears that the mutant gene contributed to tumor formation but was not causal. No mutation analyses of human *scf* in TGCT patients have been reported thus far. Fleischhacker *et al.* (27) reported the absence of point mutations from c-*kit* codon 936 (analogous to the codon of the c-*fms* gene which encodes a homologous receptor, often mutated in cellular transformation and cancer) in a series of 44 TGCTs.

Not only c-KIT/SCF interactions might play a role in the development of TGCTs and especially SEs. Recent investigations point to a possible involvement of Müllerian inhibiting substance (anti-Müllerian hormone). This factor was shown to be involved in the differentiation of murine gonocytes to spermatogonia A (62,63). Whether this factor is also involved in (human) PGC development remains to be investigated. Interestingly, in humans a peak in Müllerian inhibiting substance serum levels can be detected between 4 and 12 months of age (64), the time at which differentiation from gonocytes to spermatogonia A occurs (65). In addition, lower levels of Müllerian inhibiting substance are correlated with cryptorchidism (66), as previously mentioned a risk factor for TGCT development.

Recently, two groups reported enhanced proliferation of murine PGCs when cultured *in vitro* in the presence of tumor necrosis factor-α or retinoic acid, respectively (67,68). We have not yet studied the involvement of retinoic acid and its receptor in SE development. Concerning tumor necrosis factor-α, we did not observe enhanced SE cell survival or proliferation *in vitro* in its presence, as investigated for a few SEs (unpublished data). Although an anti-tumor effect has been attributed to the SE-infiltrating lymphocytes (69), it can be speculated that these cells support SE cell survival and proliferation through the production of certain cytokines and/or growth factors, *e.g.* tumor necrosis factor-α and SCF. In view of these findings, the fact that the gene encoding the tumor necrosis factor receptor-1 has been mapped to chromosome 12p13 (70) is interesting, since this chromosome arm is frequently overrepresented in TGCTs (71-78).

To investigate the involvement of candidate ligand-receptor interactions in the development of TGCTs and especially SEs, familial cases of TGCTs might be used in a search for mutations in ligand-receptor systems that are possibly involved in TGCT development.

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Chapter 12

DISCUSSION AND FUTURE RESEARCH

12.1. Introduction

In this chapter the results of the experiments performed on human seminomas (SEs), a subgroup of the testicular germ cell tumors of adolescents and adults (TGCTs), will be discussed. In addition, suggestions for future research will be given.

12.2. Relation between seminomas and nonseminomas

To describe the relation between the two entities of TGCTs, SEs and nonseminomatous TGCTs (NSs), two models are used: the independent origin model and the linear progression model. Neither one of these models has been proven. We tried to shed light on the pathobiological relation between SEs and NSs by analysing the glycolipid content of primary TGCTs. This investigation was based on the finding that glycolipid expression in embryonal carcinoma (EC) and other NS cell lines relates to their differentiation lineage (1-3).

Our results are in agreement with the findings on TGCT cell lines (1) and indicate a close relation between SEs and ECs. Furthermore, our findings could indicate the existence of a less and a more mature type of SEs. Mature SEs might be committed to the germ line and not able to give rise to NSs. This would only be possible for less mature SEs, which can be reprogrammed to ECs and subsequently give rise to differentiated NSs.

Studies comparing the glycolipid profile of carcinoma *in situ* and adjacent tumor, either SE or NS, should be performed to further investigate the relation between SEs and NSs, since the present data cannot confirm either model on the development of TGCTs.

12.3. Models for human seminomas

In search of an animal model for human SEs, we investigated whether canine seminomas could be used as an experimental system. In keeping with earlier suggestions by Scully (4,5), we found canine seminomas to resemble human spermatocytic seminomas and not human SEs. Recently, the occurrence of seminomatous tumors in mice infected with human papilloma virus has been reported (6). However, since the tumor cells resemble pachytene-stage spermatocytes containing numerous mitochondria, which are reportedly sparse in SEs (7) we suppose that these tumors are not primordial germ cell (PGC)-derived. Takatera *et al.* (8) claim the establishment of a human SE cell line. However, their data also point to a spermatogonial origin of the tumor cells. They do not report whether the tumor cells express germ cell-specific alkaline phosphatase, which is a marker for PGCs, CIS and SE cells. Moreover, the tumor cells do not contain the chromosomal marker of human TGCTs, isochromosome 12p (9-16), but a chromosome 1 inversion (1p13-q21) which can be related to spermatogenetical anomalies (17). The ploidy of the tumor cells was not reported. Thus, the above mentioned models appear inappropriate for human SEs.

12.4. Development of an *in vitro* culture system for human seminomas

We tried to develop an *in vitro* culture system for human SEs, because these tumors appear to represent a unique tumor type for which no animal model is known. Using the conditions that gave rise to the derivation of murine embryonic germ (EG) cell lines from PGCs (18), we could not obtain SE cell lines. A major obstacle encountered in our attempts to culture SE cells *in vitro* was the onset of apoptosis upon disruption of cell-matrix interactions prior to *in vitro* culturing. This is in keeping with the observations made by Frisch and Francis (19) and Boudreau *et al.* (20). Deprivation of growth factor(s) might also have contributed to the onset of apoptosis. This contention is supported by the observed enhanced *in vitro* survival of SE cells in the presence of stem cell factor, a growth factor known to block apoptosis of murine PGCs (21). Interestingly, SEs bearing mutant N- or K-*ras* showed enhanced survival and initial proliferation during *in vitro* culture, which might reflect the apoptosis abrogating activity of mutant *ras* (22). The biological and prognostic significance of our finding could be further studied, using TGCT (especially SE) samples from patients who did not respond to therapy.

To succeed in the development of a culture system for human SE cells and the subsequent derivation of cell lines, it appears necessary to abrogate apoptosis. This might be achieved by collection of SE cells in medium supplemented with the anti-oxidant Trolox (Hoffmann-La Roche), an apoptosis inhibitor (23), which we found to inhibit apoptotic DNA fragmentation in suspended SE cells. In addition, transfection with apoptosis blocking genes, such as *bcl*-2 and *crm*A, or direct transfer of apoptosis blocking proteins into SE cells, for example using liposomes, can be used. These attempts to intervene with apoptosis should be based on a thorough understanding of the endogenous presence in SE cells of apoptosis regulators.

The fact that, until now, no NS cells could be derived from SE cells *in vitro* does not invalidate the linear progression model, since this finding could be due to the absence of the required growth and differentiation conditions from the applied culture system. In addition, our glycolipid and *in vitro* analyses might indicate a subgroup of SEs committed to the germ cell lineage, which are no longer able to give rise to NSs.

12.5. Insights in the development of germ cell tumors

Teratocarcinomas can develop in the 129J murine strain, either spontaneously (24,25) or upon induction through transplantation of early embryo's or genital ridges to ectopic sites (26-29). This model has often been used in the study of human NSs (30-34), although it has been reported that the murine tumors merely resemble human (immature) teratomas and yolk sac tumors, occurring in the gonads and extragonadal sites in infants and children, which probably may develop from primitive germ cells and pluripotent embryonal stem (ES) cells, respectively (35,36). Recently, the derivation of pluripotent EG cells from murine PGCs upon *in vitro* culture (18) and the subsequent differentiation of the EG cells to embryonic and extra-embryonic cells, renewed the interest in a murine model of human TGCTs, and especially SEs. Matsui *et al.* (18) suggested that "reprogramming" of PGCs resulted in the formation of EG

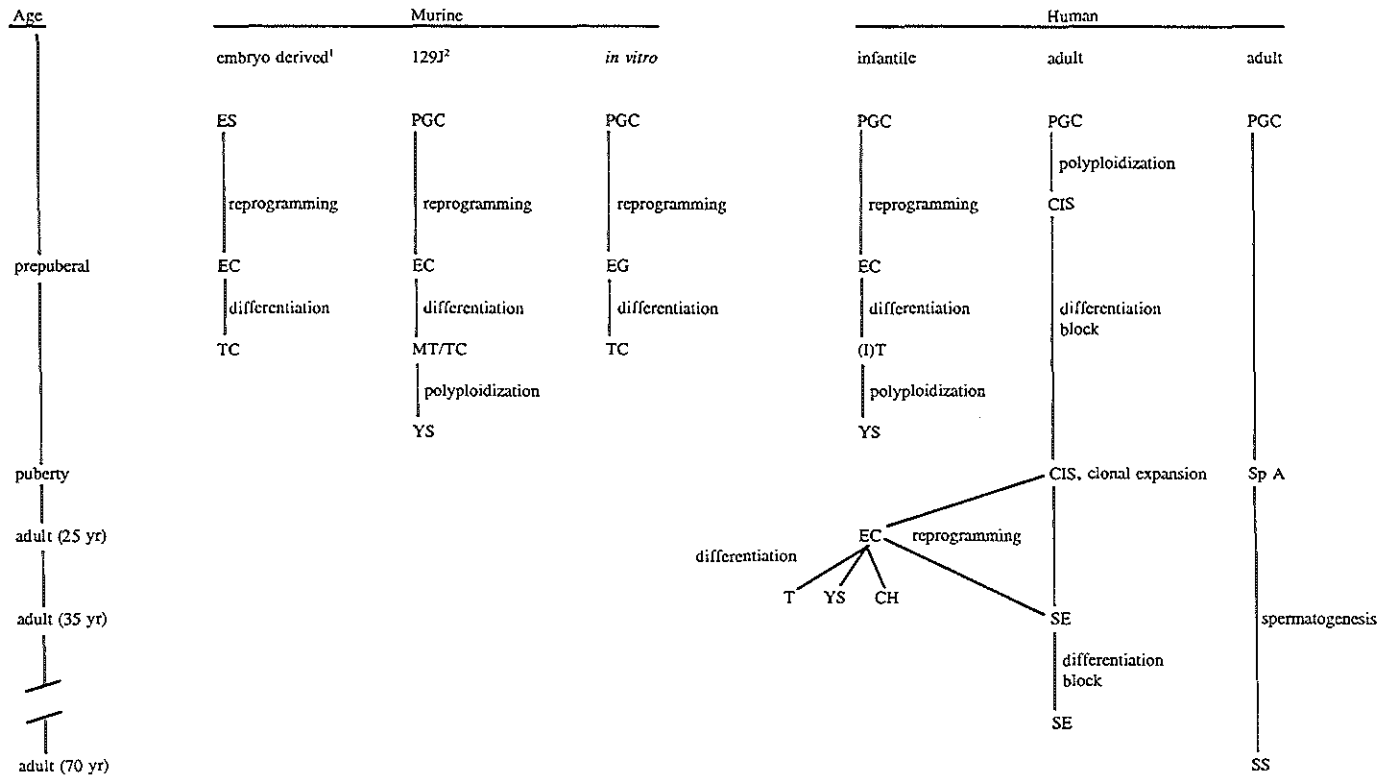

Figure 12.1 *Schematic representation of murine and human germ cell tumor development. [1]Comparable to certain human infantile germ cell tumors, e.g. sacral tumors and tumors of head and neck; [2]Comparable to human infantile germ cell tumors; CH, choriocarcinoma; CIS, carcinoma* in situ*; EC, embryonal carcinoma; EG, embryonic germ cell; ES, embryonal stem cell; (I)T, (immature) teratoma; MT, mature teratoma; PGC, primordial germ cell; SE, seminoma; Sp A, spermatogonium A; SS, spermatocytic seminoma; TC, teratocarcinoma; YS, yolk sac tumor.*

cells. In our view this process could resemble the transition from CIS or SE to EC, subsequently giving rise to the formation of differentiated nonseminomatous tissues. However, this reprogramming could also be comparable to the forementioned neoplastic transformation of pluripotent ES cells and primitive germ cells in infants and children. This latter contention is supported by data on ploidy and cytogenetics, which show that murine EG cells, tumors in 129J mice, murine embryo and genital ridge-derived tumors as well as human (immature) teratomas in infants and children are diploid. These latter tumors can give rise to yolk sac tumor upon polyploidization (18,24-29,37,38). Thus (at least) with respect to ploidy the *in vivo* and *in vitro* murine models do not resemble human TGCTs, which are reportedly near triploid (39-41). Moreover, seminomas are absent in the *in vivo* murine models. In Figure 12.1 the development of murine and human germ cell tumors is schematically depicted.

McLaren (42) and Rossant (43) suggested that PGCs are (highly) differentiated, not pluripotent cells, explaining why these cells cannot give rise to chimeras when injected into blastocysts (43). This is in contrast to murine ES, EG and EC cells which can give rise to chimeras, with ES and EG cells contributing to the germ line (18,42-48). The fact that EC cells, which are malignant cells, can contribute to normal development (except for contribution to the germ line) in chimeras is intriguing. Apparently, murine EC cells can "easily" switch from a normal developmental program to a malignant state and back to normal. This supports the suggestion put forward by Walt *et al.* (37) who state that murine germ cell tumors develop by direct activation of PGCs without an intermediate stage of neoplastic proliferation of PGCs. Therefore, no CIS or SE occur. In contrast, in adolescent and adult men a neoplastic transformation of PGCs occurs and CIS and SE develop. It remains unclear why neoplastic, reprogrammed diploid ES cells and PGCs have a limited developmental potential (giving rise to (immature) teratoma, and yolk sac tumor upon polyploidization) as compared to reprogrammed CIS/SE cells, which give rise to pluripotent EC. This might be due to differences in genomic imprinting (*i.e.* the fact that homologous genes are differentially expressed, based on their parental origin (49-51)), which may be progression and aneuploidy related.

Once apoptosis of SE cells can be abrogated, SE cell lines might be obtained. These cell lines can be used to answer questions relating to the different developmental potentials observed for murine GCTs and human infantile GCTs on the one hand and human TGCTs on the other. The involvement of genomic imprinting and genetic constitution in the establishment of the developmental potential of malignant germ cells can also be investigated.

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SUMMARY

Two hypotheses exist on the development of human testicular germ cell tumors of adolescents and adults (TGCTs). One hypothesis assumes that the two histological subtypes of TGCTs, the seminomas (SEs) and nonseminomatous TGCTs (NSs) arise from independent carcinoma *in situ* (CIS) precursor cells. The other hypothesis, the linear progression model, assumes that a CIS cell can give rise to NS through a seminomatous stage. We used various approaches, mainly aimed at the development of an *in vitro* culture system for SE cells and the derivation of SE cell lines to gain more insight into the relationship between SEs and NSs.

Information necessary to understand the rationale of the performed experiments is presented in Chapters 2 to 4. In **Chapter 2** an overview is given of the present knowledge on TGCTs. **Chapter 3** provides information on the involvement of stem cell factor and its receptor c-KIT in the development of primordial germ cells and pathology. The role of apoptosis in normal development and carcinogenesis is addressed in **Chapter 4**.

The differentiation lineage of NS cell lines has been shown to correlate with the expression of certain combinations and amounts of glycolipids on the cell surface. Whether these data are representative of the situation in primary tumors is unknown. In addition, the glycolipid profile of SEs has not been specifically studied. We analyzed the glycolipids of a large series of primary SEs and NSs, in order to shed light on the relationship between these tumor types. We used thin-layer chromatography in combination with orcinol and immunostaining to detect specific glycolipids. Our results, presented in **Chapter 6**, confirm the data on NS cell lines and furthermore show that SEs and embryonal carcinomas (ECs, containing the undifferentiated stem cells of NSs) express similar amounts of globo-series glycolipids, indicative of the presence of stem cells in these tumors. In addition, our data suggest the existence of two subpopulations of SEs, a less mature gangliosides-negative and a more mature gangliosides-positive one. We found ECs to contain the lacto-series glycolipid Le^x, which might be carried by EC cells that are derived from globo-series-expressing stem cells upon reprogramming. Our glycolipid analysis does not prove either hypothesis on the development of TGCTs.

Since an animal model representative of human SEs would facilitate the set up of experiments, we looked for such a model. The murine systems of spontaneous or induced teratocarcinomas appeared inappropriate since no SE-like tumors have ever been detected in these systems. In addition, the tumors merely resemble human (immature) teratomas and yolk sac tumors, occurring in the gonads and at extragonadal sites in infants and children, but not TGCTs. We studied a series of canine testicular tumors, comprising many seminomas, using flow and image cytometry. In addition, we performed an epidemiological study. Our data, presented in **Chapter 7**, show that canine seminomas are comparable to human spermatocytic seminomas (that were included in this study) and not to human SEs. We thus conclude that canine seminomas cannot be used as a model for human SEs.

We have previously shown that under culture conditions for NSs, *i.e.* the use of medium with fetal calf serum, SE cells die within three days. In **Chapter 8**, the development of an improved *in vitro* culture system for human SE cells is described, which could be used in the study of the pathogenetic relationship with NSs. Using a murine embryonal fibroblast (STO) feeder, a low percentage of SE cells attached to this feeder and few cells survived to day 10. However, three SEs showed a higher degree of

attachment and two of them initially proliferated. One of these two tumors was cultured using the conditions resulting in the derivation of pluripotent embryonal germ cells from murine primordial germ cells, *i.e.* the use of a STO feeder in combination with the growth factors stem cell factor, leukemia inhibitory factor and basic fibroblast growth factor, in serum-containing medium. These conditions resulted in prolonged survival, proliferation and colony formation. However, in contrast to the situation for murine primordial germ cells, no SE cell lines could be derived nor could reprogramming to a pluripotent phenotype be observed.

We investigated whether the cell degeneration described in Chapter 8 was due to the onset of apoptosis. As described in **Chapter 9**, very few apoptotic cells were detected in intact SE tissues, using *in situ* end labeling. In single cell suspensions, obtained upon disruption of the SE cell micro-environment up to 85% of the SE cells was apoptotic, based on end labeling and flow cytometric analysis. In addition, DNA ladders on electrophoresis gels, characteristic for the occurrence of apoptosis, were not detected in intact tissues, but only in cell suspensions. We conclude that disruption of the SE cell-matrix interactions results in the onset of apoptosis and that blocking of this pathway might be necessary to succeed in the development of an optimal *in vitro* culture system for human SEs. We could not immunohistochemically detect *bcl*-2 expression, which might explain the high susceptability of SE cells to apoptosis-inducing stimuli. Furthermore, SE cell apoptosis upon disruption of the micro-environment appears independent of (enhanced) *p*53 expression, since P53 could not be immunohistochemically detected.

Conflicting results on the incidence of N- and K-*ras* mutations in TGCTs have been reported. In **Chapter 10**, our analysis for N- and K-*ras* mutations in a series of 40 SEs and 60 NSs, using a polymerase chain reaction and allele specific oligonucleotide hybridization, is described. To exclude underestimation of the incidence of *ras* mutations, we additionally used a technique preferentially amplifying mutant K-*ras* (codon 12) alleles. In total, six mutant SEs and one mutant NS were detected. We conclude that N- or K-*ras* mutations are rare and apparently not essential for initiation and progression of TGCTs. Interestingly, three *ras* mutant SEs, included in the *in vitro* culture assays, were the only tumors showing enhanced attachment to feeder layer, while two of them initially proliferated (as already mentioned above). These findings are concordant with the apoptosis-abrogating activity, recently found for mutant *ras* genes. Whether the presence of *ras* mutations in TGCTs has prognostic relevance should be investigated.

Chapter 11 contains the results of unpublished studies, mainly aimed at the prevention of SE cell apoptosis. These results are discussed in view of a literature study concerning factors that possibly influence the onset of apoptosis in SE cells, as well as the possible role in SE cells of apoptosis regulators.

In **Chapter 12**, the results obtained during this project are discussed and future studies indicated.

SAMENVATTING

Twee hypotheses beschrijven de ontwikkeling van humane testiculaire kiemceltumoren van adolescenten en volwassenen (TKCT). De ene hypothese gaat er van uit dat de twee histologische subtypen van de TKCT, de seminomen (SE) en de nonseminomateuze TKCT (NS) ontstaan uit onafhankelijke carcinoma *in situ* (CIS) voorlopercellen. De andere hypothese, het lineaire progressie model, stelt dat NS ontstaat uit CIS, na doorlopen van een SE stadium. Wij hebben verscheidene benaderingen gevolgd, met name gericht op het ontwikkelen van een *in vitro* kweeksysteem voor SE en het verkrijgen van SE cellijnen, om meer inzicht te krijgen in de relatie tussen SE en NS.

Informatie betreffende de rationale van de uitgevoerde experimenten wordt gepresenteerd in de Hoofdstukken 2 tot en met 4. In **Hoofdstuk 2** wordt een overzicht gegeven van de huidige kennis van TKCT. **Hoofdstuk 3** geeft informatie over de betrokkenheid van stamcelfactor en diens receptor c-KIT bij de ontwikkeling van primordiale kiemcellen en pathologie. De rol van apoptose tijdens de normale ontwikkeling en bij tumorigenese wordt beschreven in **Hoofdstuk 4**.

De richting van differentiatie van NS cellijnen blijkt gecorreleerd te zijn met de expressie van bepaalde combinaties en hoeveelheden van glycolipiden op het celoppervlak. Of deze gegevens representatief zijn voor primaire tumoren is onbekend. Bovendien is het glycolipidenprofiel van SE niet specifiek bestudeerd. Wij analyseerden de glycolipiden van een grote serie primaire SE en NS, om meer licht te werpen op de relatie tussen deze tumortypen. Wij gebruikten dunne-laag chromatografie in combinatie met orcinol -en immunokleuring om specifieke glycolipiden aan te tonen. Onze resultaten, gepresenteerd in **Hoofdstuk 6**, bevestigen de gegevens betreffende NS cellijnen en laten zien dat SE en embryonaalcel carcinoom (EC, de ongedifferentieerde stamcellen van NS) vergelijkbare hoeveelheden globo-serie glycolipiden tot expressie brengen. Dit geeft aan dat in deze tumoren stamcellen aanwezig zijn. Bovendien suggereren onze gegevens het bestaan van twee subpopulaties SE, de ene minder uitgerijpt en zonder gangliosiden, de ander meer uitgerijpt en met gangliosiden. In EC vonden wij het lacto-serie glycolipide Le^x, dat aanwezig kan zijn op EC cellen die na reprogrammering zijn ontstaan uit globo-serie expresserende stamcellen. Onze glycolipidenanalyse kan geen van de beide modellen voor de ontwikkeling van TKCT bewijzen.

Aangezien een diermodel, representatief voor humaan SE, het opzetten van experimenten zou vergemakkelijken, hebben wij naar een dergelijk model gezocht. De muizesystemen met spontane of geïnduceerde teratocarcinomen leken niet bruikbaar, aangezien SE-achtige tumoren nooit in deze systemen zijn aangetroffen. Bovendien lijken deze tumoren meer op humane (immature) teratomen en dooierzaktumoren, optredend in de gonaden en op extragonadale locaties in pasgeborenen en kinderen, dan op TKCT. Wij bestudeerden een serie testiculaire tumoren van honden, waaronder veel seminomen, gebruikmakend van flow- en beeldcytometrie. Bovendien werd epidemiologisch onderzoek gedaan. Onze gegevens, gepresenteerd in **Hoofdstuk 7**, laten zien dat seminomen van de hond vergelijkbaar zijn met humane spermatocytaire seminomen (die ook in deze studie zijn geanalyseerd) en niet met humane SE. Wij concluderen dat seminomen van de hond niet geschikt zijn als model voor humaan SE.

In een eerder stadium hebben wij laten zien dat onder de *in vitro* kweekcondities voor NS, dat wil zeggen het gebruik van medium met foetaal kalverserum, SE cellen binnen drie dagen doodgaan. In **Hoofdstuk 8** wordt de ontwikkeling van een verbeterd

in vitro kweeksysteem voor humaan SE beschreven, dat gebruikt kan worden in de studie van de pathogenetische relatie met NS. Gebruikmakend van een muize embryonale fibroblast (STO) cellaag, bleek een laag percentage SE cellen aan deze cellaag te hechten, terwijl een deel van de cellen tot dag 10 overleefde. Cellen van drie SE, daarentegen, vertoonden een betere hechting en twee van hen prolifereerden initieel. Eén van deze twee tumoren werd gekweekt onder de condities die resulteerden in het verkrijgen van pluripotente embryonale kiemcellen uit muize primordiale kiemcellen, d.w.z. gebruikmakend van een STO cellaag in combinatie met de groeifactoren stamcelfactor, leukemie inhiberende factor en basische fibroblast groeifactor, in serumhoudend medium. Deze condities resulteerden in langere overleving, proliferatie en de vorming van kolonies. In tegenstelling tot de situatie met muize primordiale kiemcellen konden geen SE cellijnen verkregen worden, noch kon reprogrammering tot een pluripotent fenotype worden waargenomen.

Wij onderzochten of de in Hoofdstuk 8 beschreven celdegeneratie het gevolg was van het optreden van apoptose. Zoals beschreven in **Hoofdstuk 9** werden in intacte weefselstukjes van SE zeer weinig apoptotische cellen gedetecteerd met *in situ* eindlabeling. In celsuspensies, verkregen na het losmaken van de SE cellen uit hun micro-omgeving, bleek tot 85% van de SE cellen apoptotisch te zijn, gebaseerd op analyses met eindlabeling en flowcytometrie. Bovendien konden DNA ladders op electroforese gels, karakteristiek voor het optreden van apoptose, niet worden gevonden in intacte weefsels, maar alleen in celsuspensies. Wij concluderen dat verstoren van de SE cel-matrix interacties leidt tot het optreden van apoptose en dat blokkeren van deze route noodzakelijk kan zijn om te slagen in het ontwikkelen van een optimaal *in vitro* kweeksysteem voor humaan SE. Immunohistochemisch konden wij geen expressie van *bcl*-2 aantonen, wat de hoge ontvankelijkheid van SE cellen voor apoptose-inducerende prikkels kan verklaren. Daarbij lijkt apoptose van SE cellen na verstoren van hun micro-omgeving onafhankelijk van (verhoogde) expressie van *p*53, daar P53 niet immunohistochemisch aangetoond kon worden.

Betreffende de incidentie van N- en K-*ras* mutaties in TKCT zijn tegenstrijdige data gerapporteerd. In **Hoofdstuk 10** wordt onze analyse van het voorkomen van N- en K-*ras* mutaties in een serie van 40 SE en 60 NS beschreven. Hierbij is gebruik gemaakt van een polymerase kettingreactie en allelspecifieke oligonucleotide hybridisatie. Om onderschatting van de incidentie van *ras* mutaties uit te sluiten hebben wij ook een techniek gebruikt die preferentieel mutante K-*ras* (codon 12) allelen amplificeert. In totaal werden zes SE en één NS met een mutatie gedetecteerd. Wij concluderen dat N- of K-*ras* mutaties zeldzaam zijn in TKCT en niet essentieel voor initiatie en progressie van deze tumoren. Belangwekkend is het feit dat drie *ras* mutante SE die bij de *in vitro* kweekassays gebruikt zijn, als enige tumoren verhoogde hechting aan de cellaag vertoonden, terwijl twee van deze tumoren initieel prolifereerden (zoals hierboven reeds beschreven). Dit is in overeenstemming met de apoptose-blokkerende werking die recent is gerapporteerd voor mutante *ras* genen. Of het aanwezig zijn van *ras* mutaties in TKCT prognostisch relevant is, zou onderzocht moeten worden.

Hoofdstuk 11 bevat de resultaten van ongepubliceerde studies, gericht op het tegengaan van apoptose in SE cellen. Deze resultaten worden besproken in het kader van een literatuurstudie betreffende factoren die mogelijk apoptose van SE cellen veroorzaken

en de mogelijke rol van apoptose regulatoren in deze cellen.

In **Hoofdstuk 12** worden de resultaten van dit project bediscussieerd en toekomstig onderzoek aangegeven.

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Robert

CURRICULUM VITAE

Robert Olie werd op 20 oktober 1964 te Voorburg geboren. In 1983 behaalde hij het VWO-B diploma aan de Scholengemeenschap Voorburg 't Loo, waarna hij in datzelfde jaar aanving met de studie Moleculaire Wetenschappen aan de Landbouwhogeschool (de latere Landbouwuniversiteit) Wageningen. Gedurende deze studie richtte hij zich met name op biotechnologische disciplines en deed in dat kader afstudeervakken bij de vakgroep Moleculaire Biologie, onder begeleiding van dr. Pim Zabel en bij de sectie Proceskunde van de vakgroep Levensmiddelentechnologie, onder begeleiding van prof. dr. ir. Hans Tramper. Vanuit de sectie Proceskunde werd in 1989 een zesmaandse stage doorgebracht bij het Amerikaanse biofarmaceutische bedrijf Amgen, gevestigd te Thousand Oaks, California. In augustus 1989 werd het ingenieursdiploma behaald in de chemisch-biologische richting met biotechnologisch profiel. Vervolgens was Robert bijna vijf maanden als vrijwilliger werkzaam aan de Volkshogeschool van het "Centre Socio-culturel Franco-Néerlandais de Méridon" te Chevreuse, Frankrijk. Vanaf juli 1991 was hij aangesteld aan de Dr. Daniel den Hoed Kliniek te Rotterdam, waar het in dit proefschrift beschreven onderzoek werd uitgevoerd onder begeleiding van dr. Leendert Looijenga en prof. dr. Wolter Oosterhuis, in het kader van een door de Nederlandse Kankerbestrijding gefinancierd project.