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Regulation of Male Germ Cell Apoptosis by Sphingosine-1-Phosphate

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ACADEMIC DISSERTATION

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To my Mum

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Summary

Testicular tissue is extremely sensitive to external stress such as cancer treatments, which induce inappropriate germ cell apoptosis leading to defects in reproductive health (1, 2). For postpubertal men, cryopreservation of sperm or testicular tissue is a proven method for preserving fertility against the effects of these treatments (3). Unfortunately, no such method exists for prepubertal boys, whose spermatogenesis has not yet begun (3). For these boys, the best way to preserve fertility would be to protect the spermatogenic stem cells against apoptosis induced by cancer treatments *in vivo*.

Strategies aimed at cell protection should be directed at inhibiting the early signals in apoptosis cascades (4). Sphingolipids are powerful mediators of diverse cellular processes, such as apoptosis and cellular differentiation, and thus hold great promise in different fields of cancer research (5). The sphingolipid metabolites ceramide and sphingosine-1-phosphate (S1P) represent early mediators of apoptosis (6). Ceramide plays an important role in the induction of apoptosis, whereas S1P is considered to be a survival factor. S1P was recently demonstrated to block mouse oocyte apoptosis induced by cancer therapies *in vitro* and *in vivo* (7), suggesting that modulation of the sphinoglipid pathway is a reasonable attempt to protect germ cells from unwanted cell death. Little, however, is known of how S1P exerts its antiapoptotic effects. Initially, S1P was suggested to act as an intracellular signaling molecule and the balance between the intracellular levels of S1P and ceramide to be crucial in determining whether the cell will survive or die. The issue is, however, complicated by the latest findings that S1P not only acts as an intracellular second messenger but also as a ligand to its receptors, recently renamed as $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$.

In the present study, we aimed at evaluating the inhibitory potential of S1P against male germ cell apoptosis induced by external stress *in vitro* and *in vivo* and at characterizing the mechanisms, by which S1P inhibits this apoptosis. The *in vitro* studies, in which culture of human seminiferous tubules was used as a model for external stress situations, showed that during male germ cell apoptosis testicular ceramide levels increase rapidly before initiation of caspase 3 activation and apoptotic DNA fragmentation and that in cultured human seminiferous tubules, germ cell death can be inhibited by exogenous S1P. Consistent with

previous findings in the rodent testis, we detected expression of the $S1P_1$ and $S1P_2$ receptors in somatic Sertoli cells. S1P, however, appears to inhibit male germ cell apoptosis of the human testis independently of its receptors. We demonstrated that intracellularly generated S1P inhibits transcription factor nuclear factor κB (NF-κB), which in turn is regulated upstream by Akt. The transcription factor activator protein 1 (AP-1), which regulates diverse cellular pathways including apoptosis, and is suggested to play a role in the regulation of spermatogenesis, appears to play no role in S1P-related inhibition of male germ cell death.

The potential ability of S1P to protect male germ cells against irradiation-induced cell death in an animal model was also investigated *in vivo*. In mice, irradiation mainly damaged the early developmental stages of spermatogonia. After 21 d, testes treated with S1P before irradiation showed modest preservation of early spermatogonia, as detected by DNA flow cytometric analysis of 4C population of spermatocytes: the number of these cells was reduced in irradiated, but not in irradiated plus S1P-protected testes.

The present results show that apoptosis of male germ cells involves sphingolipids and can be partially inhibited by S1P. Our findings are important in understanding the mechanism of germ cell death and in attempts to find ways to prevent its inappropriate occurrence, although much work needs to be done before S1P can be considered as a potential therapeutic agent against male germ cell apoptosis. By manipulating the enzymes that regulate the intracellular sphingolipid rheostat, the intracellular level of S1P may be adjusted to the level needed for optimal inhibition of unwanted male germ cell apoptosis caused by external stress. Alternatively, other as yet unknown compounds, possibly in the sphingolipid pathway, may prove to be more effective than S1P.

Original publications

This thesis is based on the following original publications, which are referred to in the text by Roman numerals.

I Suomalainen L, Hakala JK, Pentikäinen V, Otala M, Erkkilä K, Pentikäinen MO, Dunkel L 2003 Sphingosine-1-phosphate in inhibition of male germ cell apoptosis in the human testis. J Clin Endocrinol Metab 88:5572-9

II Suomalainen L, Pentikäinen V, Dunkel L 2004 Inhibition of male germ cell apoptosis of the human testis by sphingosine-1-phosphate is independent of its receptors. Am J Pathol. In press

III Suomalainen L, Dunkel L, Ketola I, Eriksson M, Erkkilä K, Oksjoki R, Taari K, Heikinheimo M and Pentikäinen V 2004 Activation protein -1 in human male germ cell apoptosis. Mol Hum Reprod. Aug 6 Epub ahead of print

IV Otala M*, Suomalainen L*, Pentikäinen MO, Kovanen P, Tenhunen M, Erkkilä K, Toppari J, Dunkel L 2003 Protection from radiation-induced male germ cell loss by sphingosine-1-phosphate. Biol Reprod. Mar 70(3):759-67
*these authors contributed equally

In addition, some unpublished data are presented.

Abbreviations

Apaf-1	Apoptotic protease-activating factor 1
ASMase	Acid sphingomyelinase
ASMKO	Acid sphingomyelinase knockout
AP-1	Activator protein 1
cAMP	cyclic adenosine monophosphate
DMS	*N,N* dimethylsphingosine
Cyt-c	Cytochrome c
dS1P	Dihydrosphingosine-1-phosphate
EDG	Endothelial differentation gene
EMSA	Electron mobility shift assay
ERK	Extracellular signal-regulated kinase
E2	17β estradiol
FasL	Fas ligand
FB 1	Fumonisin B1
FSH	Follicle-stimulating Hormone
GnRH	Gonadotropin-stimulating Hormone
Gy	Gray
ICSI	Intracytoplasmic sperm injection
Iκβ	Inhibitory κB
Iκβα	Inhibitory κBα
IKK	Inhibitory kappa kinase
ISEL	*In situ* end labeling
JNK	c-Jun terminal kinase
KCN	Potassium cyanide
KO	Knockout
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
NAC	n-acetyl-L-kysteine
NF-κB	Nuclear Factor κB
NSMASE	Neutral sphingomyelinase
PI3	Phosphatidyl inositol
PI3K	Phosphatidyl inositol kinase
PBS	Phosphate buffered saline
PT	Permeability transition
ROS	Reactive oxygen species
S1P	Sphingosine-1-phosphate
S1PR	Sphingosine-1-phosphate receptor
SM	Sphingomyelin
Smase	Sphingomyelinase
SPHK	Sphingosine kinase
TNF	Tumor necrosis factor
WT	Wild type

Review of the literature

Introduction

Childhood cancer treatments

With advancement in cancer treatments the overall survival rate in most childhood cancers is almost 85% (1). In boys destruction of the spermatogonial stem cells in the testis is a severe side effect of chemotherapy and radiation. These treatments may lead to failure of spermatozoa formation after puberty and consequent infertility in adulthood (1), which is physiologically one of the most traumatic late complications among long-term survivors and one of the major factors decreasing their quality of life (8). Fertility in adult men can be restored by cryopreservation of spermatozoa before cancer treatments (1, 3). Prepubertal boys, unfortunately, cannot benefit from this option, due to the absence of haploid gametes (1, 3). In men preservation of fertility may also be achieved by germ cell transplantation or by *in vitro* maturation (1-3). Alternatively, rendering the testis tissue quiescent during cytotoxic treatments may protect the germ cells from subsequent damage (1, 2). Despite many attempts, however, these methods have not yet benefited prepubertal boys (1-3).

Since most cancer treatments aim at induction of apoptosis in cancer cells (9, 10), modulation of the apoptotic response by manipulating specific apoptotic signaling pathways may prove useful in attempts to protect normal cells against the side effects of these treatments (9). A body of evidence indicates that certain therapeutic approaches that regulate cell membrane-bound sphingomyelin (SM) metabolism or endogenous concentrations of its metabolites may be one such alternative (5). Promising results were obtained in female mice, in which oocytes were protected against irradiation-induced ovarian failure by means of sphingosine-1-phosphate (S1P) given before irradiation (7). The use of S1P in male germ cell preservation, however, remains unknown.

Germ cells in testes of prepubertal boys

Although the prepubertal testis does not complete spermatogenesis and produce mature spermatozoa, it displays significant cellular activity essential for normal adult function (11, 12). The testis triples its volume between birth and the onset of puberty (13). The seminiferous epithelium of normal infant and child testes generally consists of immature Sertoli cells and different types of spermatogonia (14). These spermatogonia proliferate by mitosis but do not enter meiosis. This increase in spermatogonial number is essential for the achievement of quantitatively normal sperm production in adult life (15). Although the prepubertal testis consists of some spermatocytes and spermatids and isolated phases of spermatogonial proliferation followed by the transient appearance of primary meiotic spermatocytes and spermatids exist, such germ cells degenerate and fail to develop into spermatozoa (11, 15).

Effect of anti cancer treatments on spermatogenesis

Gonadal damage in prepubertal boys may result from systemic chemotherapy or radiotherapy involving the spinal or pelvic area. These treatments lead to destruction of the spermatogonial stem cells, involving also the somatic Sertoli- and Leydig cells, although these cells are rather resistant to gonadotoxic therapy (16-18). Androgen production may therefore be restored and secondary sexual characteristics of these boys develop normally even when patients are infertile. Cancer treatments may also lead to the Sertoli cell only syndrome that represents the seminiferous tubules, in which the germ cells are completely absent (19).

Cytotoxic treatments target rapidly dividing cells and are therefore especially damaging to the differentiating spermatogonia and stem cells (2, 16). The degree of injury on the spermatogenic epithelium is dependent on the type, combination, and dosage of the chemotherapy received (18). Drugs known to cause germ cell damage include procarbazine, vinblastine, cytosine arabinoside, and the alkylating agents, particularly cyclophosphamide, chlorambucil, mechlorectamine, and the nitrosureas (18).

In prepubertal cancer patients, radiation-induced gonadal damage is most often observed following direct testicular irradiation for treatment of testicular relapse of leukemia, or following total body irradiation given prior to bone marrow transplantation (2). The most sensitive germ cells are the spermatogonia; doses of radiation as low as 0.1 Gy are known to cause damage to these cells (20-22). Doses of radiation from 0.1 to 0.3 Gy cause temporary oligospermia, and after 0.3-2.0-Gy, the recovery requires as long as 30 months. Recovery generally occurs, if the doses remain under 1-2 Gy; doses exceeding 2-3 Gy, however, induce irreversible germinal damage accompanied by increased levels of follicle–stimulating hormone (FSH) and decrease in testicular volume (18, 23). Somatic cells are more resistant to radiation therapy than germ cells, Leydig cell function is usually preserved following doses as high as 12 Gy (2).

Options for fertility preservation

Cryopreservation of testicular spermatozoa

Cryopreservation of spermatozoa is commonly used in preservation of fertility for adult cancer patients (1-3), while advances in techniques of assisted reproduction, especially intracytoplasmic sperm injection (ICSI), have enabled men with oligospermia to become fathers (3, 8). Unfortunately, no equivalent method is available for prepubertal boys with absence of haploid gametes (1-3).

Cryopreservation of testicular tissue

Testicular tissue from men was successfully cryopreserved as cell suspensions (24) or as pieces of tissue (25). Healthy offspring were born as a result of ICSI carried out using spermatozoa after both freezing methods (3). To maintain the reproductive capacity of prepubertal boys, the aim would be to cryopreserve the spermatogonia (3), which are large cells and located in the basal part of the seminiferous epithelium. Since human testicular tissue is relatively dense, its dispersion in cell suspensions is unknown (3). In theory, spermatogonia could be harvested from a biopsy and stored prior to cancer therapy, either as a segment of tissue or isolated germ cells (3). After the original disease has been cured, reimplantation of frozen thawed testicular cells back to the seminiferous tubules could be one method of initiating spermatogenesis (3). This method is possible in rodents, and attempts at injecting frozen thawed cell suspensions were carried out in human adult

males (3). In the rat model, however, injection of as few as 20 leukemic cells mixed with germ cells was able to cause leukemia in the recipient (26). Thus, if a boy has a hematological malignancy, high risk ensues of transplanting cancer cells back to him after the cure (3). For such boys, maturation of spermatozoa *in vitro* may be a better option. Clinically, however, the low number of spermatogonia makes this option extremely difficult to carry out succesfully.

In vitro maturation

Maturing frozen thawed spermatogonia *in vitro* aims at stimulating their differentation into spermatozoa under culture conditions (3). In mice, spermatogonial stem cells survive for up to four months in culture, retaining the ability to initiate spermatogenesis following transplantation back into a recipient (27). Complete spermatogenesis *in vitro* seems nevertheless a remote possibility, since it has not yet been successfully demontrated in any animal species (3).

Germ cell transplantation

The technique of spermatogonial stem-cell transplantation was introduced in 1994 (28). Injection of germ cell suspensions from donor mice into genetically sterile mice resulted in restoration of spermatogenesis from the donor stem cells (28). Similar results were demonstrated in recipient mice that received sterilizing treatment with busulfan (28). In addition, successful transfer of germ cells between species was demonstrated, with restoration of rat spermatogenesis following transplantation of rat germ cells into the seminiferous tubules of mice (29). Possibly due to presence of inappropriate microenvironments for the proliferation of donor spermatogonia, germ cell transplantation between phylogenetically more distant species, such as from rabbits and dogs into mice, has not been successful (30). Successful spermatogenesis, however, was achieved following xeno grafting of testicular tissue from mice, pigs, and goats into castrated, immunodeficient mice (31). Although the risk of reintroducing malignant cells would be eliminated, the clinical application of germ cell transplantation carries a high risk of inter species transfer of potentially pathogenic micro organisms (2).

Pharmacological protection of testicular tissue

The hypothesis that prepubertal boys have low rates of permanent chemotherapy-induced gonadal damage has led many investigators to believe

that suppression of testicular function at the prepubertal level may provide protection against cytotoxic therapy (32). Although in humans no evidence for this exists, promising results were achieved in animal studies (33). Recovery of spermatogenesis with hormone treatments was demonstrated in procarbazine-treated rats by administration of the gonadotropin-releasing hormone (GnRH) analogue Zoladex for two weeks prior to and during chemotherapy (34). Similar effects were described following the use of testosterone (35), testosterone and estradiol (36), and GnRH antagonist combined with testosterone (37) following gonadal insult with chemotheraphy or irradiation. Although these hormone treatments showed promising results in rats even when given after irradiation (38), clinical trials of hormonal manipulation in patients receiving gonadotoxic therapy failed to demonstrate any advantage (2).

Amifostine, an organic triphosphate that acts through free-radical scavenging, hydrogen donation, and inhibition of DNA damage (39) is one pharmacological compound that shows selective protection in many human tissues against the toxic side effects of radiation and cytotoxic drugs, while preserving the antitumor efficacy of the treatment (39). Reports of its effect on testicular tissue, however, are conflicting (40, 41) and, most importantly, knowledge of its usefulness in children is scarce (40).

Use and design of less toxic chemotherapeutic drugs and refined radiation techniques with less toxic long-term effects will hopefully become one future option for decreasing testicular damage (18).

Cancer treatments and apoptosis

Several cancer treatments aim at eradicating tumor cells by apoptosis (9, 42, see next chapter). Defects in apoptotic machinery or stimulation of survival pathways, however, may prevent the cells from responding to apoptotic stimuli (9). Modulation of the apoptotic response by manipulating specific apoptotic signaling pathways, therefore, may enhance the therapeutic ratios of cancer treatments (9). More importantly, such manipulation may also be useful in attempts to protect normal cells against unwanted apoptosis induced by cancer treatments (9). A growing body of evidence indicates that certain therapeutic approaches that regulate cell membrane-bound SM metabolism or endogenous concentrations of its metabolites may be useful in attempts to manipulate the apoptotic machinery induced by cancer

treatments (5). Proapoptotic ceramide and antiapoptotic S1P are metabolites of SM and represent early mediators of apoptosis (6). Promising results have been obtained in female mice oocytes, in which radiation-induced premature ovarian failure could be prevented by protecting the ovaries with S1P before irradiation. The S1P-treated ovaries retained a normal distribution of follicles as well as overall tissue mass (7). No genetic anomalies in the progeny of irradiated mothers were found (43). Sphingolipid-based therapeutics are currently being under preclinical and clinical investigation, and S1P represents the first agent capable of protecting female germ cells against exposure to irradiation (5). The potential ability of S1P to protect germ cells from apoptosis in the testis, however, remains unknown.

Apoptosis

Apoptosis, also known as programmed cell death, is a regulatory mechanism that eliminates abundant and unwanted cells in an organized fashion during embryonic development, growth, differentation and normal cell turnover (44). The morphological features of apoptosis include condensation of nuclear cromatin, blebbing of the cellular membrane, shrinkage of the cell, and fragmentation of the cell including its nucleus into small apoptotic bodies that are engulfed *in vivo* by macrophages or other cells of the mononuclear phagocyte system (45). A normal degree of apoptosis is important for adequate development of organisms and maintenance of homeostasis, whereas excessive or inadequate levels of apoptosis are associated with disease processes and are critical factors in the pathogenesis of a number of diseases (45). The capability for specifically manipulating cell death, *i.e.* increasing or decreasing its occurrence, thus holds great promise for the future development of therapies (46).

Apoptotic cell death machinery consists of a complex network of tightly controlled signaling pathways (Fig. 1). Which apoptotic signal the cell death machinery conducts is dependent on the death-inducing stimululus, the surrounding environment, and the type of cell (47).

Apoptotic pathways in general

Caspases

The caspases are a family of cysteine proteases that are able to cleave their substrates, transforming procaspase into active caspase (47-50). They can be roughly divided into initiator and effector caspases (51). The initiator caspases (caspases 2, 8, 9, and 10) interact with various caspase-activating proteins. After activation, they cleave the effector caspases (caspases 3, 6, and 7) that amplify the apoptotic signal by cleaving the substrates responsible for the morphological changes characteristic of apoptotic cell death (52). Caspases can be activated via the mitochondrial or death receptor pathways. Whatever the pathway, the final results are specific and the uniform morphologic features of apoptosis consistent in different tissues and organisms (48-50).

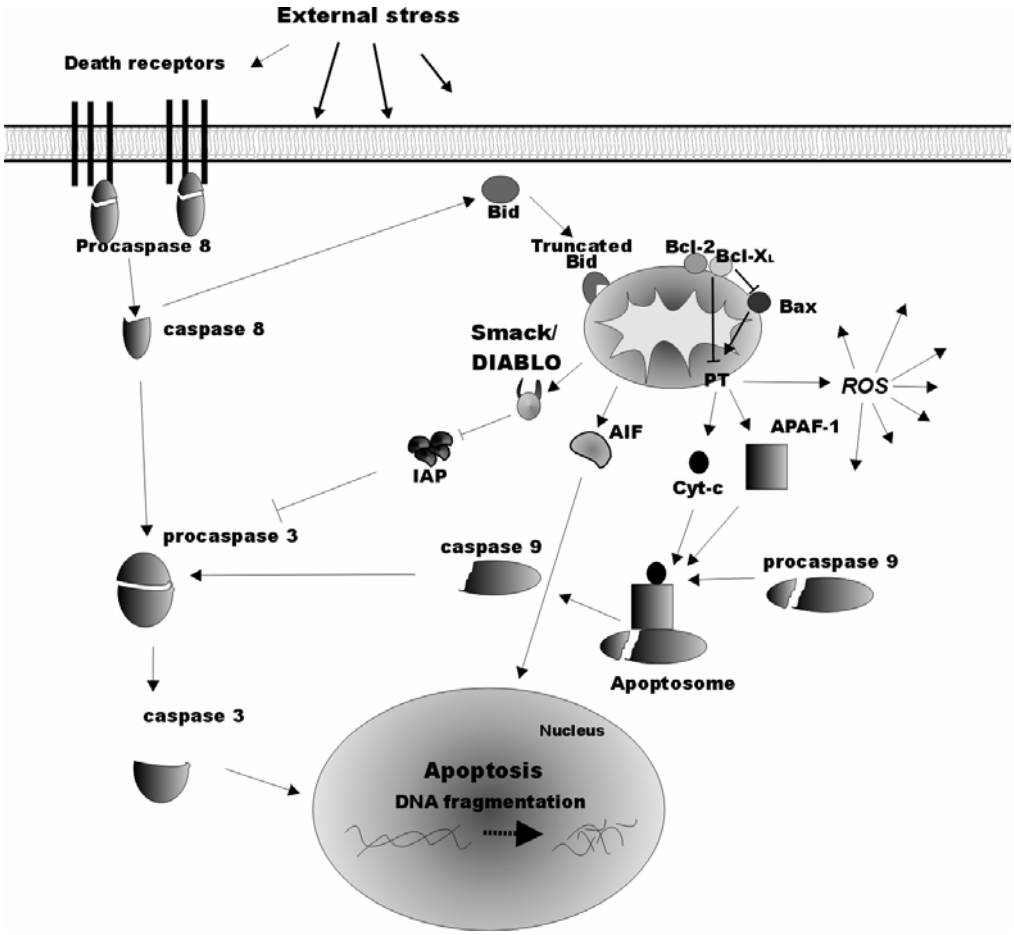

Figure 1. Schematic illustration demonstrating the cell death machinery. The death receptor pathway can be triggered by members of the death receptor superfamily, such as Fas and TNFR1. Activation of the death receptors is followed by recruitment of several adapter proteins and consequent activation of procaspase 8. In the mitochondria-independent pathway, active caspase 8 directly activates effector caspases, such as caspase 3 and 7. In the mitochondria-dependent pathway, caspase 8 cleaves proapoptotic Bid, which translocates to the mitochondria. The truncated Bid, together with other proapoptotic Bcl-family proteins, such as Bax, participates in mitochondrial events, such as membrane permeability transition (PT) and release of cytochrome c (cyt-c). The anti-apoptotic Bcl-family members (Such as Bcl-2 and Bcl- X_L) counteracts these events. Various caspase-independent stimuli can also induce the mitochondrial events and the release of cyt-c, apoptosis-inducing factor (AIF), Smack/DIABLO (second mitochondria-derived activator of caspases/direct IAP-binding protein with a low isoelectric point), and several other apoptosis-inducing factors. Reactive oxygen species (ROS), formed in the mitochondria during apoptosis, are able to regulate the cell death machinery at several levels. Cyt-c, released from the mitochonria, associates with caspase adaptor Apaf-1 and procaspase 9 to form the apoptosome. Smack/DIABLO antagonizes procaspase 3 by binding to and inhibiting the inhibitor of apoptosis proteins (IAPs).

Death receptor pathway

Apoptosis can be induced by ligation of a subset of plasma membrane tumor necrosis factor receptor (TNFR) family members, referred to as death receptors (53, 54). After binding of specific death ligands, they can activate caspases within seconds and cause apoptotic cell death within hours (53, 54). The best characterized subset of the death receptors are Fas (CD95/Apo-1) and TNFR1 (p55/CD120a) (53, 54). The ligands that bind to the death receptors, including FasL (CD95L) and TNFα, are structurally related proteins that belong to the TNF subfamily (53, 54). After ligation, the resulting complex recruits several molecules of procaspase 8, resulting in autocatalytic liberation of active caspase 8, which is considered to be the key initiator caspase in the death receptor pathway (47). After this point, active caspase 8 may activate other caspases or death messengers, mediating the apoptosis signal via separate pathways depending on the cell and stimulus (47).

Mitochondrial pathway

The mitochondria are important regulators of oxidative conditions and the main cellular source of reactive oxygen species (ROS) (55, 56). Diverse

apoptotic pathways converge toward the mitochondria and trigger permeability transition (PT) both in the inner and outer mitochondrial membranes (55, 57, 58). The Bcl-2 family members play an important role in mitochondrial apoptotic signaling pathways. The apoptotic stimuli cause a disequilibrium between the pro- and antiapoptotic family members that are located in the outer membrane of mitochondria or in the cytosol. Translocation of proapoptotic family members to the mitochondrial outer membrane leads to the release of cyt-c (or other mitochondrial proapoptotic factors) (57, 59-61). After accumulation in the cytoplasm, cyt-c binds to and oligomerizes apoptotic protease activating factor (Apaf-1) (55, 57, 58). Procaspase 9 then binds to Apaf-1 resulting in a formation of apoptosome, which is a high-molecular-mass complex that contains cyt-c, Apaf-1, and procaspase 9 (55, 57, 58). The interaction of procaspase 9 and Apaf-1 leads to formation of active caspase 9, which in turn proteolytically activates caspase 3 (57, 59, 60).

Caspase-independent apoptosis

Although caspase 3 is the primary effector caspase in most apoptotic cascades, caspase 3-independent and even caspase-independent cell deaths have been described (4, 62, 63) For example, apoptosis can be induced by overexpression of Bax (a proapoptotic Bcl-2 family member), even when the activity of caspases is blocked. This apoptosis is apparently due to mitochondrial energy depletion and generation of ROS (4, 47). Indeed, a variety of noncaspase proteases may induce apoptosis by triggering the mitochondrial pathway (47). For example, apoptosis-inducing factor (AIF) and endonuclease G may be released from mitochondria in either a caspase-independent or -dependent manner and be translocated to the nucleus resulting in chromatin condensation (64).

Activator protein 1

Activator protein 1 (AP-1) is a transcription factor involved in a broad range of biological responses such as proliferation, transformation, cell differentiation, cell migration, and apoptosis (65-67). AP-1 activity can be induced by multiple environmental insults and physiological stimuli that activate mitogen-activated protein kinase (MAPK) cascades (65, 67). Three well-characterized subfamilies of MAPKs are present in multicellular

organisms: the extracellular signal-regulated kinase (ERK 1/2), the stress-activated c-Jun aminoterminal kinases (JNKs), and the p38 enzymes (65, 67).

AP-1 comprises a family of transcription factors including Jun and Fos subfamilies (68). After dimerization, these subunits bind to a common AP-1 DNA-binding site where they regulate different target genes and thus execute diverse and distinct biological functions (65). AP-1 activation during apoptosis appears to be highly regulated in tissue -and developmental stage-specific manner (69-73). Although its activation enhances cell survival and proliferation, it also contributes to induction of apoptosis, especially under extreme conditions (65-68).

Nuclear factor kappa B

Nuclear factor kappa B (NF-κB) transcription factor proteins regulate genes that are involved in apoptosis, proliferation, migration, and stress, inflammatory, and immune responses (74-76). Prior to activation, NF-κB proteins are sequestered into the cytosol by inhibitory kappa B (IκB) proteins that release NF-κB after degradation, allowing its translocation into the nucleus (77). The degradation of IκB is controlled by the IκB kinase (IKK) complex, which in turn is regulated by several mechanisms, including the phosphatidyl inositol (PI3)/Akt kinase cascade (78).

NF-κB transcription factors play pivotal roles in the regulation of pro and antiapoptotic pathways, depending on the inducing agent and cell type (79, 80). NF-κB is usually cytoprotective and its inhibitory effect was observed in certain cells in response to certain external stimuli, such as TNF, ionizing radiation, and chemotherapeutic compounds (79, 80). A growing body of evidence, however, indicates the presence apoptosis-inducing functions in NF-κB (81-83). NF-κB-related control of apoptosis is important in normal processes, such as liver development, immune balance and homeostasis, as well as in pathological conditions such as cancer, and neurodegenerative diseases (79, 80). Therefore, agents that affect the ability of NF-κB to control apoptosis are likely to be clinically relevant (84).

Phosphatidyl inositol kinase

The PI3 kinases are a family of lipid kinases that are involved in cell survival and proliferation in several cell types. An important result of PI3 phosphorylation is activation and relocalization of cytoplasmic Akt (also known as PKB) at the plasma membrane (78, 85-87). The PI3/Akt kinase cascade regulates multiple cellular processes such as transcription, proliferation, angiogenesis, motility, survival, and apoptosis (78). Akt regulates apoptosis at multiple sites by directly phosphorylating components of the cell death machinery or by indirectly changing the levels of expression of genes that codes for the components of cell death machinery (78). The PI3/Akt kinase cascade is able to regulate NF-κB activity by regulating IKK (78, 88).

The sphingolipid pathway

Sphingolipids are ubiquitous components of eukaryotic cell membranes, which by regulating apoptosis act as rheorstats that determine the fate of the cell (89). SM, located in the outer layer of the plasma membrane, comprises more than half of all plasma membrane-bound phospholipids. A variety of stimuli can induce enzymatic cleavage of SM, resulting in the release of bioactive messenger molecules that influence proliferation, differentation, and survival of various cell types (Fig. 2) (90-92).

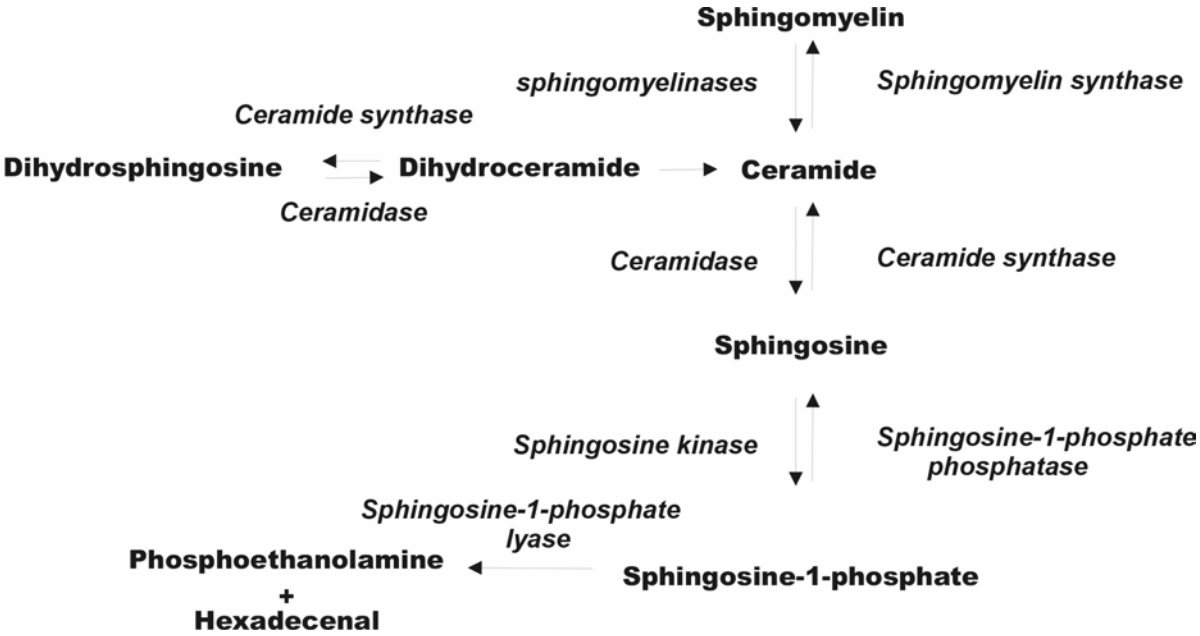

Figure 2. Sphingolipid metabolism

Ceramide

Ceramide acts as a ubiquitous second messenger in a number of evolutionary conserved signaling systems that regulate diverse cellular processes including apoptosis, the cell cycle, and cellular differentiation (93). It is a main metabolite of SM and can be formed from SM by sphingomyelinases (SMases) or *de novo* by the enzyme ceramide synthetase. Whatever ceramide pathway is activated, is highly dependent on the cell and stimulus (94). SMases that hydrolyze SM rapidly are classified into two main groups according to the pH optimum. Acid sphingomyelinase (ASMase) is a lysosomal enzyme that displays a pH optimum of 4.5 (92). Deficiency of ASMase causes Niemann-Pick disease in humans (95). Several reports provided evidence that ASMase is the main enzyme for generating ceramide and mediating cell differentiation and apoptosis (96-98). Neutral sphingomyelinase (NSMase), with a neutral pH optimum has an association different from that of ASMase in the plasma membranes. Two different forms of NSMase are known: a membrane-associated Mg 2^+-dependent and a cytosolic Mg 2^+-independent NSMase (92). Activation of NSMase may be modulated by the cellular redox state, with glutathione being a prominent negative regulator of NSMase activity (99). NSMase-related ceramide formation plays an important role in induction of apoptosis in many cell types (100).

Alternatively, ceramide can be synthesized at the initial step of the SM signaling pathway by enhanced *de novo* synthesis from sphingosine or palmitoyl-CoA and serine by the enzyme ceramide synthetase (92). This pathway can be stimulated by drugs and ionizing radiation and it usually results in a prolonged ceramide elevation (91). Indeed, several anticancer agents act by increasing tumor cell ceramide via this pathway (91).

Increased ceramide levels and exogenously added cell-permeable ceramide analogs induce apoptosis in several cell lines (101, 102). A wide range of inducing agents, which are considered cytotoxic or stressful, accumulate ceramide in the cell. These agents include TNFα, Fas ligation, interleukin-1, serum deprivation, heat shock, ischemia, ultraviolet light, radiation, and chemotherapeutic agents such as cytosine arabinoside, vincristine, and daunorubicin (91, 93, 103). Ceramide regulates apotosis via multiple signaling pathways and activates a number of enzymes involved in stress signaling

cascades (93). In mitochondria, it is able to form large stable pores in mitochondrial membranes, allowing cyt-c to be released in the cytosol (104). In addition, it may influence mitochondrial apoptosis signaling via the proapoptotic Bcl-2 family and generation of ROS (105). Whether it acts upstream or downstream caspases, remains unclear (106). Ceramide can be converted back to SM by SM synthase or alternatively into a variety of metabolites including glucosylceramides, ceramide-1-phosphate, sphingosine and S1P (91).

Sphingosine

Sphingosine is degraded from ceramide by ceramidase (107). It´s functions in the cell are controversial, depending on the enzyme activities, it may act as an inductor of apoptosis and negative regulator of cell proliferation or as a mitogen (92). The level of sphingosine in the cell is extremely low, since the entzyme sphingosine kinase (SPHK) is able to rapidly phosphorylyze sphingosine into S1P and thus catalyze its mitogenic functions (92, 107). Dephosphorylation of S1P back into sphingosine by S1P phosphatase, in turn, potentiates apoptosis (92). Which molecule will finally predominate and initiate the cell response is dependent of the rapid interconversion of the lipid molecules ceramide, sphingosine and S1P, which vary depending on the expression pattern of the cell and activity of the converting enzymes (92, 107).

Sphingosine kinase

SPHK is predominantly a cytosolic enzyme and critical regulator of the sphingolipid rheostat (108). SPHK is encoded by a highly conserved gene family (109) and is activated by numerous external stimuli, in which growth and survival factors are the most prominent (110). Increase in SPHK activity leads to increased level of intracellular S1P, with concomitant decrease in the level of sphingosine and, to a lesser extent, ceramide (111, 112). SPHK and its activation, therefore, play a central role in controlling the cellular effects of S1P.

Sphingosine-1-phosphate

S1P potentiates cell survival and inhibits apoptosis in several cell types (89, 113-115). Initially, S1P was proposed to act as a direct intracellular second messenger, and the balance between S1P and its antagonist ceramide to determine whether the cell will survive or undergo apoptosis (116). More recently, the extracellular first messenger effects of S1P have been discovered; it acts as a ligand for a family of G-protein-coupled receptors that are expressed widely and differentially in several tissues (117). Depending on the abundance of S1P receptors (S1PR) and associated G-proteins, S1P is able to activate and regulate diverse signal transduction pathways in different cell types (110).

S1P as a direct intracellular second messenger

A variety of growth factors and cytokines activate SPHK thereby increasing the intracellular levels of S1P (115). This intracellularly generated S1P acts as a second messenger in cellular proliferation and survival as well as in protection against ceramide-mediated apoptosis (116, 118-121). The basal cellular level of S1P is low (115, 122) and tightly regulated by the balance between synthesis and degradation (115, 122). The inhibitory effect of S1P against ceramide-mediated cell death was originally proposed to be due to intracellularly generated S1P (116), supported by findings that the effects of S1P on ERK and JNK (116), and on caspases (118) are opposed by elevated levels of intracellular ceramide. Secondly, dihydroS1P (dS1P), an analog of S1P that binds to S1PRs but does not act as an intracellular second messenger, is not able to mimic all of the effects of S1P, especially those related to cell survival (119). For example, induction of ceramide-mediated apoptosis by the anticancer drug doxorubicin in unfertilized mouse oocytes is blocked by S1P, but not by dS1P or the ceramide synthetase inhibitor Fumonisin B1 (FB1) (7). Moreover, the protective effect of S1P appeared entirely independent of the S1P receptors coupled to G_i (123). Although several effects of intracellularly generated S1P have been elucidated, the direct intracellular targets of S1P remain unclear.

Receptor-dependent signaling of S1P

The prototype of the endothelial differentation gene (EDG) -receptor family, EDG-1, was cloned in 1990 and soon after, the G-protein-coupled receptors EDG -1,3,5,6, and 8 were found to be specific for S1P (113, 124). The high affinity S1P receptors EDG -1,3,5,6, and 8, were recently renamed as $S1P_{1,}$ $S1P_{2,}$ $S1P_{3,}$ $S1P_{4,}$ and $S1P_5$, respectively (125). Most tissues express one or more S1PR subtypes that couple differentially to the G-proteins $G_1/G_{0,}$ G_q, G_{12}, and G_{13} with high affinity (115, 117). Binding of S1P to its receptors activates diverse signaling pathways, including MAPK and PI3K (126), second messenger systems such as cyclic adenosine monophosphate (cAMP) (6) and Ca^{2+} (127), and regulation of the Rho family of small GTPases (important in carcinogenesis, proliferation, apoptosis, cytoarchitecture, migration, and regulation of transcription factors) (128, 129). Despite the diversity, all S1PR affect cell motility, which is an important feature of many physiological and pathological processes (110). Indeed, many significant functions of S1P such as angiogenesis, vascular maturation, neurite retraction, and heart development are mediated through S1PR (130, 131). Whether the regulation of apoptosis by S1P is related to its receptors, however, remains unclear.

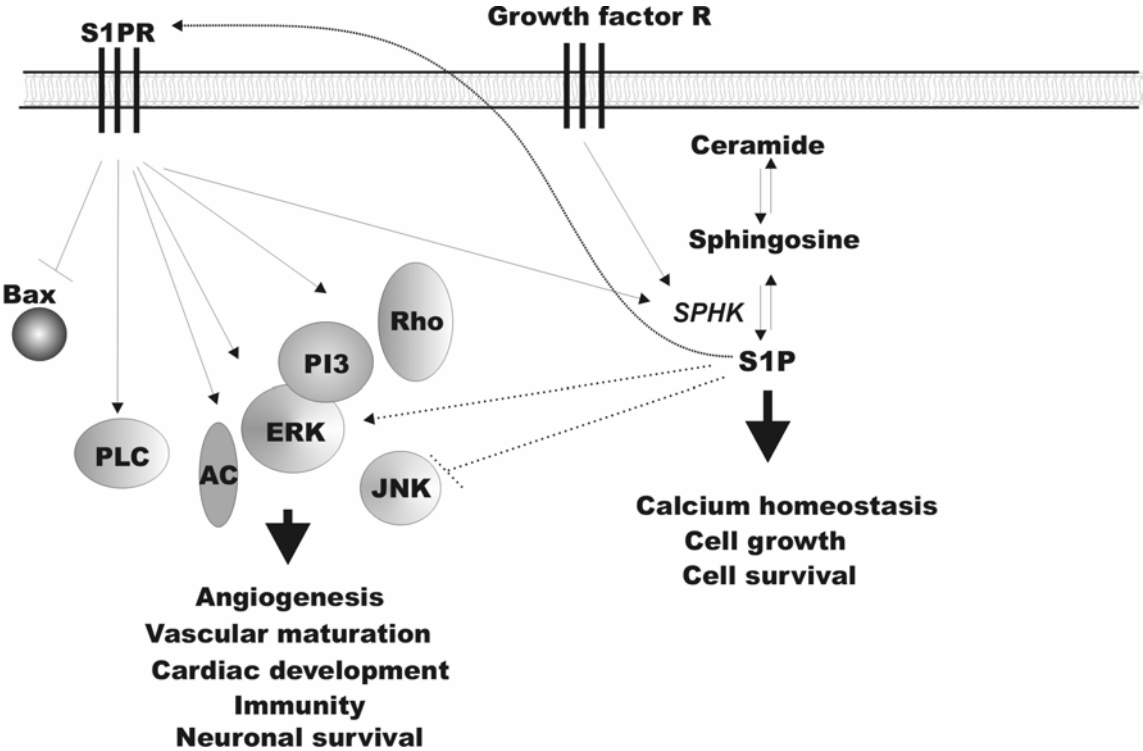

Figure 3. Major signaling pathways of S1PR and intracellularly generated S1P. Several important functions of S1P are mediated inside-out through S1PR. These include activation of different pathways such as of Rho, adenylatee cyclase (AC), PI3, ERK, and phospholipase C (PLC). Intracellularly generated S1P that acts as a second messenger contributes to many cellular effects often associated with cell survival independently of S1PR. The specific targets of intracellularly generated S1P, however, remain unknown.

Sphingolipids as therapeutic agents

Regulation of the sphingolipid rheostat has implications for the treatment of cancer, since many cancer treatments, including chemotherapeutic drugs and irradiation, cause accumulation of ceramide (91, 110, 132). Manipulation of ceramide levels enhance the effectiveness of some cancer therapies by selectively enhancing apoptosis of tumor cells. For example, in the liver, potent ceramidase inhibitor B13 increases the ceramide content of tumor cells and tumor cell apoptosis without affecting the ceramide level or survival of normal cells (91). Furthermore, dysregulation of ceramide metabolism may

contribute to multidrug, -and radiation resistance of various cancers, since increased levels of glycosylceramide and decreased levels of ceramide are found in drug-resistant tumors (91). Preventing the degradation of ceramide to non toxic metabolites such as glycosylceramide, may therefore increase the potency of some chemotheraputic agents (91). Finally, the ability of ceramide and ceramide-generating drugs to induce cytotoxity in cancer cells lacking p53 suggests that ceramide-based therapeutics are active in some cases, where resistance to DNA-damaging agents is high (91).

Blockade of S1P biosynthesis by SPHK inhibitors may be useful in amplifying ceramide-mediated apoptosis signals (110). Inhibition of SPHK increases induction of apoptosis in cancer cells and enhances sensitivity to gamma radiation (110). An inhibitor of SPHK, *N,N* dimethylsphingosine (DMS) is ubiquitous inductor of apoptosis in cancer cells (110). In addition, an analog of sphingosine, L-threo-dihydrosphingosine (known as safingol), which also acts as an inhibitor of SPHK, enhances doxorubicin accumulation and sensitivity in drug resistant breast cancer cell line MCF-7 (110).

In several cell types, radiation acts directly on the plasma membrane, activating ASMase, which in turn generates ceramide (132). Recent *in vivo* studies showed that radiation targets ASMase pathway of microvascular endothelial cells in the lungs, intestines, brain, and oocytes, initiating the pathogenesis of tissue damage (132). In the intestines of mice, the lethal gastrointestinal syndrome that limits the efficiency of radiation and chemotherapy, could be prevented by genetic inactivation of ASMase and by basic fibroblast growth factor (bFGF) that acts in part by suppressing ASMase activity (133). In the ovaries of mice, ASMase-deficient oocytes cultured with doxorubicin failed to show the signs of apoptosis seen in wild-type (WT) mice (7). More importantly, the equivalent level of reduced ovarian germ cell apoptosis was achieved by genetic disruption with ASMase and treatment with 10 μmol/L S1P *ex vivo* (7). Consequently, radiation-induced germ cell apoptosis was effectively inhibited by *in vivo* administration of S1P prior to 0.1 Gy radiation, the amount that usually results in destruction of the primordial follicle reserve within 2 weeks (7).

Male germ cell apoptosis

Spermatogenesis

Spermatogenesis occurs in the seminiferous tubules of the testis, which comprises the seminiferous epithelium, the basement membrane and the surrounding peritubular myoid cells (Fig. 4). It is characterized as a continuous and elaborate maturation process of germ cells towards the center of the seminiferous tubules; haploid spermatozoa are produced from diploid spermatogonia through mitosis and meiosis (134, 135). Germ cells at various phases of differentiation are arranged in defined cellular associations, *i.e.* stages that enter the spermatogenic process at regular time intervals (136, 137). The time interval between appearance of the same stage at certain points in the tubule extends to about 70 d in humans and 35 d in mice (138, 139) and constitutes a single cycle of the seminiferous epithelium (134). The number of stages differs between species; 6 stages have been defined in humans (134) and 12 in mice (140). In rodents, each particular stage occupies a relatively long segment of a seminiferous tubule and therefore only one stage is seen in a tubule cross-section making the stages easily defined (141). In contrast, stages in the human testis are spirally oriented, leading to the typical finding of several irregular cellular associations (138).

Physiological male germ cell apoptosis

Germ cell renewal to achieve homeostasis, proliferation, export and apoptosis must be finely regulated (135). Apoptosis that aims at selective removal of dysfunctional or damaged germ cells and at limitation of the germ cell number is thus a prerequisite for continuous spermatogenesis (135, 137). The first wave of spermatogenesis is initiated when the gonocytes differentiate into spermatogonia. While some spermatogonia become self-renewing spermatogonial stem cells, most differentiate into spermatocytes, and at puberty develop by meiosis in to haploid spermatids (135).

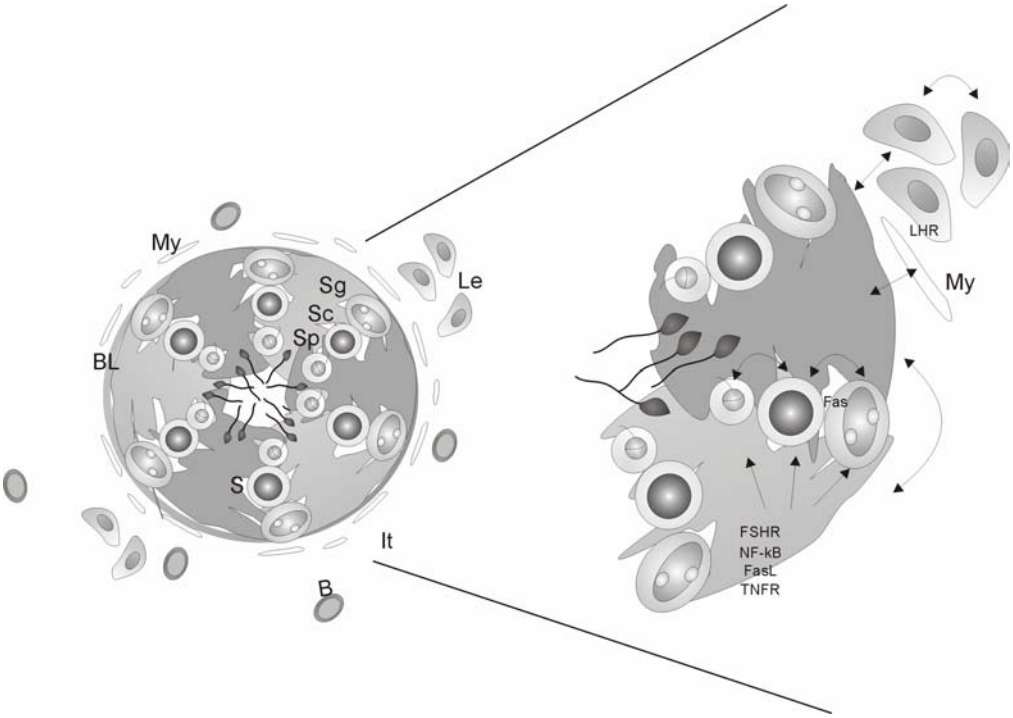

Figure 4. Schematic illustration of the structure of seminiferous epithelium and the interactions between different cell types. Modified from Toppari and Huhtaniemi (142). Spermatogonia (sg), spermatocytes (sc) and spermatids (sp) are in close contact with the supporting Sertoli cells (S). The cells of the seminiferous epithelium are separated from the interstitial tissue by the basal lamina (BL) and peritubular myoid cells (My). Le = Leydig cells, B = blood vessel. The arrows demonstrate the paracrinical and/or autocrinical regulation of the cells.

The first wave of spermatogenesis involves extensive germ cell apoptosis and is mediated by signals derived from closely associated Sertoli cells and by signals that originate outside the testis (135). In the adult, up to 75% of potential spermatozoa degenerate (135). The exact incidence of adult male germ cell apoptosis, however, remains unclear, since some dying germ cells do not show the classical signs of apoptosis (135).

The early apoptotic wave of spermatogenesis may be required to regulate the number of differentiating spermatogonia to fit the capacity of each Sertoli

cells to support the developing germ cells (135). The Sertoli cells, possibly together with the adjacent basement membrane, create a particular microenvironment, termed "niche", which controls the renewal and differentiation of the germ cells by providing nutrition, adhesion and several transport functions (135, 138, 143).

Inappropriate male germ cell apoptosis

In male reproductive health, dysregulation of apoptosis is a cause of several pathological conditions such as germ cell tumors, cryptorchidism, testicular torsion and infertility (144-147). The pathogenesis of these situations involves either the inappropriate occurence of apoptosis, or the absence its appropriate occurence. In addition, several external disturbances such as chemotherapy, radiation, ischemia, toxicant exposure, alterations in hormonal support, and elevated temperature may cause excessive male germ cell death leading to germ cell loss and infertility (23, 148-153).

Hormonal regulation of male germ cell apoptosis

Gonadotropins (FSH, LH), and testosterone are important regulators of germ cell apoptosis (135, 154). Their removal induces apoptosis, which occurs presumably through indirect effects, since hormone receptors are present on somatic cells (135).

FSH

FSH is generally considered to be involved in the initiation of pubertal spermatogenesis. It regulates DNA synthesis, proliferation, and differentiation of spermatogonia and spermiogenesis (15). Observations with FSH-β subunit knockout (KO) mice (155) and patients with homozygous mutation of the FSH receptor (156), indicate that spermatozoa can be produced without FSH, but the number remain small. Thus, while FSH is essential for obtaining a quantitatively normal sperm production, it may not be essential for the qualitative completion of spermatogenesis (15).

FSH inhibits male germ cell apoptosis in cultured rat seminiferous tubules (157), partially via stem cell factor (SCF) produced by Sertoli cells, and interacts with the c-kit receptor in the germ cells (158). This mechanism may involve changes in the Bcl-2 family members, since in cultured rat

seminiferous tubules either FSH or Sertoli cell-derived SCF can regulate antiapoptotic Bcl-w (159, 160).

Androgens

Testosterone, produced by the Leydig cells, plays an indispensable role in spermatogenesis (15). High intratesticular, rather than circulating, levels of testosterone and adequate expression of androgen receptors in Sertoli cells are necessary for the onset of puberty (15). In the human testis, testosterone is effectively able to inhibit *in vitro*-induced apoptosis of spermatocytes and spermatids (161). The antiapoptotic action of testosterone may also be regulated by some of the testicular metabolites of testosterone, such as dihydrotestosterone and estrogens (15).

Estrogens

Estrogens are potential regulators of male reproduction and germ cell death. Low concentrations of 17β estradiol (10^{-9} and 10^{-10} mol/L) are able to inhibit male germ cell apoptosis in cultured human seminiferous tubules (162). Estrogens can also cause alterations in circulating concentrations or gonadotropins and testosterone and thus affect apoptosis in germ cells indirectly (163, 164).

AP-1 in the testis

Several studies suggest the presence of diverse functions of AP-1 in testis development and functions. In the germ cells of mouse testis, AP-1 proteins are expressed in a developmental stage-specifical manner (165). JNK participates in the regulation of mouse spermatogenesis (166) whereas ERK1/2 is induced in sperm maturation (167) and capacitation (168).
JunD -/- male mice exhibit multiple age-dependent defects in reproduction, hormone imbalance, and impaired spermatogenesis, with abnormalities in head and flagellum sperm structures (169). The role played by AP-1 in the regulation of male germ cell apoptosis remains unclear.

NF-κB in the testis

In the rat testis, NF-κB is expressed in the nuclei of Sertoli cells at all stages of spermatogenesis, the intensity of expression varying between the stages

(170). TNFα induces activation of NF-κB, which may lead to the activation of cAMP response element binding protein (CREB) that has been suggested to be a regulator of spermatogenesis (171). In the human testis, low basal NF-κB DNA-binding activity is present in the seminiferous epithelium, and is localized in the Sertoli cells (83). During *in vitro*-induced apoptosis of seminiferous tubules, the Sertoli cell nuclear NF-κB expression and entire seminiferous tubule NF-κB DNA binding activity increase strongly and rapidly before the onset of nuclear germ cell apoptosis (83). Induction of NF-κB DNA binding is completely blocked by the anti-inflammatory drug sulfasalazine concomitantly with suppression of germ cell apoptosis (83).

Sphingolipid pathway in the testis

The SM pathway regulates several functions of somatic testicular cells. In porcine Sertoli cells, it participates in the production of lactate, a crucial metabolite for germ cells (172). In rat Leydig cells, the ceramide-dependent pathway regulates hCG-stimulated Leydig-cell steroidogenesis at the level of cAMP production and at post-cAMP events (173). In the same cells, ceramide generation is completely blocked by the ceramide synthetase inhibitor FB1 and exogenous ceramide itself induces apoptosis (174). The role played by the SM pathway in the regulation of spermatogenesis has remained unclear. ASMKO mice lacking one of the enzymes that convert SM to ceramide, have pathological testicular tissue and sperm due to lipid accumulation (175); the germ cells, however, appear normal (175).

Aims of the study

Sphingolipids are powerful mediators of diverse cellular processes and thus hold great promise in different fields of cancer research (5). The sphingolipid metabolite S1P is able to inhibit apoptosis in several cell lines and appears to be promising in the means of protection for ovaries against cancer treatments (7). The best way to preserve fertility of prepubertal boys would be to protect the spermatogenic stem cells against apoptosis induced by cancer treatments *in vivo.* The present series of studies therefore aimed at elucidating the inhibitory potential and inhibitory mechanisms of S1P on male germ cell apoptosis and the ability of S1P to prevent radiation-induced male germ cell loss. In particular, the following issues were addressed:

1. The inhibitory effect of S1P *in vitro* on male germ cell apoptosis of the human testis in a culture of seminiferous tubules.

2. Characterization of the mechanisms, by which S1P inhibits male germ cell apoptosis *in vitro*, with emphasis on the roles of S1PRs and intracellularly generated S1P, and on transcription factors involved in the regulation of male germ cell apoptosis.

3. The potential ability of S1P *in vivo* to protect against radiation-induced male germ cell death in an experimental animal model.

Materials and treatments

I In vitro studies

Patients

Testicular tissue was obtained from 47 men aged 59-80 years of age undergoing orchidectomy as a treatment for prostate cancer. These patients had received no hormonal, chemotherapeutic, or radiotherapeutic treatments before the operations. They had no endocrinological disease and none had suffered from cryptorchidism. The testicular tissue was prepared for culture immediately after the operations, which were performed between December 1999 and August 2003 at the Department of Urology, Helsinki University Central Hospital (Helsinki, Finland). The ethics committees of the Hospital for Children and Adolescents and the Department of Urology, University of Helsinki, approved the study protocol.

Study design

Tissue culture. Germ cell apoptosis of the human testis was induced by incubating segments of seminiferous tubules under serum-free conditions. To maintain the physiological contact between the Sertoli cells and the germ cells, segments of seminiferous tubules rather than isolated testicular cells were cultured. The testicular tissue was microdissected in a Petri dish containing culture medium supplemented with 0.01% human serum albumin and 10 μg/mL gentamicin. Segments (~2 mm in length) of seminiferous tubules were transferred to a Petri dish containing the same serum-free culture medium and incubated for 0-10 h at 34 °C in a humidified atmosphere containing 5% CO_2. Since human testicular tissue is rather dense, some interstitial tissue with occasional Leydig cells were present in the culture. Immunohistochemical studies from cultured samples (II-III) were performed from the small (0.05-0.85 g) tissue samples that were cultured under serum-free conditions as described. Since the testis tissue was not homogenous, the immunohistochemically best part of the slide was used for further evaluation.

Treatments

Descriptions of the use of the following compounds are detailed in the original publications (I-III). The effects of these compounds on germ cell apoptosis were studied by adding them to the culture medium prior to the 5-h culture under serum-free conditions at the concentrations indicated: S1P (1 μmol/L, 10 μmol/L, 20 μmol/L), the ceramide synthetase inhibitor FB1 (100 μmol/L, 250 μmol/L), N-Acetyl-L-cysteine (NAC; 100 mmol/L), potassium cyanide (KCN; 50 mmol/L), desipramine (10 μmol/L, 50 μmol/L), imipramine (10 μmol/L, 30 μmol/L), recombinant human FSH (0.01 IU/ml, 0.1 IU/ml, 1.0 IU/ml), 17 β estradiol (E2; 10^{-10} mol/L), specific JNK inhibitor SP600125 and specific MEK inhibitor PD98059 (10 μmol/L, 50 μmol/L, 100 μmol/L), dS1P (1μmol/L, 10 μmol/L, 20μmol/L).

II In vivo study

Mice

WT C57BL/6 young adult male mice, 8-10 weeks of age, were obtained from the University of Helsinki experimental animal facilities. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Wihuri Research Institute, Helsinki Finland.

Study design

In the experiments, the mice were anesthetized by intraperitoneal injection of 600–800 μL 1.25% Avertin [2,2,2-tribromoethanol in *tert*-Amyl-Alcohol; 1600 mg/mL] 1-2 h before irradiation, after which they received a dose of 0, 0.1, 0.5, 1.0, or 2.0 Gy whole-body irradiation at the Department of Oncology, Helsinki University Hospital, Helsinki, Finland. Irradiation was performed with a Varian Clinac 600C linear accelerator using a 6-MV photon beam and a dose rate of 2 Gy/min. The mice were placed in the prone position in a plastic box and irradiated by means of a single posterior field covering the entire box plus a 2-cm margin to achieve maximum uniformity of dose distribution. The absorbed dose was calculated at a depth of 2 cm. In addition, a 1.5-cm-thick plexiglass absorber was added at the entrance side of the field to obtain a full dose build up. Sixteen hours after irradiation, the tissue samples were fixed in 2.5% glutaraldehyde for electron microscopic studies and after 21 d, the testes were weighed and DNA flow cytometric analyses were performed.

Irradiation experiments: At the 16-h time point after 0, 0.5, or 1.0 Gy irradiation, the mice were anesthetized by CO_2 and sacrificed by cervical dislocation. The tissue samples were collected in 2.5% glutaraldehyde for electron microscopic studies. At the 21-d time point, after 0, 0.1, 0.5, 1.0, or 2.0 Gy irradiation, the mice were sacrificed as described, the testes weighed and after stage-specific preparation of seminiferous tubules, DNA flow cytometric analyses were performed.

S1P experiments: Description of the use of S1P is detailed in the original publication (IV). For the control group of animals, the vehicle (PET-PBS) was injected intratesticularly into the right testis, while the left testis remained as an untreated control. For S1P-treated mice 30 μL of 50 μmol/L S1P in PET-PBS was injected into the right testis and 30 μL of 200 μmol/L S1P in PET-PBS into the left testis.
At the 16-h timepoint, the effect of S1P on 0.5 Gy radiation-induced rapid germ cell death was studied with *in situ* end-labeling (ISEL) analysis of DNA fragmentation. After radiation doses of 0.5 and 1.0 Gy, the tissue samples were also collected in 2.5% glutaraldehyde for electron microscopic studies. At the 21-d timepoint, after stage-specific preparation of seminiferous tubules, the long-term effects of S1P on 0.5 Gy radiation-induced apoptosis were studied with DNA flow cytometry.

Seminiferous tubule preparations. Sixteen hours or 21 d after total body irradiation, the testes were decapsulated in PBS in a Petri dish, the seminiferous tubules were gently teased apart, and three 1-mm-long segments of seminiferous tubules at each of the stages II–V, VI–VIII, and IX–XII per mouse were prepared under a transillumination stereomicroscope (140, 176, 177).

Methods

Laboratory analyses

Analysis of testicular levels of ceramide and sphingomyelin

The levels of SM and ceramide were measured from seminiferous tubules after a 5-h induction of apoptosis. Small samples of human testis tissue were homogenized in homogenization buffer and the supernatants collected for determination of protein concentration by the DC protein assay. Human testis tissue lipids were extracted by the modified method of Bligh & Dyer (178). The lipids were dissolved in chloroform:methanol (2:1, v/v) and analyzed by high performance thin-layer chromatography (HPTLC) on an HPTLC silica gel 60 plate. Densitometric scanning of the bands and evaluation of the data were performed with an automatic plate scanner (CAMAG TLC Scanner no. 3) and CAMAG TLC Software, respectively. The amounts of ceramide and SM were compared with the total amount of cell protein, and the ceramide:SM weight ratio was calculated.

In vitro acid sphingomyelinase activity assay

Small samples of human testis tissue were homogenized in 1000 μL of homogenization buffer and the supernatants were collected for protein concentration determination with the DC protein assay. The protein homogenate (100 μg) was mixed with 100 μL of ASMase buffer containing 40 000 cpi [^{14}C] SM and incubated for 1 h at +37 °C. The reactions were stopped by addition of 1.5 mL chloroform:methanol **(**2:1, v/v**)** plus 200 μL of distilled water and vortexing. After centrifuging at 1800 *g* for 5 min, 300 μL of the upper aqueous phase containing the released radioactive phosphorylcholine was transferred to scintillation vials for determination of radioactivity in a liquid scintillation counter. Negative controls containing no enzyme were assayed simultaneously.

Detection of apoptosis

Southern blot analysis of apoptotic DNA fragmentation. DNA was extracted with the apoptotic DNA Ladder Kit (Roche Molecular Biochemicals, Mannheim, Germany) as described (162). After the DNA

was quantified spectrophotometrically, 1 µg of the total DNA from each sample was subjected to 3´end-labeling with digoxigenin-dideoxy-UTP (dig-dd-UTP) in the terminal transferase reaction. The DNA samples were electrophoresed on 2 % agarose gels, blotted onto nylon membranes, and cross-linked to the membranes with UV irradiation. The membranes were then washed and blocked with 1% blocking reagent in maleic acid buffer. The 3' end-labeled DNA in the membranes was localized and the bound antibody was detected with the chemiluminescense reaction (CSPD).

Caspase 3 activation. The activity of caspase 3 was measured with the Caspase 3 fluorometric assay kit (RD Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, samples of human testis tissue were homogenized in lysis buffer, centrifuged at 17 000 *g* for 20 min, and the supernatants collected for determination of protein concentration using the DC protein assay. Thereafter, 100 µg of protein homogenate in 50 µL lysis buffer, 50 µL of reaction buffer 3, and 5 µL Caspase-3 fluorogenic substrate (DEVD-AFC) were added to 96-well plates, and the plates were incubated at + 37 °C for 2 h. Finally, the fluorescence was measured on a fluorescence microplate reader (Perkin Elmer HTS 7000 Plus Bio Assay Reader) using 405-nm excitation and 505-nm emission filters. For negative controls, the fluorescence was measured from wells containing no substrate or no protein homogenate.

Nonradioactive in situ 3´end-labeling of DNA. Small segments of seminiferous tubules (~1-2 mm in length) were squashed under coverslips to produce a monolayer of cells, and the preparations were fixed and rehydrated as previously described (177). After incubation for 10 min with terminal transferase reaction buffer, the apoptotic DNA was 3´end-labeled with dig-dd-UTP by the terminal transferase reaction. For the negative controls, the terminal transferase enzyme was replaced with the same volume of distilled water. The dig-dd-UTP was detected with the antidigoxigenin antibody conjugated with horseradish peroxidase. For location of the antibody, 0.05% diaminobenzidine substrate was added. Light counterstaining was performed with hematoxylin, and the samples were dehydrated and mounted.

Immunohistochemistry

The immunostainings were performed on 5-h cultured or non cultured paraffin-embedded sections of formalin-fixed adult testicular tissues. The paraffin sections were deparaffinized in xylene and the sections were then

rehydrated and microwaved in citrate buffer for antigen retrieval. The samples were subjected to immunohistochemistry by the antibodies described in Table 1 and the antibodies were used at concentrations of 0.5-0.8 μg/mL. The primary antibodies were added to the samples and incubated overnight at 4 °C and detected with biotin-conjugated goat anti-rabbit or rabbit anti-mouse IgG from the ABC-Elite kit (Vector laboratories, Inc., Burlingame, CA, USA), followed by incubation with ABC solution. For location of the secondary antibody, 0.05% diaminobenzidine substrate was added. For the negative controls, the primary antibodies were replaced with nonimmune rabbit or mouse IgG.

Table 1. Antibodies used in immunohistochemistry, EMSA, and Western blotting

Antibody	**Clonality**	**Source**	**use**
p-c-Jun	PC	Santa Cruz Biotechnology;sc-822	IH, EMSA
c-Fos	MC	Santa Cruz Biotechnology;sc-52	IH, EMSA
JunD	MC	Santa Cruz Biotechnology;sc-74	IH, EMSA
JunB	PC	Santa Cruz Biotechnology;sc-46	EMSA
EDG-1($S1P_1$)	PC	Santa Cruz Biotechnology;sc-16070	IH
EDG-1($S1P_2$)	PC	Santa Cruz Biotechnology;sc-16085	IH
p-ERK1/2	PC	Cell Signaling Technology 9910	IH
ERK	PC	Santa Cruz Biotechnology;sc-94	IH
IκBα	PC	Santa Cruz Biotechnology;sc-847	WB
Akt	PC	Cell Signaling Technology 9271	WB
p-Akt	PC	Cell Signaling Technology 9271	WB

MC = Monoclonal
PC= Polyclonal
IH = Immunohistochemistry
EMSA = Electric mobility shift assay supershifts
WB = Western blotting

Electron microscopy

Segments of the seminiferous tubules were fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer, dehydrated, and embedded in epoxy resin. The tissue blocks were sectioned at 50 nm with an ultramicrotome and stained with uranyl acetate and lead citrate. The samples were examined with a JEOL JEM 1200 EX transmission electron microscope (JEOL, Tokyo, Japan) at the Institute of Biotechnology, Electron Microscopy Unit, Finland.

Protein extracts

For cytoplasmic and nuclear protein extracts, the seminiferous tubules were gently homogenized with a tight-fitting Potter-Elvehjelm homogenizer into ice-cold hypotonic buffer, and the protein extracts were prepared as previously described (179). For whole-cell protein extracts, small tissue sections were homogenized with an Ultra-Turrax T8 homogenizer on ice in homogenization buffer. The protein concentrations from the supernatants were determined using the DC protein assay.

Western blotting

The proteins (50 μg) were loaded onto SDS-polyacrylamide gels and electrophoresis was performed at 180 V. The proteins were transferred to polyvinylidene difluoride membranes by electrophoresis for 2 h at 4 °C in transfer buffer at 100 V. The transfer was checked by staining with 0.2% Ponceau S in 3% trichloroacetic acid. The primary antibodies (Table 1) against the proteins under investigation were used at 0.2 μg/mL. The primary antibodies were followed with peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG. The bound secondary antibodies were located with an electron chemiluminescence (ECL) detection kit.

Electron mobility shift assay

The AP-1 and NF-κB DNA binding activities were assayed by DNA probe containing the consensus AP-1 site 5´GATCTATCTGAGTCAGCAG-3 (180) and the consensus κB enhancer element 5´AGTTGAGGGGACTTTCCCAGGC-3. The probes 5´end-labeled with [γ-^{32}P] ATP using polynucleotide kinase (15 000-30 000 cpm) were incubated with testicular protein nuclear extracts (10 μg) and thereafter, the reaction products were separated on 4% polyacrylamide gels at 200 V at room temperature. In the competition experiments, a 100-fold molar excess of unlabeled probe was added prior to the labeled probe. After electrophoresis, the gels were dried and visualized by autoradiography. In the supershift assays, 2 μg of an affinity-purified polyclonal antibody (Table 1) was added after the binding reactions, and incubation was further continued for 1 h at room temperature.

DNA flow cytometry

DNA flow cytometric analyses after irradiation were performed as described previously, with some modifications (140, 181). Stage-specific 1-mm-long single segments of seminiferous tubules were treated with a detergent and ribonuclease A for 10-15 min at 4 °C, followed by a 10 sec vortex mix and incubation for 10 min at 37 °C. Propidium iodide (25 μg/mL) was added to the samples for staining of the nuclei, and 10 μL of diluted fluorescent particle solution (500 beads/μL, TrueCount Beads, Becton Dickinson, Mountain View, CA, USA) was added to each sample as an internal volume standard for the quantification of absolute cell numbers. The samples were filtered through 45-μm metal strainers and 200 μL of PBS was added to each sample to increase the volume. The samples were analyzed with a FACSCalibur flow cytometer (Becton Dickinson) using an excitation wavelength of 488 nm. A total of 5000 fluorescent impulses were counted, excluding the beads that were gated out of the rest of the sample. The number of nuclei in each peak of the DNA histograms were calculated using CellQuest Pro software (Beckton Dickinson) and converted to absolute numbers using the internal standard. The 1C population consisted of step I-XII spermatids. The 1C´ population consisted of step XIII-XVI spermatids that bound less propridium iodide than step I-XII spermatids and therefore formed a hypohaploid peak. The 4C population consisted mainly of primary spermatocytes (except for preleptotene spermatocytes that were dispersed in the S-phase and the 2C peak, depicting chromatin synthesis and diploid chromatids, respectively) but also of spermatogonia (G_2/M) (140).

Quantitative analyses of x-ray films

In the original publications (I-III), the X-ray films exposed to chemiluminescence (Southern blots), autoradiography (EMSA), or ECL (Western blots) were scanned with a tabletop scanner (Hewlett Packard Scan Jet 6300C) and the digital image was analyzed with the gel plot 2 macro for Scion image β 4.0.2. (Scion Corp., Frederic, MD, USA) analysis software. For the Southern blots, digitized quantification of the low-molecular-weight DNA fragments (< 1.3 kB) in the sample cultured for 10 h (in the time-course analyses of nuclear apoptosis) or for 5 h (in the S1P-, FB1-, desipramine-, imipramine-, FSH-, NAC-, E2-, dS1P-, PD 98059-, and SP 600125-treated samples) without treatments was taken as 1.0

(100% apoptosis), and the amounts of low-molecular weight DNA fragments in the other samples were expressed in relation to this. For time-course analysis of AP-1 activation (EMSA), digitized quantification of the specific AP-1 band in the sample cultured for 5 h was set as 1.0 and the intensities of the bands in other samples were expressed in relation to this.

Statistics

I In vitro studies

Statistical analyses were performed based on at least 3 independent experiments. Data obtained from 3-11 cultures (mean ± SEM) were analyzed by one-way analysis of variance (ANOVA), and if significant differences existed this was followed by comparison of the groups with the two-tailed unpaired Student t-test. A value of $P < 0.05$ was considered statistically significant.

II In vivo study

Sixteen hour time point. The Mann-Whitney test was used to test differences in apoptotic cell counts between the S1P-treated and non-treated groups.
Twenty-one-day time point. Differences in cell numbers between the radiation dose groups within the 3 stages among the 1C and 4C populations, effect of S1P on cell numbers and the effect of radiation dose on testicular weight were tested with ANOVA followed by Dunnett's test.
Values of $P < 0.05$ were considered significant. Results were expressed as mean ± SEM.

Results

Induction of human male germ cell apoptosis in vitro

Germ cell apoptosis was induced by incubating the seminiferous tubule segments under serum-free culture conditions. The different types of human testicular cells undergoing apoptosis were previously characterized by ISEL and electron microscopy: apoptosis occurs mainly in the germ cells *i.e.* the spermatids and early spermatocytes, whereas the Sertoli cells are rather resistant to apoptosis (161, 182-185).

Low-molecular-weight DNA fragmentation. Culturing the seminiferous tubule segments under serum-free conditions resulted in induction of apoptotic DNA fragmentation within 5 h, which further increased at 48 h as measured with Southern blot analysis of the low-molecular-weight DNA fragmentation (Fig. 1C in I, Fig. 1A in II).

Ceramide levels. During the seminiferous tubule culture the level of ceramide increased within 1 h and at 2.5 h, it had almost attained maximum (Fig. 1A-B in I).

Caspase 3 activity. The time course activation of caspase 3 during human testicular apoptosis was measured from samples incubated under serum-free conditions from 15 min to 10 h. Caspase 3 activity began to increase after 1 h (Fig. 1B in I, Fig. 1C in III).

Activator protein 1 DNA-binding activity. AP-1 DNA-binding activity was clearly increased at the 2.5 h time point and was further elevated at the 5-h time-point, after which it bagan to decrease (Fig 1B in II, Fig. 3B in III).

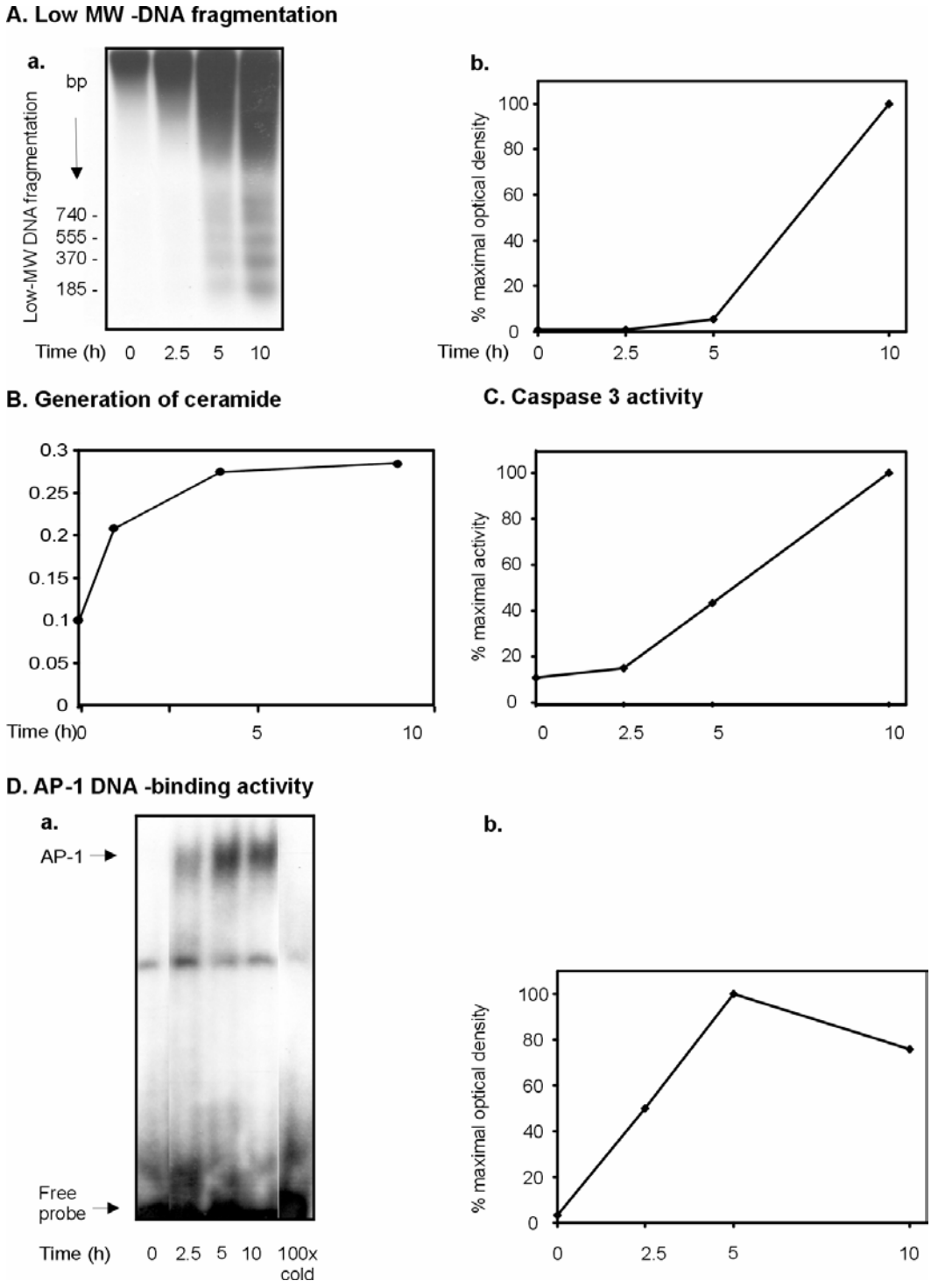

Figure 5. Time course of activation of *in vitro* -induced human testicular apoptosis. Segments of seminiferous tubules were cultured under serum-free conditions for 0-10 h to induce apoptotic cell death, after which apoptotic DNA fragmentation, AP-1 DNA-binding activity, and caspase 3 activation were measured. **A a:** Southern blot analysis of low-molecular-weight DNA (< 1.3 kb) fragmentation. DNA was extracted, after which 1 µg of the total DNA from each sample was 3´end-labeled with dig-dd-UTP and subjected to electrophoresis. The labeled DNA was detected with chemiluminescence. **b**: Low-molecular-weight DNA (< 1.3 kb) fragmentation was quantified from the radiographs

and plotted as a function of time. Induction of apoptotic DNA fragmentation occurred within 5 h and further increased at 10 h. The digitized quantification of the low-molecular-weight DNA fragments in the sample cultured for 10 h (maximal optical density) was taken as 100%, and the amounts of low-molecular-weight DNA fragments in the other samples were expressed in relation to this. **B**. Lipids from seminiferous tubules cultured for 0-10 h were extracted and ceramide and SM in the samples quantified. **C.** Time course activation of caspase 3 during human testicular apoptosis from samples incubated under serum-free conditions for 0-10 h. Caspase 3 activity increased steadily after 1 h**.** Data represent 2 independent experiments. **D a:** EMSA demonstrating the increase in AP-1 DNA-binding activity during *in vitro*-induced testicular apoptosis. Nuclear protein extracts (10 μg) from the seminiferous tubules were incubated with ^{32}P-labeled AP-1 oligonucleotide, and the DNA-protein complexes formed were resolved on polyacrylamide gel electrophoresis. **b**: AP-1 DNA binding was quantified from the radiographs and plotted as a function of time. The digitized quantification of the specific AP-1 band in the sample cultured for 5 h (maximal optical density) was taken as 100% and the intensities of the other bands were expressed in relation to this.

Generation of ceramide

Role of acid sphingomyelinase

Although ASMase activity was suppressed by 38.5 % and 17.6 % with desipramine and imipramine, respectively, neither of the ASMase inhibitors used significantly effected testicular germ cell apoptosis or the level of testicular ceramide (I).

Effect of Fumonisin B_1

No significant effects of FB1 (100 μmol/L or 250 μmol/L) on germ cell apoptosis were observed in Southern blot analysis of DNA fragmentation (Fig. 2 in I). The levels of ceramide and SM in FB1-treated seminiferous tubules were decreased by 25% and 24%, respectively (P < 0.01). However, the ceramide:sphingomyelin ratio did not differ from that of control samples cultured under serum-free conditions without treatments.

Potassium cyanide and N-Acetyl-L-cysteine

After 5-h culture, NAC and KCN suppressed low-molecular-weight DNA fragmentation by 78% and 64%, respectively (P < 0.05), but had no significant effect on testicular ceramide levels (Figs. 3B and 4B, I).

Effect of S1P in vitro

Role of intracellularly generated S1P

Addition of exogenous S1P to the culture caused evident inhibition of germ cell death. The most effective antiapoptotic concentration was 10 μmol/L, which suppressed low molecular weight DNA fragmentation by 30% ($P < 0.001$; Fig. 3A in I, Fig. 2A in II). ISEL analysis consistently showed a reduced number of apoptotic cells in the samples incubated with exogenous S1P, with no apparent abnormalities in the morphology of the germ cells (Fig 3. in I). To quantify the effect of S1P with yet another independent mechanism, we found that S1P was able to inhibit caspase 3 activation by 15% ($P < 0.05$) (Fig. 3 in I).

In contrast, dS1P, a S1P analog that like S1P activates the S1PRs but unlike S1P does not penetrate the cell membrane, did not inhibit apoptotic DNA fragmentation when used at a concentration (10 μmol/L) similar to that of S1P. The amount of DNA fragmentation in dS1P-treated samples was 108% compared with the non treated, 5-h-cultured samples (*P = NS*; Fig. 2 in II).

Expression of $S1P_1$ and $S1P_2$ receptors

Antibodies against $S1P_1R$ and $S1P_2R$ showed similar cytoplasmic staining patterns. Positive cytoplasmic immunostaining in the Sertoli cells was observed in the majority of the cross sections of the seminiferous tubules. Occasional germ cells, mainly spermatogonia and early meiotic spermatocytes, also expressed weak cytoplasmic immunostaining (Fig. 1 in II).

S1P but not dS1P inhibits NF-κB DNA binding and phosphorylation of Akt

Seminiferous tubules cultured for 5-h with 10 μmol/L S1P showed almost complete suppression of NF-κB DNA-binding activity as measured by EMSA (Fig. 3A in II). In contrast, in tubules cultured for 5 h with 10 μmol/L dS1P, no effect on NF-κB DNA-binding activity could be seen compared with the non treated samples (Fig. 3A in II).

Western blot analysis of the cytoplasmic extracts from uncultured seminiferous tubules and seminiferous tubules cultured for 5 h in the

presence or absence of 10 μmol/L S1P or dS1P showed intensive IκBα expression in the uncultured tubules and clearly diminished IκBα expression in the tubules cultured for 5 h without treatments. In the tubules treated with S1P, inhibition of IκBα degradation was more evident than in the tubules treated with dS1P (Fig.4 in II).

Western blot analysis of Akt showed that active, *i.e.* phosphorylated Akt (p-Akt), was strongly expressed in uncultured seminiferous tubules, whereas in the 5-h-cultured tubules the expression of p-Akt was clearly diminished. In the S1P-treated, 5-h-cultured tubules, the expression of p-Akt was almost totally absent. In contrast, phosphorylation of Akt was evident in the dS1P-treated, 5-h-cultured samples. Equal amounts of inactive Akt were detected in the uncultured, 5-h-cultured untreated, and 5-h-cultured, S1P- or dS1P-treated seminiferous tubules (Fig. 4 in II).

AP-1 activity in the human testis

AP-1 DNA-binding activity increased at the 2.5 hour time point during culture of seminiferous tubules, and was further elevated at the 5 h time point, after which it began to decrease as measured by EMSA (Fig. 1B in III). All the AP-1 proteins detected at various time points gave a similar staining pattern. After 2.5-h or 5-h-culture under serum-free conditions, the Sertoli cells in most of the seminiferous tubules expressed nuclear AP-1 (Fig. 3 in III). In non cultured seminiferous tubules, some spermatogonia and early meiotic spermatocytes expressed cytoplasmic immunostaining for JunD and c-Fos. Participation of the AP-1 transcription factors c-Jun, JunD, and c-Fos in formation of the DNA-protein complexes was further elucidated by the EMSA supershift assays. Incubation of the nuclear extracts with antibodies against c-Jun, JunD, and c-Fos, but not with antibody against JunB, resulted in supershifts of the basal DNA-protein complexes (Fig. 2 in III).

To specify the effects of MAPK kinases on testicular tissue, we tested the effects of the specific inhibitors of ERK and JNK, PD98059 and SP600125, respectively, during induction of human male germ cell apoptosis. As measured in Southern blot analysis of DNA fragmentation and with AP-1 DNA-binding activity, neither PD98059 nor SP600125 affected male germ cell apoptosis or AP-1 DNA-binding activity (unpublished data).

S1P and activation of ERK

In non cultured seminiferous tubules, some Sertoli cells expressed weak cytoplasmic immunostaining of the phosphorylated, *i.e*. active ERK protein (p-ERK). After 5 h culture in the presence or absence of S1P or dS1P, the Sertoli cells in most of the seminiferous tubules detected expressed cytoplasmic p-ERK. However, positive staining was more intense in tubules treated with S1P and dS1P, especially with the latter (Fig. 5 in II). Expression of ERK representing the overall levels of ERK was similar in the samples cultured for 5 h with or without treatments; strong expression of ERK was found in the cytoplasm of both the germ cells and Sertoli cells (Fig. 5 in II).

Effects of FSH, NAC, E2 and S1P

FSH, at 1.0 IU/mL inhibited testicular apoptosis by 35% ($P < 0.05$) and concomitantly suppressed AP-1 DNA-binding activity (Fig. 4A-B in III). In the samples from 2 independent cultures, however, we observed that although FSH suppressed AP-1 DNA-binding activity, it did not affect NF-κB DNA-binding activity (Fig. 4C in III).

E2 at a concentration of 10^{-10} mol/L inhibited apoptosis by 56% but did not reduce the AP-1 DNA-binding activity below the level detected in control samples (testicular tissue cultured for 5 h without treatments). Likewise, E2 did not affect NF-κB DNA-binding activity (Fig. 5 in III). At a concentration of 100 mmol/L, NAC inhibited apoptosis by 78% and concomitantly suppressed AP-1 DNA-binding activity almost to the basal level (Fig. 5 in III). We previously showed that NAC does not affect NF-κB DNA-binding activity (83).

Neither S1P nor dS1P affected AP-1 DNA-binding activity; similar activity was detected in tubules cultured for 5 h under serum-free conditions with or without 10 μmol/L S1P or dS1P (Fig. 3 in II).

Table 2. Regulation of *in vitro*-induced male germ cell death

Compound	Function	Male germ cell death	Generation of ceramide	AP-1 activity	NF-κB activity
Sphingolipid pathway					
S1P	Survival factor	↓	**?**	↔	↓
dS1P	S1P analog that binds to S1PR	↔	**?**	↔	↔
Fumonisin B1	Inhibitor of *de novo* ceramide pathway	↔	↔	**?**	**?**
Imipramine	Inhibitor of ASMase	↔	↔	**?**	**?**
Desipramine	Inhibitor of ASMase	↔	↔	**?**	**?**
Hormones					
17β estradiol	Survival factor	↓	**?**	↑	↔
FSH	Survival factor	↓	**?**	↓	↔
Regulators of ROS					
NAC	Glutathione precursor, antioxidant	↓↓	↔	↓	↔
KCN	Regulator of ROS	↓↓	↔	**?**	**?**
MAPK inhibitors					
PD 98059	MEK inhibitor	↔	**?**	↔	**?**
SP 600125	JNK inhibitor	↔	**?**	↔	**?**

↔ No effect
? Not tested

In vivo effect of S1P on radiation-induced male germ cell loss

Sixteen hours after irradiation

Effect of S1P. As detected by electron microscopy, S1P in itself dit not appear to induce morphological changes in S1P-treated segments of seminiferous tubules, since although occasional apoptotic cells (mainly spermatogonia) were observed, most of the germ cells had retained their normal appearance (Fig. 2D-E in IV).

In stage-specific squash preparations for ISEL of DNA fragmentation, no statistically significant differences were found between the non treated and 50 μmol/L or 200 μmol/L S1P-treated groups, although a statistically non significant trend towards lower amounts of ISEL-positive cells was detected in stages II–V and IX–XII (Fig. 5A in IV).

Twenty-one days after irradiation

Testicular weight. The weights of the testes decreased 21 d after irradiation. A significant decrease in testis weight was already seen after 0.5 Gy radiation, the weight being 77% ($P < 0.05$) of the non irradiated testes. After 2.0 Gy, the decrease in testis weight was 44% ($P < 0.05$) (Fig. 1 in IV).

Effect of S1P. The number of cells in the 1C and 4C populations markedly decreased with increasing dose of irradiation. S1P was not able to inhibit the reduction of apoptotic cells in the1C population. In contrast, the numbers of cells in the 4C population in samples from irradiated plus S1P-treated animals were not significantly lower at any stage (II–V, VII–VII, and IX–XII) than the numbers of 4C cells in non-irradiated controls (Fig. 6 in IV), indicating that S1P was able to inhibit apoptosis of the early spermatogonia. More precisely, at stages II–V the cell numbers from S1P-treated animals were 16% and 34% higher after treatment with 50 μmol/L and 200 μmol/L S1P, respectively, than in irradiated and vehicle-treated animals. At stages VII–VIII this effect of S1P treatment was clearer, the total cell numbers being 35% and 47% higher. Finally, at stages IX–XII, the cell numbers in S1P-treated animals were 40% and 38% higher than in vehicle-treated animals.

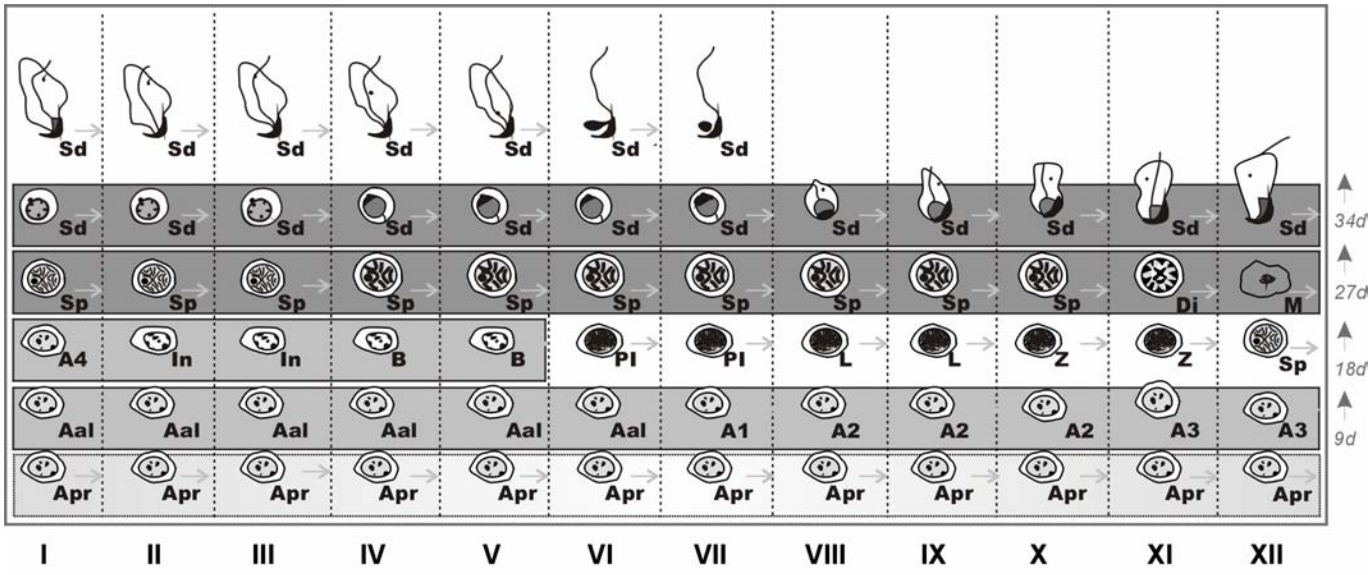

Figure 6. Schematic illustration of the structure of mouse seminiferous epithelium. Modified from Pentikäinen *et al.* (162) and Yan *et al.* (186). Differentiation of early spermatogonia to spermatocytes and to spermatids lasts approximately 9 and 18 d, respectively (arrows). Spermatogonia dying of radiation-induced apoptosis are shown with a light gray background. Twenty-one days after irradiation, apoptosis of spermatogonia damaged at the time of irradiation is observed as a reduction in the 4C population of spermatocytes and the 1C population of spermatids (dark gray background). The population of spermatogonia (Apr) that are preserved against radiation–induced germ cell damage by S1P are presented with the lightest gray. The preservation of these cells is observed as a non reduced number of 4C population of spermatocytes (dark gray) 21 d after irradiation. The specific cell associations in the vertical columns represent spermatogenic stages of the epithelial cycle (Roman numerals). Apr, A-paired spermatogonia; Aal, A-aligned spermatogonia; A1-A4, type A1-4 spermatogonia; In, intermediate spermatogonia; B, type B spermatogonia; PI, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; Sp, spermatocytes; Di, diplotene spermatocytes; M, meiotically dividing spermatocytes; Sd, spermatids.

Discussion

Methodological aspects

In vitro studies

Male germ cell death was induced in a culture model that has many limitations. The model was most likely not capable of supporting the process of spermatogenesis, thus representing a dying model. It, however, aimed at representing a stress situation in which the reactions of different cell types in the seminiferous epithelium during severe stress could be evaluated. Although the primary inductor of cell death was serum deprivation, other simultaneous inductors, such as relative hyperoxia, activated parallel apoptotic pathways. In addition, prolonged stress most probably led to activation of secondary apoptotic pathways and the cells treated with antiapoptotic compounds most likely died of delayed kinetics. Thus, in the present model, multiple parallel pathways were most likely induced and each specific pathway may have had multiple inducers. In addition, various stimuli may have activated certain pathways differently in different types of testicular cells.

Due to the density of human testicular tissue, the presence of interstitial tissue with Leydig cells and their possible effects could not be ruled out, whereas in the similar rat model, interstitial tissue can be separated from the seminiferous tubules (152). For immunohistochemistry from cultured samples, we had to use small pieces of testicular tissue, which may have differed in size, rather than small segments of seminiferous tubules, leading to inhomogenous fixation. Human seminiferous tubules contain several spirally oriented stages of spermatogenesis, in which the number of apoptotic cells varies considerably. The length of the stage within the seminiferous tubule was not known and the representativeness of each stage remained unclear. Therefore, in our studies the quantification of immunohistochemical studies and ISEL analyses was difficult to conduct. The ISEL-positive cells in individual slides showed variation, which was independent of the treatments, especially in the squash preparations.

The testis tissue samples were received from patients undergoing orchidectomy as a treatment for prostate cancer. Despite the advanced age of most of these patients, germ cells at all phases of maturation were

found during light microscopic and electron microscopic evaluation of the tissue. Samples from prepubertal boys or from younger men are scarce and were only available as small testicular biopsies taken for infertility or suspected disease, or as testis tissue removed for testicular cancer. These samples were therefore not suitable for our studies, since they were either too small or the tissue was disturbed by disease. Despite its limitations, this *in vitro* model maintained physiological contacts between the germ cells and the supporting Sertoli cells, thus enabling us to evaluate apoptotic mechanisms that involve interactions between different cell types within seminiferous tubules. Since the cellular responses have potential species specifity, results from animal studies may not apply to humans. With the aid of this model, we were able to examine the apoptotic mechanisms representative of the human testis.

In vivo study

Germ cell apoptosis in the rat testis, which resembles that of the mouse, is known to begin approximately 8 h after irradiation (146). The number of apoptotic cells increases until about 16 h, when mostly degenerating step 1 spermatids and spermatogonia can be found. After 42 h, mainly meiotically dividing cells and early spermatids are undergoing apoptosis and after approximately 66 h the number of degenerating cells returns to control levels (146). Since physiological death of selected spermatocytes and spermatids is a common feature of normal spermatogenesis (136, 187), a large number of physiologically apoptotic spermatocytes and spermatids is present in the testis. In the present *in vivo* study, the total amount of dying germ cells at the 16-h time point may have masked the protective effect of S1P on spermatogonia and thus interfered the interpretation of the results. A more detailed analysis of radiation-induced germ cell death in the mouse testis at several time points and a larger number of animals could have clarified the events occuring in the early phases of radiation-induced germ cell death. However, since stage-specific preparing of the squash preparations is time consuming, it is technically impossible (or at least extremely demanding) to make numerous samples from tubule segments in a short time period, which, however, is a prerequisite for maintenance of reliable incubation times.

Generation of ceramide

Ceramide can be formed at the initial step of the signaling pathway by enhanced *de novo* synthesis or by degradation of SM due to activation of

NSMase or ASMase (6). Whatever ceramide pathway is activated is highly dependent on the cell and stimulus. In our study, neither the ceramide synthetase inhibitor FB1, nor 2 different inhibitors of ASMase (desipramine and imipramine) significantly affected male germ cell apoptosis or testicular ceramide levels. More importantly, both ASMase inhibitors decreased the ASMase activity to the same level as was detected in nontreated controls. These results suggest that activation of the NSMase instead of ASMase contributes to the observed increase in ceramide levels during culture of the seminiferous tubules. Unfortunately, no specific NSMase inhibitors were available to further determine the role played by NSMase in the regulation of male germ cell apoptosis. The assumption of NSMase playing an important role in generation of ceramide, however, is supported by a number of observations showing a critical role for NSMase instead of ASMase in cell death (188-190). Somewhat surprisingly NAC, which effectively inhibits apoptosis (184), did not affect ceramide levels, although it is a glutathione precursor and glutathione is an inhibitor of NSMase (99, 189). This, however, may have been due to inability of the exogenous NAC to restore appropriate glutathione levels during *in vitro* culture conditions.

ASMKO mice were recently found to have pathological testicular tissue and sperm due to lipid accumulation, although the germ cells appeared normal (175). Our studies on ASMKO mice (191) support the minor role of ASMase in the regulation of male germ cell apoptosis. We found ASMKO testes to contain significantly elevated levels of SM at the age of 8 wk. Concomitantly, the lipid vesicles resided in the Sertoli cells and in the interstitium, but not in the germ cells. When germ cell apoptosis was induced *in vivo* by irradiation, similar degrees of apoptosis were observed in the ASMKO and the WT testes (191). Similarly, under serum-free cultures both ASMKO and WT germ cells showed massive apoptosis after culture for up to 48 h and in both groups the ceramide levels became significantly elevated (191). Thus, ASMKO testes were able to produce ceramide, suggesting that apoptosis pathways other than ones dependent on ASMase, may be utilized for male germ cell apoptosis.

Increased ceramide levels and exogenously added cell-permeable ceramide analogs were reported to affect mitochondrial functions (104, 192). Therefore, to further study the intracellular target of ceramide in the apoptotic cascades, we tested the ability of KCN, a specific inhibitor of oxidative phosphorylation (complex IV) and a mitochondrial poison (193) to influence the testicular levels of ceramide. Although KCN inhibited germ

cell apoptosis effectively, it had no effect on the level of ceramide. This finding suggests that in human testicular germ cells, ceramide functions either upstream of or in parallel to mitochondrial complex IV and most likely upstream of ATP production, thus serving as an early intracellular effector of male germ cell apoptosis.

S1P in inhibition of germ cell apoptosis of the human testis in vitro.

S1P is an important regulator of stress-induced apoptosis (115), which acts as an intracellular signaling molecule or as a ligand to its cell-membrane bound $S1P_{1-5}Rs$ (115). A central finding of the present thesis was that exogenous S1P, a plausible antagonist of ceramide, inhibited germ cell apoptosis of the human testis *in vitro.* In this culture model, we previously showed by electron microscopy, that the morphological signs of apoptosis is most often seen in spermatocytes and spermatids (161, 182-185, 194). Whatever type of germ cell S1P is able to protect against apoptosis unfortunately was unclear and remains to be elucidated in future studies.

Although several findings support the role of S1P as an antiapoptotic second messenger (119, 195), most tissues were recently shown to express one or more S1PR subtypes (196). The role played byS1P in the direct signaling or as a classic second messenger has thus become unclear, suggesting that highly cell-specific effects of S1P occur in the regulation of apoptosis (117, 197-199). We therefore wished to clarify the role of S1P in regulation of male germ cell death by evaluating the effect of dS1P (an S1P agonist that binds to S1PR but does not have the second messenger effect of intracellularly generated S1P) on this process. In contrast to S1P, dS1P did not inhibit male germ cell death, which suggests that the inhibitory effect of S1P on male germ cell death is a second messenger, receptor-independent effect. These findings may have been further elucidated with the aid of pertussis toxin or suramin, which are able to block the activity of S1PR at different levels. By adding S1P and one of these inhibitors simultaneously to the tissue culture, the second messenger effects of S1P could have been evaluated independently of S1PR.

Caspase 3

Inhibition of apoptosis by S1P was related to caspase 3 in several cell lines (200-204). In our study, however, S1P suppressed the caspase 3 activation only by 15% although it was able to inhibit apoptotic DNA laddering in testicular germ cells by 30%. This difference in degree of inhibition of caspase activation and DNA laddering by S1P may reflect the presence of parallel apoptotic pathways in the present model. Thus, simultaneous inductors other than serum deprivation may activate parallel apoptotic pathways some of which are not blocked by S1P. Finally, prolonged stress may lead to activation of secondary apoptotic pathways that lead to death of the S1P-treated cells with delayed kinetics. The regulation of caspase 3 by S1P may be regulated through sphingolipid metabolism (204) or through binding of S1P to S1PRs (202). In the human testis, caspase 3 activation may occur in the Sertoli cells and can be suppressed via the S1PR expressed in the Sertoli cells. This hypothesis is supported by the previous observation that although caspases play a central role in the apoptosis of human Sertoli cells, germ cell apoptosis appears to be caspase-independent (205). In our study model, however, apoptosis occurs mainly in the germ cells, whereas Sertoli cells are rather resistant to apoptosis (162, 184, 206). The role of caspase 3 in the Sertoli cells is therefore controversial. That apoptosis occurs mainly in the germ cells and caspase 3 is activated, although rather late, during induction of male germ cell apoptosis (I) suggests that caspase 3 activation may also occur in the germ cells.

Since we observed no expression of S1PR in the germ cells, inhibition of germ cell apoptosis by S1P may be, at least partly, independent of caspase 3 inhibition. Caspase 3 activation may also occur only in the germ cells and not in the Sertoli cells, which is supported by the apoptosis-resistant charasteristic of the Sertoli cells. S1P, via its receptors, may thus regulate the Sertoli cells, which paracrinically regulate caspase 3 activation in the germ cells. This regulation, however, would not be related to inhibition of male germ cell apoptosis, since dS1P does not inhibit male germ cell apoptosis. Indeed, although caspase 3 is the primary effector caspase in most apoptotic cascades, alternative caspase pathways, such as activation of caspase 7 or caspase 8 may be involved (64, 207). In certain stress or *in vitro* situations, the caspase 3 apoptosis cascade may be compensated for by activation of other caspases; *e.g.* caspase 3-deficient female germ cells are able to undergo apoptosisis possibly by compensatory induction of caspase 7 (207).

NF-κB and Akt

NF-κB activates rapidly during the induction of human male germ cell apoptosis (83). In our study we found that S1P effectively inhibited NF-κB DNA-binding activity, whereas it only inhibited male germ cell apoptosis by 28%. Our results are surprising, since in this culture model, the anti-inflammatory drug sulfasalazine was recently found to block both apoptosis and NF-κB DNA-binding activity almost totally (83). It thus appears that NF-κB may not play a central role in inhibition of male germ cell apoptosis and that parallel apoptosis inhibitory pathways may exist. Secondly, S1P, together with NF-κB inhibition, may induce parallel proapoptotic pathways, possibly by binding to its receptors, since dS1P did not inhibit apoptosis or NF-κB activity.

The best characterized IκB protein, IκBα, is degraded in the seminiferous tubules during germ cell death in the human testis (83). In the present study, inhibition of NF-κB DNA-binding activity by S1P was associated with inhibition of IκBα degradation, which suggests that S1P regulates NF-κB activity by inhibiting the degradation of IκB proteins. Since Akt may regulate NF-κB via the IKK-IκB regulatory cascade, we studied whether this cascade was involved in the S1P-induced inhibition of human male germ cell death. We found that S1P inhibited the phosphorylation of Akt and the degradation of IκBα concomitantly with inhibition of NF-κB activation and apoptosis. In contrast, dS1P did not have an inhibitory effect on these processes (Fig. 7). Our findings indicate that the regulation of apoptosis by S1P is unusual and involves a regulatory cascade consisting of various intracellular components.

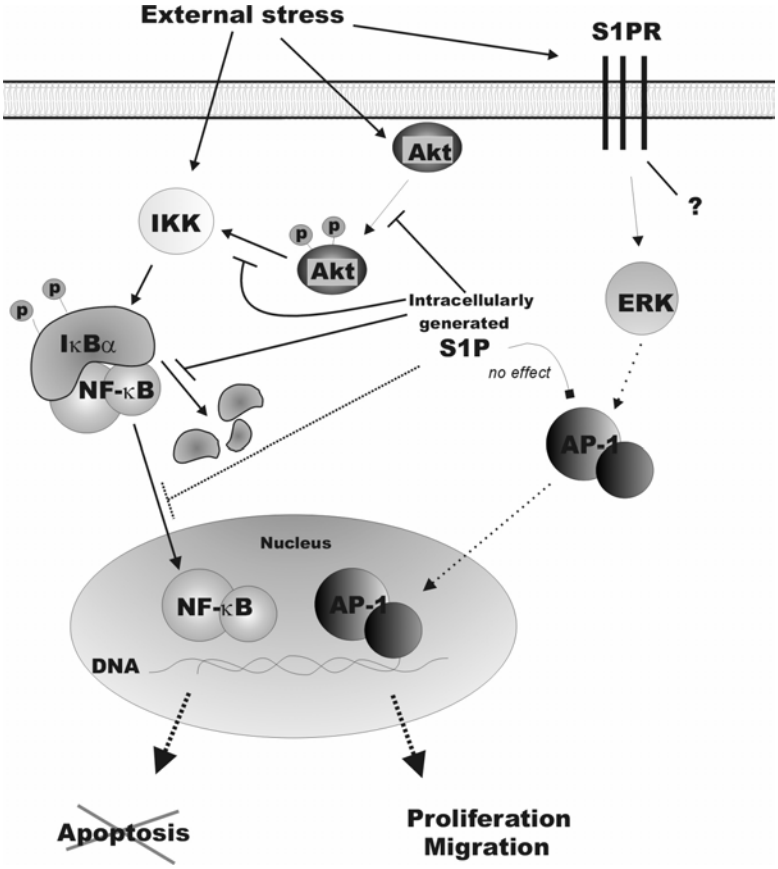

Figure 7. Schematic illustration demonstating the hypothetical inhibitory pathway of S1P on germ cell apoptosis of the human testis.To inhibit male germ cell apoptosis, S1P suppresses activation of NF-κB upstream by inhibiting the phosphorylation of Akt and the degradation of IκBα. S1P is generated intracellularly by phosphorylation of sphingosine (which is degraded from ceramide by ceramidase) by the enzyme SPHK. Intracellularly generated S1P inhibits phosphorylation of Akt, which results in inhibition of IKK. IKK, in turn, regulates degradation of IκBα, which sequesters NF-κB in the cytosol. Since IκBα degradation is suppressed due to the inhibition of IKK, entry of NF-κB into the nucleus, and thus male germ cell apoptosis, is inhibited. S1PR appears to play no role in the inhibition of male germ cell death.

AP-1

In the present culture model, AP-1 activity began to increase in the testicular Sertoli cells earlier than the beginning of caspase 3 activation and apoptotic DNA fragmentation. AP-1, however, was activated later than NF-κB, which in this model activates strongly and rapidly in the Sertoli cells within 30 min (83). Considering the relatively slow initiation of AP-1 activity as compared with the rapid activation of immediate mediators such as NF-κB, the role of AP-1 activation in germ cell apoptosis is not fully clear. Activation of AP-1 before caspase 3 and low-molecular-weight

DNA fragmentation suggest that AP-1 activation is associated with induction of apoptosis. Most likely, AP-1 is involved in apoptotic pathways that are induced in the present model after the initial rapid pathways. During severe stress, AP-1 in somatic Sertoli cells may thus induce transcription of Sertoli cell gene(s) that are able to mediate germ cell death.

That S1P did not affect AP-1 activation, although it effectively inhibited NF-κB activation, suggests that AP-1 is not a universal regulator of human male germ cell apoptosis. Yet, AP-1 activation is not a secondary phenomenon, which always accompanies apoptosis, since concomitantly with inhibition apoptosis FSH was able to inhibit AP-1 activation. Thus, in the human testis many parallel apoptotic cascades may be present and the activation of AP-1 appears to be specifically regulated, depending on the stimulus.

The finding that specific MAPK inhibitors, PD 98059 and SP 600125 did not affect apoptosis or AP-1 DNA-binding activity suggests that these inhibitors were not activated under the present culture conditions or that MAPK plays no role in the regulation of male germ cell death. The hypothesis that MAPK may play a minor role in the regulation of this apoptosis is supported by our finding in which ERK activation was increased in the cytosols of the Sertoli cells and the primary spermatocytes both in S1P- and dS1P-treated seminiferous tubules (II). More importantly, the expression was greater in the dS1P-treated than in the S1P-treated samples. Since dS1P does not appear to regulate male germ cell apoptosis, this finding suggests that the function(s) of ERK in the cultured testis are connected with the S1PRs. Thus, the regulatory function of ERK appears to be focused on events other than the regulation of male germ cell apoptosis.

S1P and radiation-induced germ cell apoptosis in vivo

The aim of the present study was to evaluate the potential ability of S1P *in vivo* to preserve male germ cells against irradiation-induced apoptosis. With flow cytometry and by back-calculation of spermatogenic stages, we indirectly showed that the number of apoptotic spermatogonia increases with increasing doses of radiation. The differentiating spermatogonia were the cell types most vulnerable to radiation-induced damage, since 21 d after irradiation, spermatocytes and spermatids representing the 4C population were decreased considerably, depending on the dose of

irradiation. S1P was able to modestly protect these early spermatogonia against 0.5 Gy radiation (observed as a normal 4C population that represented early spermatogonia at the time of irradiation). The finding suggests that apoptosis at these early developmental stages is more dependent on the SM pathway than apoptosis of germ cells in the later steps of spermatogenesis. Combining the therapeutics that preserve different stages of spermatogonia could be one possible methodfor to amplifying the effects of S1P on male germ cell preservation.

By injecting S1P intratesticularly prior to irradiation, we aimed at delivering therapeutic concentrations of S1P at the site of testicular damage. Since metabolism and interactions of sphingolipid second messengers, as well as their ability to transit through membranes remains unknown, the intratesticular concentration of S1P may have been incorrect, and the effect of S1P therefore remained modest. The modest effect of S1P *in vivo* may also be explained by the hypothesis that the effect of intratesticular injection of S1P *in vivo* was mediated via S1PRs, which may activate regulatory pathways other than those related to the apoptosis machinery. We also recently demonstrated that after irradiation *in vivo*, the level of ceramide rises neither in ASMKO nor WT mice testes, although similar degrees of apoptosis are observed (191) .Since various stimuli appear to induce different apoptotic pathways, it is possible that the sphingolipid pathway does not play a universal role in radiation-induced apoptosis, although it is important in male germ cell death induced by serum deprivation *in vitro* (I).

Conclusions and future prospects

S1P is a sphingolipid, which effectively suppresses stress-induced apoptosis in several cell types and has a potential ability to inhibit germ cell loss caused by cancer treatments. The present series of studies aimed at elucidating the role played by S1P in inhibition of male germ cell death *in vitro* and *in vivo*. The main conclusions of this thesis are as follows:

1. In a culture of human seminiferous tubules used as a model for stress situations, increase in intracellular ceramide appeared early in the apoptosis cascades supporting the concept that sphingolipids are early mediators of apoptosis and that ceramide plays a role in the induction of male germ cell death *in vitro*.

2. S1P, a plausible antagonist of ceramide, was able to inhibit *in vitro*-induced male germ cell apoptosis of the human testis.

3. Concomitantly with inhibition of apoptosis, S1P was able to inhibit the transcription factor NF-κB, which appears to be a crucial step in the induction of male germ cell apoptosis of the human testis.

4. Although the expression of the $S1P_1R$ and $S1P_2R$ was detected in the somatic Sertoli cells of the human testis, only intracellularly generated S1P appears crucial in the regulation of male germ cell apoptosis, whereas S1PRs are not indispensable.

5. In an animal model S1P, injected intratesticularly into the mouse testis prior to 0.5-Gy irradiation, was able to modestly inhibit radiation-induced apoptosis of the A-paired spermatogonia. The result suggests that apoptosis at the very early developmental stages of spermatogenesis may be more dependent on the SM pathway than apoptosis of germ cells at the later steps of spermatogenesis.

Sphingolipid therapeutics hold great promise in different fields of cancer research (5). In basic science, characterization of the sphingolipid pathway, and elucidation of the dynamics between

sphingolipid metabolites therefore remain a promising field of investigation. Future challenges will include characterization of the specific roles of the various S1PRs as well as identification of the intracellular targets of S1P. The development of antagonists or agonists of S1PRs or of inhibitors or activators of enzymes that affect intracellular concentrations of S1P may enable amplification of S1P function in the human testis for therapeutic purposes. Alternatively, other as yet unknown compounds, possibly in the sphingolipid pathway, may prove to be more effective than S1P in protecting male germ cells against apoptosis caused by external stress.

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