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MECHANISMS INFLUENCING ACTIVATION AND SURVIVAL OF NORMAL AND MALIGNANT LYMPHOID CELLS IN THE TESTIS
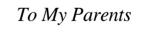
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Stockholm 2002

To My Parents

ABSTRACT

Acute lymphoblastic leukaemia (ALL) is the most common childhood malignancy and the testis is the third most common site of relapse after treatment. Testicular function is regulated by pituitary hormones, but the testicular microenvironment is also locally regulated through cell-cell interactions and by paracrine factors. Such factors could be of importance for the tendency for testicular relapse of ALL.

The aim of this thesis was to obtain a better understanding of the contribution of testicular paracrine factors to the tendency for relapse of treated childhood ALL.

Seminiferous tubules were shown to produce factors that inhibit lymphocyte proliferation in a stage related way. The characteristics of these factors indicate that they could be isoforms or multimers of bioactive transforming growth factor β (TGFβ). Seminiferous tubule extracts contain a factor that showed response in the CCL-64 mink lung epithelial cell line TGFβ bioassay, indicating that the factor was TGFβ. Neutralizing TGFβ antibodies could reverse suppression of lymphocyte proliferation induced by testis protein extracts as further evidence that the activity is mediated by TGFβ. TGFβ dose-dependently inhibited rat testicular interleukin-1 (IL-1) driven proliferation in a mouse thymocyte IL-1 bioassay and polyclonal mitogen stimulated rat PBL proliferation.

In co-cultures, Leydig cells suppressed polyclonal mitogen induced lymphocyte proliferation and suppressed recombinant IL-1α and IL-1β as well as rat testicular IL-1α bioactivity in a mouse thymocyte IL-1 bioassay. The suppressive effect was not mediated by testosterone.

A single injection of hCG induces a rapid surge of expression of pro-inflammatory cytokines in the rat testis. The reaction was not detectable in Leydig cell depleted rats, confirming that this inflammatory reaction is hormonally regulated and mediated via hypophyseal hormonal regulation of Leydig cells. As has been previously shown, this acute reaction can be mimicked by testicular injection of IL-1β but not IL-1α.

IL-1β was detectable in macrophages but not in Leydig cells after hCG dosing, indicating that Leydig cells can regulate inflammatory responses by activation of macrophages in the testis.

Using a rat leukaemia model and human leukaemic cells recovered from patients with childhood leukaemia, we could demonstrate that leukaemic cell proliferation can be regulated by testicular constitutive factors.

In conclusion, the present data suggest that testicular proteins can influence proliferation of normal as well as malignant lymphocytes. In the present study IL-1 and TGFβ have been especially implicated - IL-1 as a proliferation-inducing factor and TGFβ as a proliferation suppressive factor that might render malignant lymphocytes less sensitive to cytotoxic effects of chemotherapy. Production of such factors is, at least in part, under hormonal regulation by pituitary hormones. These results support a role for the testicular paracrine factors in the tendency for testicular relapse of ALL.

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LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Pöllänen P, **von Euler M**, Sainio-Pöllänen S, Jahnukainen K, Hakovirta H, Söder O, Parvinen M. Immunosuppressive activity in the rat seminiferous tubules. J Reprod Immunol. 1992 Aug; 22(2): 117-26.

II. Jahnukainen K, Attermo P, Saari T, Salmi TT, **von Euler** M, Pöllänen P, Söder O. Effects of testicular cytokines on proliferation of rat T-leukaemic lymphoblasts in vitro. J Reprod Immunol. 1995 Mar; 28(3): 263-73.

III. **von Euler M**, Söder O: Proliferative effects of cytokines and testicular proteins on human leukemic cells in vitro. Manuscript. 2002.

IV. **von Euler M**, Svechnikov K, Assmus M, Pöllänen P, Söder O: Leydig cells suppress testicular interleukin-1 induced lymphocyte activation. Manuscript 2002.

V. Pöllänen P, **von Euler** M, Jahnukainen K, Saari T, Parvinen M, Sainio-Pöllänen S, Söder O. Role of transforming growth factor beta in testicular immunosuppression. J Reprod Immunol 1993 Jul; 24(2): 123-37

VI. Assmus M, Svechnikov K, **von Euler M**, Setchell B, Sultana T, Zetterström C, Holst M, Kiess W, Söder O: Chorionic gonadotropin-induced surge of proinflammatory cytokine expression in rat testis. Manuscript 2002.

CONTENTS

LIST OF ABBREVIATIONS

aa	amino acids
ALL	acute lymphoblastic leukaemia
AML	acute myeloic leukaemia
ALC	adult Leydig cells
AMH	anti Müllerian hormone
ABP	androgen binding protein
BMP	bone morphogenetic proteins
BSA	bovine serum albumin
cAMP	cyclic adenosine mono phosphate
Con-A	concanavalin-A
cpm	counts per minute
CNS	central nervous system
DNA	deoxyribonucleic acid
EDS	ethane dimethane sulphonate
EGF	endothelial growth factor
FCS	fetal calf serum
FLC	fetal Leydig cells
FITC	fluorescein isothiocyanate
FSH	follicular stimulating hormone
GM-CSF	granulocyte-macrophage colony stimulating growth factor
GDF	growth/differentiation factor
HPLC	high-performance liquid chromatography
HR	high risk
hCG	human chorionic gonadotropin
3β-HSD	3β-hydroxysteroid dehydrogenase
IGF-1	insulin-like growth factor 1
IL	interleukin
IL-1R	interleukin-1 receptor
IL-1ra	interleukin-1 receptor antagonist
ISF	interstitial fluid
kDa	kilo Dalton
LC	Leydig cells
LH	luteinizing hormone
LHRH	luteinizing hormone release hormone
Ly	lymphocytes
M	molar (mole/litre)
M_r	relative molecular mass
m	milli (10^{-3})
mRNA	messenger ribonucleic acid
μ	micro ($10^{-6)}$
MHC	major histocompatibility complex
n	nano (10^{-9})
PBL	peripheral blood lymphocytes
PBS	phosphate-buffered saline

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PDGF	platelet derived growth factor
PHA	phytohemaglutinin
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
TβR	transforming growth factor β receptor
TGF	transforming growth factor
TNFα	tumour necrosis factor α
^{3}H-TdR	tritium marked thymidine
SR	standard-risk
VHR	very high-risk
WBC	white blood count

INTRODUCTION

The testis is the third most common site of relapse after treatment of childhood acute lymphoblastic leukaemia (ALL) (1, 2). Despite improvements in therapy, testicular relapses continue to pose a clinical problem. The mechanisms behind the tendency for testicular relapse of ALL have been linked to the immunosuppression present in the testis. The testicular immunosuppression seems to involve both physical and regulatory components and is probably critical for the ability of the testis to produce viable gametes throughout life. However, several hormonal and regulatory factors and cytokines with effects that could influence localisation, proliferation and resistance to cytotoxic agents are produced in the testis. This study focus on the local mechanisms influencing activation and survival of normal and malignant lymphoid cells in the testis and their relevance for testicular relapse of childhood ALL.

CLINICAL BACKGROUND

Acute lymphoblastic leukaemia

Acute lymphoblastic leukaemia is the most common childhood malignancy with a reported incidence of 3.4 to 3.9/100000 (1, 2, 3). It has been the focus of intense study since the first major advances were recorded using chemotherapy in the early 1960's (4, 5). Treatment results have improved greatly over the last 30 years but relapse of ALL still affects around 30% of all children diagnosed with the disease which makes relapsed ALL to the 4th most prevalent childhood malignancy (0.9/100000). Survival of children with ALL has increased from almost non-existent 1962 to greater than 70% in 1991 (6). In a recent overview of treatment results at a major cancer centre nine-year overall survival was as high as 84% for the entire population, 93% for standard risk (SR) children, and 79% for high risk (HR) and very high risk (VHR) children (7). Even though there is no clear and uniform international consensus on risk stratification of children with ALL, use of risk stratification is a uniformly adopted strategy that has contributed considerably to the improvement of treatment results. Several features are used to distinguish patients of increased risk for relapse and death and many newer laboratory investigations are under evaluation for use as risk determinants. The most uniformly accepted risk criteria are age, white blood count (WBC) at diagnosis, existence of extramedullary leukaemia, immunophenotype, and sex (8). The sex difference only appears after about 2 years and indicates that boys are more prone to late relapse (6). The improvement in therapy of ALL has benefited both girls and boys, but the difference persists. (8, 9) To some extent, this difference is explained by testicular recurrences but in part, it is due to aggregation of other factors suggesting poorer outcome such as WBC at diagnosis, immunophenotype and DNA index (6, 10). Lymphoblastic leukaemia in infants under 1 year of age is associated with unfavourable clinical features, most notably a high leukocyte count and predilection to central nervous system (CNS) disease. Age of 2-10, female sex and WBC <5,000/mm^3 constitute a favourable group. Boys older than 10 with WBC>50,000/mm^3 and extra medullar infiltrates constitute high-risk patients (8 Robinson). Even if induction of remission is achieved in over 80% of patients, children with ALL who experience

hematologic recurrence during initial therapy or shortly after its cessation still have low cure rate (11).

Testicular relapse in acute lymphoblastic leukaemia

Testicular relapse is the second most common site of relapse outside the bone marrow in boys (12). Before 1960 and the start of modern chemotherapy, testicular involvement was rarely observed but with the significant improvement of treatment outcomes in the past decades, an increase in the testicular recurrence rate occurred. (13). The reported frequency of overt testicular recurrence in children with ALL ranges between 0.9–16% (2). Testicular involvement in post-mortem studies has been reported as high as 28-92% (12). The isolated occurrence of testicular leukaemia in boys with ALL is considered to precede generalized recurrence, and therefore signal poor outcome (4). Improvement in treatment results has significantly increased event free survival, indicating a reduction in the frequency of testicular relapse over time (14). Most testicular relapses occur shortly after treatment is stopped (15, 16, 17), indicating that the relapse arises from residual leukaemic cells that have been suppressed, but not eradicated, lying dormant in the testis during maintenance therapy (18). A recent review has demonstrated that the 6-year survival rate for isolated testis relapse was 70%, demonstrating more favourable outcome than isolated bone marrow relapse (20%) or CNS relapse (48%) (19). This is probably due to the increased use of post induction intensification treatment protocols (19) and that testicular relapse indicates the survival of cells of less aggressive nature than in other cases. This would indicate that cells surviving in the testis have less proliferative drive or that they are subject to less treatment pressure than cells in other locations. Combined relapse in testes and bone marrow continues to carry same poor outcome as isolated bone marrow relapse (19). It is not clear to what extent the source of a combined relapse is the testis or the bone marrow.

The increased risk associated with less than 1 year of age and older than 10 coincides with periods of increased endocrine activity in the testis. Taken together with the sex difference in prognosis and testicular relapses it is clear that even if the clinical problem caused by testis relapse has been reduced, the role of testicular factors in ALL is of considerable theoretical interest. The mechanisms for the testicular relapse of ALL is still not well understood and are the focus of this thesis.

THE TESTIS

The testis' two main functions, sperm production and androgen production, are physiologically separated into the seminiferous tubules and the interstitial tissue (20). However, throughout evolution the two functions are closely interlinked and interdependent (21). In broad terms regulation of testicular function is exerted through the hypothalamic - hypophyseal - gonadal axis via luteinizing hormone (LH) and follicular stimulating hormone (FSH) mainly acting on Leydig cells and Sertoli cells respectively. Germ cells lack receptors for these hormones so the fine-tuning of the various cellular functions in the testis depend also to a large extent on paracrine factors and direct cell to cell interactions as well as the relative maturity of the organ (20, 22, 23).

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SEMINIFEROUS TUBULES

The seminiferous tubule is the organisational compartment for the spermatogenesis and represents 60-80% of the testicular volume (20). Germ cells and Sertoli cells are the main cell types in the tubuli. Peritubular cells make up several cell layers outside the tubules. The organisation of tubuli varies in different species as the steps in the very precise and tightly regulated process of spermatogenesis varies. All germ cells pass through a precise pattern of developmental stages, probably regulated through interactions with Sertoli cells. Each Sertoli cell nurse germ cells in several stages of development, which have a constant and precise relationship to one another (24). The spermatogonia and the Sertoli cell have direct contact with the basal membrane in the outer part of the tubule. Toward the lumen, spermatocytes and innermost haploid spermatids are found. The specific types of germ cells are always found in the same associations. This leads to the emergence of the stages of the seminiferous epithelium (24). The rat has XIV defined stages and the human only VI. The morphological appearance is of a wave like pattern that is species characteristic and can be observed through a transillumination microscope (20, 27, 38, 91).

Germ cells

The mature sperm have undergone a complicated and well-regulated development process; form the division of stem cells through meiosis and spermiogenesis. This process is regulated through interaction between the developing germ cells and the Sertoli cell and from the Sertoli cells on the other testicular cells. Testosterone is the most important regulating factor, driving spermatogenesis, but many other factors have been shown to be involved. IL-1 has been suggested to be an important growth factor for developing sperm.

Peritubular cells

Peritubular cells are poorly differentiated myocytes that cover the tubuli and have the ability for spontaneous contraction. The have important functions as producers of several soluble factors involved in paracrine regulation of the testis as well as of several matrix proteins important for the maintenance of the interstitial tissue (3).

Sertoli cells

Sertoli cells are somatic cells located within the germinal epithelium in the seminiferous tubuli. Located on the basal membrane, they are organised in a polarised fashion extending to the lumen. They comprise the morphological compartment and supporting structure for the maturing sperm (25). Specialised tight junctions between Sertoli cells form the blood-testis barrier that allows the formation of a lumen strictly separated from the interstitial compartment (36). This barrier will exclude larger molecules and particles from the tubular lumen, protecting germ cells from toxic substances and immunological reactions as well as creating a well-controlled environment for the germ cells (26). The formation of tight junctions between Sertoli cells occurs at the timing of puberty and is therefore very varying in different species (36). After onset of meiosis and for the duration of their complete development, the germinal cell are surrounded and supported by a Sertoli cell. In addition to physical support and protection, Sertoli cells act as communicating links between the germ cell

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and its environment providing all required nutrients and growth stimulatory and regulatory influences (25). Sertoli cells regulate the spermatogenic process through interactions with germ cells, but Sertoli cell function is tightly regulated by influences from the maturing germ cells. It is well established that the structure and function of the Sertoli cells change according to the stage of the seminiferous cycle (36). Both germ cells and Sertoli cells produce and secrete cytokines and growth factors that serve to maintain a very well coordinated and uniform maturation pattern. In order to function as a communication link to other cells in the testis the Sertoli cell can secrete their products both to the lumen and to the interstitial compartment. This is important since the tubular fluid has a unique composition and higher pressure than the surrounding tissue (27). FSH from the pituitary regulates Sertoli cell maturation. After onset of puberty, testosterone from Leydig cells becomes relatively more important for the regulation of Sertoli cell function (25). Important secretory products of Sertoli cells are androgen binding protein (ABP) in the rat, transferrin, inhibin, activin and IL-1.

INTERSTITIAL TISSUE

Leydig cells

Leydig cells, the androgen producing cells, constitute more than 50% of the interstitial cell mass (28), with great variations in numbers and organization in different species (29). The Leydig cells are generally organised in clusters, often in close proximity to blood or lymph vessels and have close direct contacts with adjoining Leydig cells through gap junctions (30). Leydig cells and macrophages are also often found adjacent to one another with Leydig cell plasma membrane processes frequently inserted into macrophage membrane invaginations (31, 32), indicating a close functional relationship. A physiological relationship is also suggested by the finding that lymphocyte and macrophage motility can be inhibited by non-specific adhesion to the Leydig cell surface membrane in vitro (33, 34, 35). Many macrophage secretion factors have been shown to be important modulators of Leydig cell function and many macrophage secretion products have been shown to be regulated via Leydig cells (see below).

The principal activity of Leydig cells is the production of testosterone in response to luteinizing hormone (LH) secretion from the pituitary. High concentration of testosterone is needed to induce and maintain spermatogenesis (36) and this is in part achieved by the close proximity of the Leydig cells to the seminiferous tubules (37).Testosterone levels are usually 2-3 times higher in interstitial fluid compared to testicular venous blood (36) and 20-100 times higher than in peripheral blood (38). The actual levels of testosterone present in the interstitial tissue is difficult to assess precisely and has been reported to be in the range from 0.1 - 3 μM (36) and varies greatly between species, individuals and studies. The actual mechanism maintaining this gradient is not fully understood. Leydig cells depend on LH stimulation for testosterone release and production, but LH is also required to maintain their structure, number and other differentiated functions (30).

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Fetal Leydig cells

The ontogenesis of Leydig cell function involves at least two generation of cells: fetal (FLC) and adult (ALC) Leydig cells. The fetal type Leydig cells are responsible for the fetal androgen production that leads to sex differentiation and development of secondary sex characteristics. Fetal Leydig cells are probably recruited from mesenchymal cells during early fetal development independent of any stimulating factors (39), but from later fetal development are differentiating under the influence of insulin like growth factor–I (IGF-I) (40). It has been widely accepted that fetal Leydig cells regress during the neonatal period, probably in response to paracrine regulation. However, there are now data to suggest that FLC are present in the adult rat testis (41, 42). The fetal Leydig cells do not seem to be precursors of adult Leydig cells; rather, these are two distinct cell populations (39, 41). In humans, as in all other primates, there is a second surge of androgen production in the perinatal period, under hypophyseal LH regulation resulting in almost adult levels of circulating androgens (39). The significance of this perinatal androgen surge is not completely understood but significant androgen levels are present neonatally in many species (36, 41). The absence of virilising signs as a consequence of this androgen surge is probably due to a relative insensitivity of target organs at this stage of development and to a high level of sex hormone binding globulin in serum (43).

Pubertal development

Onset of puberty is stimulated from the pituitary through increased production and release of LH and FSH. At onset of puberty, adult Leydig cells start to develop from mesenchymal precursors. In rats and other fast maturing species, onset of puberty starts already in the neonatal period. From postnatal day 14, there is onset of pubertal changes such as development of ALC and formation of tight junctions between Sertoli cells in the seminiferous tubules (36). However, the ALC are not capable of producing testosterone until day 35 so it is probable that the FLC are the main source of testosterone until day 35. Unlike the fast maturing species like rat and mouse, in humans and other species with a defined infancy, there is a long period of testicular quiescence, before the third surge of Leydig cell numbers and androgen production starts. There are now several suggestions that the maintenance of Leydig cell quiescence during this period is dependent on TGFβ expression (39, 44). In humans, this period is unusually long compared to other mammals and from around age 1 to around age 10 the testes are quite inactive (38). At onset of puberty increased LH and FSH initiates the maturation of the testes, which continues under the influence of the hypothalamic – hypophyseal – gonadal axis until after puberty (43).

Adult Leydig cells

In addition to circulatory hormones, locally produced growth factors have been shown to have important effects on the differentiation and maturation of the adult Leydig cell population, with different factors regulating each maturation stage of the adult Leydig cell lineage. Five main cell types are present in the adult Leydig cell lineage: mesenchymal precursor cells, progenitor cells, newly formed adult Leydig cells, immature Leydig cells, and mature Leydig cells (45). Differentiation from mesenchymal precursor to lineage committed Leydig progenitor cells is the first step in

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the prepubertal development (30, 46). Transforming growth factor (TGF) α, IGF-I, platelet derived growth factor A (PDGF-A) IL-1β, and thyroid hormone, but not LH, has been implicated in the mesenchymal precursor recruitment and differentiation while LH is critical for further differentiation (30, 36, 39, 40, 47). Androgens, estrogens and anti-Müllerian hormone (AMH) act inhibitory on this differentiation. The newly formed Leydig cells display steroidogenic organelles such as abundant smooth endoplasmic reticulum and mitochondria with tubular cristae (20) and produce androgens, mainly androstenedione. Immature Leydig cells develop a fully functional steroidogenic pathway but metabolise most of the produced steroids. (45, 48). LH and IGF-I influence proliferation and maturation of these cells into mature adult Leydig cells, acquiring the features of testosterone production and release (41, 45, 48). Testosterone and estrogen are inhibitory to the onset of precursor cell differentiation, and these hormones produced by the mature Leydig cells may be of importance to regulate differentiation of precursor cells to Leydig cells in the adult testis to maintain a constant number of Leydig cells (45).

LH is the main regulatory hormone for Leydig cell steroidogenesis. It is a glycoprotein regulation of steroid production hormone secreted by the pituitary in response to LH release hormone (LHRH) stimulation from the hypothalamus. LHRH release is highly pulsatile, and it is the frequency and amplitude of the LHRH pulses that determine the amount of released LH. Half-life of LH is about 20 minutes and its release is pulsatile with each pulse being released in response to an LHRH pulse. Testosterone is the major factor regulating LH secretion through a negative feedback both at the hypothalamus and the pituitary (20). The LH receptor is a G coupled transmembrane receptor containing an extensive extra cellular peptide that serves as the hormone binding domain, seven transmembrane domains and a cytoplasmic region containing a putative phosphorylation site (30) with cyclic AMP (cAMP) serving as second messenger. The receptor binds LH and human chorionic gonadotropin (hCG) with equal affinity. Stimulation of the LH receptor results in two main intracellular effects in Leydig cells - an acute increase in cAMP and steroid production without any corresponding RNA synthesis and a long term trophic effect on cell structure and function requiring RNA synthesis (30).

In addition to LH/hCG, many intratesticularly produced factors have been shown to act on Leydig cells, regulating steroidogenesis or modulating other differentiated functions. Factors that have been demonstrated in vitro or in vivo to affect Leydig cell function include LH, IGF-I, activin, inhibin, endothelin, endothelial growth factor (EGF), TGFα, TGFβ, interleukin 1 (IL-1) α and β, PDGF, tumour necrosis factor α (TNFα) (30).

Effects of human chorionic gonadotropin

Human chorionic gonadotropin (hCG) has been clinically used in supra-physiological concentrations to stimulate testicular descent in boys with retentio testis. HCG treatment is known to induce inflammation-like adverse effects in the testis (49, 50, 51) including elevated intratesticular pressure, vascular leakage and an increased apoptotic rate of spermatogonia (50, 51, 52). In humans, inflammatory changes after hCG treatment have been noticed during orchidopexy in boys suffering from retentio testis. The observed phenomena were reversible although some changes of the vasculature may be persistent (52).

Macrophages

Resident macrophages constitute approx 20% - 25% of the testicular interstitial cell mass (20). Macrophages are located in the testicular interstitium and the lamina propria of the seminiferous tubules, but never *in* the seminiferous tubules (53). Resident testicular macrophages are probably recruited from circulating haematopoietic precursor cells that migrate to the testis (20). Resident macrophages express the resident macrophage surface antigen recognised by monoclonal antibody ED2 (54, 55, 56). In addition, these cells appear identical to resident macrophages in other tissues, expressing the same functional markers, including Fc receptors and major histocompatibility complex (MHC) class II antigens. They have the capacity for antigen presentation and normal phagocytic, cytotoxic and bactericidal properties (56). Also small numbers of monocyte type macrophages (ED2⁻) can be found in the normal adult rat testis (55). Leydig cells and macrophages develop in a co-ordinated fashion, suggesting a close interdependency between these cells. (23, 57, 58). Macrophages secrete inflammatory cytokines, some with profound effects on Leydig cell function (21, 23, 28), e.g., TNFα (59), IL-6 (60), IL-1α (61, 62), IL-1β (61) and granulocyte-macrophage colony stimulating growth factor (GM-CSF) (63). In animals that have been depleted of macrophages in the testis, testosterone production is severely depressed (20). On the other hand, there is evidence that Leydig cells play a role in the regulation of resident macrophages. Inhibition of Leydig cell function through testosterone implants or ethane dimethane sulphonate (EDS) treatment reduces the number of resident macrophages (64,56). It is probable that Leydig cells are involved in regulation of testicular macrophage proliferation since hCG stimulation increase the mitotic index of these cells (20). Treatment with hCG has also been shown to induce production of inflammatory cytokines by macrophages, influencing vascular permeability and leukocyte migration (58).

IMMUNE PRIVILEGE IN THE TESTIS

The testis is a complex tissue from an immunological point of view. The spermatogenic cells reside in the testis throughout life without eliciting any immune reactions, despite the presence of auto-antigens on these cells (65, 66). Several investigations have demonstrated that the testis can function as a privileged site where allografts and even xenografts can survive for long periods (67, 68, 69, 70). In contrast to other immunologically privileged sites in the body the testes have the elements required for eliciting an immune response: efficient lymphatic drainage as well as the presence of macrophages expressing MHC class II molecules and a small number of T and B cells (53, 32, 69, 71, 72). However, it has also been demonstrated that the testis has an effective efferent immune function, able to reject donor tissues as well as give rise to normal immune responses (67, 69). When the testicular immune tolerance breaks down as in autoimmune orchitis or after testicular injury or vasectomy the result is permanent impairment or total loss of fertility (73). Leydig cells have also been reported to suppress polyclonal mitogen or allogenic cell induced lymphocyte proliferation in vitro (74). Prolonged transplant survival has been shown with rat testicular islet allografts in diabetic rats who were either hypophysectomized, or had abdominally placed testes and were chronically treated with a LHRH-analogue and also in rats treated with EDS to deplete the testes of Leydig cells, demonstrating that long term allograft survival can

occur in testes deprived of spermatogenesis and Leydig cells under special circumstances (75, 76).

Thus, it appears that multiple factors are involved in the creation and maintenance of the special intratesticular milieu that protects the reproductive functionality. The basic physical feature is the blood – testis barrier, which might be better referred to as blood – tubule barrier, created by tight junctions between Sertoli cells, establishing a physical protective barrier for the maturing sperm. In addition a specific immunologically tolerant immunological milieu is established through the activity of several locally produced factors as well as special functions of the local immune cells - macrophages and dendritic cells - and the Fas system (77).

TESTICULAR PARACRINE FACTORS

The local proximity of sex steroid-producing cells and spermatogenic cells allows multiple cellular interactions to occur and thereby facilitates the modulation and/or synchronization of both testicular functions. Factors that are involved in local regulatory functions of steroidogenesis and spermatogenesis are often also recognised as factors that can influence immune functions and are often produced by immune cells (21, 78). Local regulatory factors include cytokines, growth factors, neuropeptides and steroids (79). The source of these cytokines in the testis is believed to be local resident macrophages (59, 61, 80; 81), Sertoli cells (82), germ cells (83) and Leydig cells (60, 84, 85). Several pro-inflammatory cytokines, including IL-1α, interleukin-1 receptor antagonist (IL-1ra) IL-18 and TNFα have been shown to be constitutively produced in the normal adult testis without signs of inflammation and are suggested to have physiological roles as growth factors (30, 59, 86, 87, 88, 61, 89, 90) and as regulators of Leydig cell function and steroidogenesis (91, 92, 136). IL-1α is produced in the seminiferous tubules in a stage and age dependent manner that suggests a physiological role in regulating germ cell deoxyribonucleic acid (DNA) synthesis (see below). IL-1β and IL-6 are mainly produced in response to inflammatory or immunological and/or immunological reactions (86, 89). Various members of the TGFβ growth factor family, including activin and inhibin, have also been suggested to be involved in the paracrine regulation of testicular function. The sources of these constitutive cytokines is believed to be mainly non-immune cells like Sertoli cells and germ cells whereas inducible cytokines like IL-1β have been traced to resident testicular macrophages activated by various stimuli (63, 93). It is easy to imagine that the very precise nature of the cellular mechanisms involved in gamete production and steroid synthesis require a very close local interaction and co-ordination of cellular activity. It is, however, not clear if this tightly regulated and closed system functions as a cascade of events, each dependent on the previous or if it is a highly conserved and genetically programmed series of events. What is clear is that the organization of the androgen production and the gamete production in close proximity is a highly conserved model throughout evolution (94).

Interleukin-1

Interleukin-1 is a family of pro-inflammatory cytokines that in the testis are suggested to act as growth factors in addition to their well-known functions in inflammatory and immunological processes. (89, 95, 96). The IL-1 family consists of two agonist

proteins, IL-1α and IL-1β, which are encoded by different genes but share the same trans membrane receptor and activity, and one antagonist, IL-1ra, which blocks the IL-1 receptor competitively. In most studies, their biological activities are indistinguishable. IL-1 affects nearly every cell type, often in concert with another pro-inflammatory cytokine, TNFα. Although IL-1 can upregulate host defences and function as an immunoadjuvant, IL-1 is a highly inflammatory cytokine (97). Interleukins were originally considered as signalling mediators in the immunoinflammatory system (98) but they have since been found to be expressed in many tissues under physiological conditions, e.g. kidney (99) and testis (100, 101). The macrophage is a primary source of IL-1, but also epidermal, epithelial, lymphoid, and vascular tissues synthesize IL-1. In the circulation, IL-1 acts like a hormone and induces a broad spectrum of systemic effects in neurological, metabolic, hematologic, and endocrinologic systems. Some IL-1 remains associated with the plasma membrane of the synthesising cell and induces changes in local tissues without producing systemic responses (102).

In the testis, IL-1α has been found to be constitutively produced at both the messenger RNA (mRNA) and protein levels and to possess non-inflammatory properties (87). The expression of IL-1α has been localized to Sertoli cells (103), without any obvious contribution from macrophages (86). In fact, testicular macrophages do not seem to produce IL-1 under normal conditions (81, 104; 105). IL-1α shows evidence of a developmentally regulated production pattern with detectable mRNA from postnatal day 15 in rats and extractable protein found from day 20 (106, 107, 108). IL-1α production correlated with the stage of the seminiferous epithelial cycle and thus with the phases of spermatogenesis showing a low or absent production in rat stage VII and a stable production through the other stages. IL-1α is proposed to act as a growth factor for germ cells in the testis since the stage specific levels of IL-1α correlate with germ cell DNA synthesis (88, 91, 109). IL-1α protein can be detected both in interstitial fluid (ISF) and in rete testis fluid, demonstrating that IL-1 is bi-directionally produced by the Sertoli cells (86, 109). The mechanisms allowing IL-1α to be produced and secreted in bioactive amounts in the adult testis without signs of local inflammation are not understood. It is interesting that injection of recombinant IL-1α as well as semi purified testicular IL-1 intratesticular fail to induce any local inflammatory response while injection into an extra testicular site, indeed evoke local inflammation. IL-1β, on the other hand, injected into the testis induces a vigorous testicular inflammation (110). These observations suggest the existence in the testis of other paracrine factors inhibiting the proinflammatory actions of IL-1α but still allowing such actions of IL-1β. Our understanding of the molecular mechanisms involved in this regulation is presently incomplete. Recent studies have demonstrated a constitutive production in the intact adult testis of IL-1ra, both at the mRNA and protein levels (111). These findings demonstrate that both forms of IL-1 agonists (IL-1α; IL-1β) and the IL-1 antagonist (IL-1ra) are produced in the testis under physiological and pathophysiological conditions, but do not give any clues to the mechanisms behind the differential effects of IL-1α and IL-1β in the testis (111). Recent data have demonstrated the existence of at least three distinct bio- and immunoreactive IL-1α iso-forms in the testis and that they may serve as paracrine mediators under physiological or pathophysiological conditions (96). The function of this testicular IL-1 agonist-antagonist network is

currently unclear but the constitutive testicular expression of IL-1ra, is suggesting that the IL-1 activity in the testis may be specifically regulated by paracrine mechanisms.

Transforming growth factor β

The transforming growth factor β (TGFβ) is a superfamily of cytokines which we now know include some 30 members that can be divided into two main branches, the TGFβ/Nodal/Activin branch and the BMP/GDF branch, based on their signalling pathway (112). Several of the members of the TGFβ superfamily have been shown to be active in the testis. TGFβs exert a wide range of biological responses on a variety of cells belonging to different organ types. They are involved in the regulation of proliferation, differentiation, survival, extra cellular matrix formation, bone, cartilage and skeletal development, wound healing, haematopoiesis, immune and inflammatory cell responses as well as reproductive and nervous system development (113, 114). TGFβ is central in cell growth regulation and differentiation.

TGFβ consists of three isoforms, TGFβ1, TGFβ2 and TGFβ3 encoded by different genes on different chromosomes, but characterized by their structural similarities as well as their signalling cascades (113, 115). Each TGFβ-isoform shows a distinct spatial and temporal expression pattern during development and adult life. During development, TGFβ expression is found in many tissues including testis, muscle, heart, blood vessels, haematopoetic cells, lung, kidney, gut, liver, skin, and the nervous system (113, 116). In the testis, there is now evidence of TGFβ expression in Sertoli cells, Leydig cells and developing germ cells (117) and that TGFβ is involved in the paracrine regulation of Leydig cell function including inhibition of steroid production. (112, 118, 119)

All the TGFβs signal via a ligand induced receptor complex consisting of one TGFβ receptor type II unit (TβR-II) that activates one TGFβ receptor type I unit (TβR-I) (117) Signal transduction is mediated through activation of Smad receptor substrates (R-Smad), specific for the two main branches of the TGFβs. The Smad proteins move into the nucleus, where they activate target gene transcription. The distinct response depends on the cells genetic makeup and what other influences the cell is under at the specific time (115). The signal transduction process might be simple but the specific response is under intricate control by a variety of regulators, suggesting that (ref.) the TGFβ signalling pathways are fundamental signal conveying systems, the response to which will be determined by the specific circumstances under which the signal is received in the nucleus. TGFβ regulated growth control is mainly exerted through inhibition of cell proliferation through inhibition of cyclin dependent kinases or down regulation of c-myc. (112) but apoptosis induced by TGFβ is an essential component of normal development of many tissues including gonocytes in the fetal testis (113). In murine B-cells TGFβ1 has been shown to induce cell cycle arrest through different mechanisms than in epithelial cells and act through p27Kip1 induction, leading to increased association with its G1 target Cdk2 resulting in inhibition of cyclin E-Cdk2 complexes (120)

In the present study, the role of TGFβ in the testicular immune suppression was evaluated by investigating the effects of neutralizing anti-TGFβ antibodies on the suppression of peripheral blood lymphocyte proliferation by testis extracts. Also the presence of bioactive TGFβ in the seminiferous tubules and the effect of recombinant TGFβ1 (rTGFβ1) on testicular IL-1α stimulated thymocyte and polyclonal mitogen

stimulated peripheral blood lymphocytes (PBL) proliferation were investigated to see, if TGFβ has a role in the testicular immunoregulation.

AIMS OF THE THESIS

The aim of this thesis was to obtain a better understanding of the testicular paracrine factors that contribute to the relapse of treated childhood acute lymphoblastic leukaemia. The mechanisms behind testicular relapse of ALL are not understood and are of considerable theoretical and clinical interest and are the focus of this study. Specific aims were to:

- Investigate testicular cellular, paracrine and endocrine factors effect on normal and malignant lymphocytes
- Attempt to characterize factors of importance for regulation of cell proliferation in the testis
- Investigate how these factors might be regulated

MATERIALS AND METHODS

This section describes important principles of the used methods. For details, see the individual papers referenced by roman numerals.

HORMONES, CYTOKINES, ANTIBODIES AND GROWTH FACTORS

To elucidate the mechanisms for immunosuppression in the testis we tested the activity of hormones, cytokines, growth factors, and specific neutralizing antibodies to factors known to be active in the testis, on lymphocyte proliferation in vitro.

Since testosterone is released by Leydig cells into the interstitial tissue for transport through lymphatic drainage to the blood stream concentrations are much higher interstitially than in plasma. We therefore wanted to test very high concentrations for influence on lymphocyte proliferation (I, IV). This has previously been difficult to achieve, but with a new water-soluble testosterone preparation, we could test a wide range of concentrations up to 100 μM (IV). These very high concentrations were tested so that the concentrations that could possibly be present in the immediate proximity of the Leydig cell membrane were covered.

Dilution series of IL-1α containing testis extracts from rat and human testes as well as recombinant human IL-1α, IL-1β, IL-6, TGFβ1 and TGFβ2 were used to determine effect and dose response in culture on normal and leukaemic lymphocytes (I, III, IV, V). These cytokines were included in the test panels since they are known to be produced in the testis or as controls to validate test systems and bioassays. In some experiments combinations of several cytokines were used since it is expected that tissue protein extracts will contain a mixture of several cytokines (II). To specifically assess the biologic effects of TGFβ in protein tissue extracts, samples were tested in the presence and absence of anti-TGFβ neutralizing antibodies (I, V). As control, an irrelevant rabbit-anti-mouse antibody was used (V).

To study hormonal control of cytokine production in the testis in vivo hCG was used to treat rats before evaluating effects on production and expression of cytokines in the testes. LH receptors bind LH and hCG with equal potency. HCG can therefore be used interchangeably with LH. HCG was given as a single subcutaneous injection of 100 U in 0.5 ml isotonic saline or the same volume of saline as control.

ANIMALS

Male Sprague Dawley rats (III, IV, VI), Wistar rats (I, II, V), and Lewis rats (I) were used as donors of tissues and lymphocytes. Piebald variegated rats were used to propagate the rat leukaemic cell line by peritoneal passage every 17 days as described in (II). For thymocyte bioassay, NMRI/KI mice were used as donors of thymocytes. They are locally bred and have been shown to be highly responsive to IL-1 (III, IV, V, VI). In experiments investigating the pro-inflammatory effect of hCG, we used male Sprague Dawley rats, some of which had been pre-treated with EDS to remove all Leydig cells (VI). EDS is a cytotoxic substance that selectively kills Leydig cells in rats (37, 121) and several other species (122). Estrogen treatment leads to disruption of the hypophyseal - gonadal axis resulting in disruption of spermatogenesis. It has been

shown that estrogen treatment of leukaemia bearing rats leads to a decrease in the infiltration of leukaemic lymphocytes into the testis (123). It was therefore of interest to evaluate what impact estrogen treatment had on the production of testicular immunosuppressive factors in normal rats. Wistar rats were treated with estrogen (estradiol benzoate 250 µg daily) to study if intact spermatogenesis was important for the production of immunosuppressive factors in the testis (II). In some experiments, disruption of spermatogenesis was achieved by making Wistar rats unilaterally cryptorchid since this has been reported to also enhance the production of immunosuppressive activity in the testis (II, V). Unilateral cryptorchidism was created by suture of the testis capsule to the abdominal wall through an abdominal incision under pentobarbital anaesthesia. A Leydig cell tumour cell line was maintained by subcutaneous implantation in the neck of Male Sprague Dawley rats approximately every 6-8 weeks.

Animals were kept in standard cages under controlled conditions in the animal house of the university. They were sacrificed through CO_2 inhalation. All experiments were approved by the relevant animal ethics committees

CELL CULTURES

In general, short-term cultures of various lymphocytic cells were established under aseptic conditions and used to investigate effects of tissue proteins, cytokines, hormones, growth factors or cells on lymphocyte proliferation. At the end of culture, tritium marked thymidine or (^{3}H-TdR) was added to cultures and after a suitable time cultures were harvested onto glass fibre filters. The incorporation of radioactivity labelled thymidine into DNA was assessed by liquid scintillation counting. Activity was expressed in counts per minute (cpm) and taken as a measure of lymphocyte proliferation.

Lymphocytes

Peripheral blood lymphocytes were isolated by Ficoll-Paque gradient centrifugation. Thymus and spleen lymphocytes were prepared from aseptically removed organs. The tissue was cut into small pieces and the cells released by washing on top of a coarse nylon net and filtered through very fine mesh nylon filters to remove connective tissue and cell aggregates. After washing, the cells were suspended in cell culture medium and diluted to the desired concentration.

Human leukaemic cells were isolated from blood or bone marrow aspirates obtained in standard heparin tubes. After centrifugation, cells were suspended in cell tissue medium and separated on Ficoll-Paque as described above. Lymphocytes were cultured in controlled environment in flat-bottomed tissue culture plates and harvested after a terminal pulse of ^{3}H-TdR.

Human leukaemic lymphocytes

Human leukaemic cells from newly diagnosed or relapsed patients were obtained from the Department of Paediatrics at Karolinska Hospital. Both bone marrow aspirates and peripheral blood was used. Samples were obtained in standard heparinized tubes. After

centrifugation, cells were suspended in cell culture medium and separated on Ficoll-Paque as described above.

Normal rat Leydig cells

Normal rat Leydig cells were enriched through Percoll density fractionation from crude interstitial cell preparations obtained through gentle collagenase treatment of aseptically removed and decapsulated testes. The crude interstitial cells suspended in the supernatant were carefully washed and then separated on Percoll gradients as described in detail in paper IV. Purified Leydig cells were recovered from the Percoll gradients and used after washing. Typically, the purified Leydig cell preparations contained 70-90% Leydig cells as determined by 3β-hydroxysteroid dehydrogenase (3β-HSD) staining.

Tumour Leydig cells

The purified Leydig cell preparations from normal rats contain a fraction of interstitial cells other than Leydig cells that could theoretically be responsible for some of the effects observed. To corroborate the conclusions on effects ascribed to normal Leydig cells a Leydig cell tumour (H-540) was used. The transplantable rat Leydig cell tumour, a generous gift from Prof. M. Niemi, Dept. of Anatomy, Turku University, Turku, Finland, was grown in Sprague-Dawley rats and transferred to new host animals approximately every 6-8 weeks. The cell line originally arose spontaneously in a male Fisher rat and has been extensively described by B. Cooke et al. (124) and Erichsen et. al. (125, 126, 127, 128, 129, 130, 131). It is essentially unchanged during multiple transfer generations and is shown to generate cells with essentially normal Leydig cell properties. To prepare tumour cell suspensions, the rat was killed by CO_2 inhalation and viable parts of the tumour were cut out and placed in medium. Free cells were obtained by carefully sucking tissue fragments in and out of a 2 ml syringe several times. The cell suspension was washed twice, resuspended in medium and diluted to the desired concentration.

CELL CULTURE ASSAYS OF HORMONES AND VARIOUS FACTORS

Effect of testosterone on lymphocyte proliferation

Testosterone was tested for influence on lymphocyte proliferation using alcohol soluble testosterone in concentrations up to 2μM and with water-soluble testosterone in concentrations up to 100μM. Testosterone was tested in the standard IL-1 assay and in assays, using anti-CD3 activated T cells in the absence and presence of recombinant IL-1.

IL-1 bioassay

To identify IL-1 bioactivity a highly specific sensitive IL-1 bioassay was used. This assay has been well characterised (132) and is based on proliferation of thymocytes from NMRI/KI mice, a locally bred NMRI mouse strain. Thymocytes were prepared as described above, from six-to-ten weeks old NMRI/KI mice, cultured in the presence of a suboptimal concentration of phytohemaglutinin (PHA) in 96 well flat-bottomed

micro-titer plates. Test material was added from the start of culture. In some experiments, the assay was used to study effects on IL-1 activity. In those experiments, a suboptimal dose of IL-1 was used and the test sample added in serial dilutions. The cultures were harvested at 48h after pulse labelling with ^{3}H-thymidine during the last 2 h of culture. The incorporated radioactivity was measured using liquid scintillation and used as measure of IL-1 activity.

TGFβ-bioassay

To identify TGFβ bioactivity a well-characterised sensitive and specific bioassay (133) based on the TGFβ-responsive CCL-64 mink lung epithelial cell line was used (V). The cell line was maintained in cell culture by weekly splitting. For the bioassay, 6 x 10^4 cells were added to standard 96-well flat-bottomed culture plates with the addition of the test samples. The cultures were harvested after 24h following a 2 h pulse of ^{3}H-TdR. Radioactivity was measured using scintillation counting and the radioactivity expressed as cpm was used as an estimate of TGFβ bioactivity.

Neutralising TGFβ activity

To qualify as a paracrine agent it should be possible to block the expected activity using specific neutralising antibodies. We therefore attempted to block the immunosuppressive activity of testis extracts using two rabbit-anti-mouse anti- TGFβ antibodies, BDA 1 and BDA19. Serial dilution of the antibody was added to cultures of peripheral blood lymphocytes supplemented with testis extract or rTGFβ1 and harvested at 64 h following a 16-hour pulse of ^{3}H-TdR. The BDA 19 antibody was added to control cultures without testis extract to see if inhibition of rat PBL proliferation was due to endogenous production of TGFβ by the cultured lymphocytes. Cultures with medium and cultures with an irrelevant rabbit-anti-mouse antibody were used as controls. Radioactivity was measured using scintillation counting and the radioactivity expressed as cpm was used as an estimate of proliferative activity.

CO-CULTURES

Co-cultures of seminiferous tubules and lymphocytes

To assess the contribution of seminiferous tubules to the testicular immunosuppression co-cultures of seminiferous tubule segment and lymphocytes were established (I). Seminiferous tubule segments of defined stages of 2mm length were isolated using transillumination-assisted microdissection as described by Parvinen and Ruokonen 1982 (136) and cultured with normal rat PBL stimulated with Con-A and FCS for 72h. Cultures were harvested following a 16-hour pulse of ^{3}H-TdR. Radioactivity was measured using scintillation counting and the radioactivity expressed as cpm was used as an estimate of proliferative activity.

Leydig cell co-cultures

To study the effect of Leydig cells on lymphocyte proliferation co-cultures of lymphocytes and Leydig cells were established (IV). Various number of Leydig cells were added to stimulated lymphocyte cultures established as described above in the

presence or absence of relevant test substances in sub-optimal concentrations. Pure lymphocyte and Leydig cell cultures were used as controls.

IMMUNOHISTOCHEMICAL AND ENZYMATIC STAINING OF CELLS

Viability of cells for cell culture was assessed by Trypan blue dye exclusion test. Leydig cells were identified by demonstrating presence of 3β-HSD activity (IV). Leydig cells were also detected using immunohistochemistry: polyclonal antibodies against steroid enzymes recovered from a patient with autoimmune Addison's disease (134) was used to incubate Leydig cells before staining with a fluorescein isothiocyanate (FITC) conjugated donkey anti-human antibody (cover picture). To confirm the cellular origin of IL-1β in testicular interstitial cell preparations a triple staining method allowing simultaneous staining of IL-1β protein and ED2 antigen (a rat macrophage marker) as well as Leydig cells was used (VI). Slides were incubated with a primary polyclonal goat anti-rat IL-1β antibody and a polyclonal mouse anti-rat macrophage ED 2 antibody before staining with FITC conjugated donkey anti-goat antibodies and Gy3-conjugated donkey-anti mouse antibodies. After washing and drying, slides were stained for 3β-HSD to identify the Leydig cells. Testicular histology was examined after staining of sections with Crysyl violet.

TISSUE EXTRACTS

Whole testis extracts

For preparation of whole testis extracts, testes were decapsulated, weighed and frozen immediately after sacrificing. After thawing tissues were homogenized in saline. After centrifugation, the supernatants were collected, frozen, thawed and cleared from membranes and debris by ultracentrifugation. Supernatants were collected and the low-molecular weight substances (< 5 K) removed by Sephadex G-25 PD-10-column chromatography. Protein concentration was assessed by the Bradford method (135) with bovine serum albumin (BSA) as standard.

Seminiferous tubule extracts

To characterize the testicular immunosuppressive activity originating in the seminiferous tubules, segments of defined stages of the seminiferous epithelial cycle were isolated using transillumination-assisted micro dissection (136) for preparation of tissue extract for analysis. A total length of one meter of 2 mm tubule segments in stages IX-XII of the seminiferous epithelial cycle was prepared and homogenized in saline. After repeated centrifugation, the supernatants were used for high-performance liquid chromatography (HPLC) size exclusion chromatography.

RT-PCR ANALYSIS OF CYTOKINE mRNA

For reverse transcriptase polymerase chain reaction analysis, RNA and complementary DNAs were prepared using standard protocols (for details see paper VI). PCR amplification was carried out on a Perkin-Elmer GeneAmp PCR System 2400 using a

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standard profile. Twenty µl of the PCR product were run on a 1% agarose gel with ethidium bromide and quantified after subtraction of blank background. Expression of the housekeeping gene G3PDH was used to normalize cytokine expression.

HPLC SIZE EXCLUSION CHROMATOGRAPHY

Samples were applied in aliquots of 100 µl to an TSK G3000SW (7,5 x 600 mm, Pharmacia-LKB, Uppsala, Sweden) gel filtration column. Fractions of 0.5 ml were eluted with phosphate-buffered saline (PBS) using a flow rate of 1.0 ml/min. The absorbance of the eluent was monitored at 280 nm. K_{av} values were calculated for the eluted peaks and their corresponding M_r's estimated from the standard curve prepared from the relative molecular mass standards used

STATISTICAL ANALYSES

Differences analysed using analysis of variance (ANOVA), Student's t-test and Tukey's test

RESULTS AND DISCUSSION

IDENTIFICATION OF IMMUNOSUPPRESSIVE ACTIVITY IN THE RAT TESTIS AS TGFβ (PAPER I AND V)

Co-culture of seminiferous tubules and PBL

Rat seminiferous tubule segments of the epithelial cycle were co-cultured together with polyclonal mitogen stimulated PBL to study the immunosuppressive activity in seminiferous tubules. A stage dependent reduction of lymphocyte proliferation was observed and it was concluded that this effect was probably due to secretion of an immunosuppressive factor into the medium. The greatest suppression was seen in stages II-VIII. Some stages displayed stimulatory effects in the system, especially in stage IX-XII. To further study the immunosuppressive effect, single stage segments of stages II - VII were prepared. It was found that the greatest suppressive activity was exerted in stage II. The variation in immunosuppressive activity in different cell associations might indicate a physiological role, maybe involved in regulation of stage related growth factor and cytokine production (95, 107, 137, 138). To further characterize the immunosuppressive factor(s) we used extracts of tubule segment of stages II - VIII for size exclusion chromatography using HPLC. The gel filtration chromatography under non-denaturizing conditions confirmed the existence of immunosuppressive activity in stages II-VIII with apparent M_r of 25 kDa and 65 kDa suggesting that they could represent isoforms or multimers of TGFβ (139).

Neutralizing TGFβ activity on PBL

The role of TGFβ in the testicular immunosuppression was further evaluated by investigating the effects of neutralizing anti-TGFβ antibodies on the suppression of PBL proliferation by testis protein extracts and the presence of bioactive TGFβ in seminiferous tubules. Using previously described methods we established that proliferation of polyclonal mitogen stimulated rat peripheral blood lymphocytes was dose dependently inhibited by the addition of testis extract or rTGFβ1 to the lymphocyte cultures (for details of extraction and culture conditions see V, 140, and 141). Using two rabbit-anti-mouse anti- TGFβ antibodies, BDA 1 and BDA19, we could reverse the inhibitory effect of testis extract on lymphocyte proliferation. An irrelevant rabbit-anti-mouse antibody was used as control and had no effect. There was no effect of the BDA 19 antibody in control cultures, demonstrating that the observed inhibition of rat PBL proliferation was not due to endogenous production of TGFβ by the cultured lymphocytes. From these results, we conclude that the active material in the testis extract suppressing PBL proliferation in our test system is most probably TGFβ.

TGFβ suppression of cytokine activity

Since suppression of inflammatory effects of constitutively produced pro-inflammatory cytokines might be one of the possible physiological effects of immunosuppressive factors in the testis, we investigated if TGFβ can suppress the effect of testicular IL-1α (tIL-1) on lymphocyte proliferation. A dose-response curve of tIL-1 bioactivity was

established in a murine thymocyte proliferation assay using the highly sensitive IL-1 responsive NMRI/KI mouse strain. A sub optimal dose was selected for culture in the presence or absence of various concentrations rTGFβ1. In this system, rTGFβ1 suppressed both tIL-1-unstimulated and tIL-1-stimulated murine thymocyte proliferation in a dose dependent way suggesting that TGFβ1 could be involved in the regulation of the testicular IL-1 effects on lymphocytes.

TGFβ in seminiferous tubules

Based on these findings, we proceeded to investigate the presence of bioactive TGFβ in seminiferous tubules using a TGFβ bioassay. Samples of gel filtration fractions of rat seminiferous tubule extracts were added to the TGFβ-responsive CCL-64 mink lung epithelial cell line growth inhibition assay. This is a well-characterised standard TGFβ bioassay (133). There was suppression of proliferative activity in the tested fractions, demonstrating the presence of a TGFβ activity in the samples. A peak of inhibiting activity could be observed at ~25 K which coincides with the reported Mr (25 K) of the bioactive rTGFβ dimer (142). These results are consistent with earlier findings of a~25 K peak of immunosuppressive activity present in abdominal testis extracts (146). The interpretation of the increase in lymphocyte inhibiting activity seen in short term experimental cryptorchidism (146) is unclear and might be a transient phenomenon related to the surgical procedure itself or the massive germ cell death and other signs of a localized inflammatory reaction that can be observed after experimental cryptorchidism (143). In a long-term cryptorchidism experiment no increase in lymphocyte inhibiting activity was seen, instead a reduced concentration was observed. This might be related to increased interstitial fluid volume and simply reflects dilution (144) or it might reflect a changed ratio of suppressive and stimulatory factors.

INFLUENCE OF INFLAMMATORY CYTOKINES ON THE PROLIFERATION OF LEUKEMIC CELLS (PAPER II AND III)

Effect of temperature on proliferation of rat lymphoblasts

Using in-vitro cell culture techniques we studied the ability of testicular extracts and cytokines known to be expressed in the testis to influence the spontaneous proliferative activity of rat and human leukaemic, cells. Roser's rat lymphoblastic leukaemic cell line cells have a high spontaneous proliferation that diminishes after 24h in culture in line with decreased cell viability. To avoid interference of decreased cell viability on the results we used 24h cultures as standard to assay effects of testicular extracts and cytokines on these cells. Effect of temperature on the in vitro system was investigated since it is known that at least one G protein signal transduction mechanism is only effective at normal testis temperature (34°C) and not at normal body temperature (25). Spontaneous proliferation of the rat lymphoblasts was not influenced by incubation at 37°C or 34°C. The growth inhibition exerted by testicular extracts was not affected either. However, viability during longer-term cultures was affected by temperature, with viability being higher at 34°C than at body temperature. Even if it is difficult to know what the physiological significance is of this result, it is still an interesting observation that could be of importance for long-term survival of lymphocytes in the testis.

Spermatogenesis is not required for testicular leukaemia

Estrogen treatment leads to disruption of the spermatogenesis through inhibition of LH production from the pituitary. In earlier studies (123), it was shown that estrogen treatment of tumour bearing rats could lead to a decrease in the testicular infiltration of rat leukaemic cells. It has also been shown that disruption of spermatogenesis by estrogen treatment; hypophysectomy or experimental cryptorchidism can increase the immunosuppressive activity of testis extracts (146). We therefore wanted to investigate the influence of testis extracts from rats without germ cells on leukaemic cell proliferation. Testis extracts from both estrogen treated and cryptorchid rats were found to inhibit spontaneous proliferation of the leukaemic lymphoblasts. The inhibitory activity was greater in both the estrogen treated and the abdominal testis extracts than in the controls. However, the sensitivity to these extracts appear to be less pronounced than on polyclonal mitogen stimulated normal rat PBL (V). Since previous studies had indicated that the physical characteristics of the immunosuppressive activity is similar with TGFβ, we tested the effects of this factor on the rat leukaemic T-cells. TGFβ₁ was shown to inhibit lymphoblast proliferation and this effect could be reversed with an anti-TGFβ1 antibody.

Influence of cytokines on human leukaemic cell proliferation

Human leukaemic peripheral cells and/or bone marrow cells were recovered from 10 patients diagnosed with childhood acute leukaemia at Paediatric Oncology Department at the Children's Hospital, Karolinska Hospital, Stockholm, Sweden. Eight patients had ALL and two had acute myelocytic leukaemia (AML). Cells remaining after diagnostic workup were cultured in vitro in the presence of rat and human testis extracts known to contain high amounts of IL-1α as well as recombinant hIL-α, hIL-1β, hIL-6 and hTGFβ1. Recombinant hTGFβ1 was suppressive in all experiments except one when no effect was observed. The inhibition was not due to decreased viability as determined by trypan blue exclusion. Recombinant hIL-1α and hIL-1β was stimulatory in 4 resp 5 experiments, was inhibitory in one and had no effect in 5 resp 6. Human testis extract showed stimulation in most experiments, whereas rat testis extract showed a mixed response, with both stimulation and suppression. Testosterone did not show any effect over a wide range of doses

LEYDIG CELLS SUPPRESS TESTICULAR INTERLEUKIN-1 INDUCED LYMPHOCYTE ACTIVATION (PAPER IV)

It is well known that Leydig cells can interact with lymphocytes under varying experimental conditions, inducing a suppression of the lymphocyte proliferative activity (33). These Leydig cell effects might well play a role in the immunological sanctuary observed in the testis under experimental conditions. The physiological importance of this might be related to control of the proliferative properties of constitutively produced pro-inflammatory cytokines and these effects might be of importance for suppressing leukaemic cell proliferation in the testis. Since most testicular relapses of ALL occur shortly after treatment is stopped (15, 16, 17), the relapse could arise from residual leukaemic cells that have been dormant in the testis

during maintenance therapy (18). To investigate the cellular origin and possible regulation of the underlying mechanisms for testicular immunosuppression we investigated the role of the Leydig cells.

Leydig cells recovered after Percoll fractionation contained 60-85% Leydig cells as confirmed by 3β-HSD staining. In co-cultures with Leydig cells and polyclonal mitogen stimulated rat spleen cells or rat thymocytes there was a dose dependent suppression of lymphocyte proliferation. This suppressive effect was evident already at 1:100 Leydig cell to lymphocyte ratio indicating that a soluble factor might be involved, in addition to direct cell-to-cell mediated effects. This assumption is supported by preliminary observations that separation of lymphocytes and Leydig cells in co-culture by a cell impermeable filter insert abolished the production of the suppressive effect. Spent medium from pure Leydig cell cultures also had no suppressive effect. Since 15-35% of the cells in the purified Leydig cell preparations were 3β-HSD negative, and most probably macrophages, it could not be ruled out that the effects observed could be attributed to other cell types. However, other Percoll fractions that contain much less Leydig cells were less suppressive or even stimulatory. To further verify the conclusion that the effect was indeed Leydig cell mediated we repeated the experiments using Leydig cells recovered from a Leydig cell tumour cell line grown in a heterotopic site in rats. The same dose dependent suppression pattern was found confirming that the effects are attributable to Leydig cell effects. Based on these findings the suppressive effect was tested on lymphocytes stimulated by IL-1α in the murine thymocyte assay. Purified Leydig cells were added in increasing numbers to the cultures stimulated by a suboptimal dose of IL-1α. There was a dose dependent inhibition of IL-1 driven thymocyte proliferation evident from a 1:20 Leydig cell to lymphocyte ratio. This effect was seen with testicular IL-1α as well as recombinant human IL-1α and β. Testosterone, the main secretion product of Leydig cells did not show any effect over a wide range of concentrations including very high concentration (up to 100μM).

HORMONAL INFLUENCE ON THE CYTOKINE EXPRESSION IN THE TESTIS (PAPER VI)

In order to understand how production of proliferative factors in the testis might be regulated, we studied the effects of hCG on expression of testicular cytokines.
After treatment with hCG testes showed macroscopic signs of inflammation with tissue oedema and reddish colour evident 8 to12 hours after dosing. Accordingly, testicular venous blood sampling 8 hours after hCG indicated a decreased blood flow when compared to untreated controls.

hCG induces pro-inflammatory cytokines in the testis

In intact control testes, constitutive expression of IL-1α, splice variant 24KproIL-1α, IL-1ra, and TNFα mRNA was detectable by RT-PCR. Slight but significant increase of the expression was found four to eight hours after hCG. No IL-1β or IL-6 mRNA was found in normal control testes but already 4 hours after hCG, IL-1β and IL-6 mRNA was readily detectable. Peak levels of all analysed proinflammatory cytokine mRNAs

were found 8-12 hours after hCG dosing. Decreased RT-PCR signals were noted after 24 hours, although not yet reaching control values for any cytokine except for TNFα. Treatment with hCG also induced a transient increase of bioactive IL-1 protein and the response mirrored that of the mRNA expression with a peak activity at 9 hours after hCG dosing and returns to basal level after 24 hours.

Leydig cell depleted rats were used to study if Leydig cells mediated the observed effects. Macroscopically, testes from rats treated with EDS with or without hCG appeared normal. Microscopically, as expected, Leydig cells were absent in testes of EDS treated rats. EDS and hCG treatment had the expected effects as verified by measuring testosterone in systemic and testicular venous blood. After EDS treatment testosterone levels were low or undetectable irrespective of injection of hCG. In contrast to in control animals, hCG did not induce IL-1β mRNA expression in the testes of EDS treated rats in vivo indicating that Leydig cells are required for the induction of IL-1β in the testis. Despite inducing maximal testosterone production, hCG failed to induce any IL-1β mRNA expression in isolated Leydig cells in vitro as assessed by RT-PCR verifying that Leydig cells do not produce IL-1β, at least not in our system.

IL-1β in testicular macrophages

Using 3-β HSD staining for Leydig cells and immunohistochemical double staining for IL-1β and macrophages (ED 2), it was evident that IL-1β was localized in testicular macrophages. Since hCG is known to stimulate immigration of leukocytes into the testis (110), we examined the number of lymphocytes and macrophages in the testis tissue before and eight hours after hCG treatment. The number of ED2 positive cells in testes of hCG treated animals did not differ from controls whereas the number of lymphocytes increased significantly after hCG treatment.

In summary, we have demonstrated that a single injection of hCG induced a rapid surge of expression of proinflammatory cytokines in the rat testis. The cytokine response was accompanied by an inflammatory reaction that could be seen macroscopically. The reaction was not detectable in EDS treated rats and thus required Leydig cells. Therefore, it is possible that inflammatory effects of hCG are mediated by increased IL-1 activity in the testis (85). This supports the hypothesis that cytokines play a role in the endocrine regulation of the testis and thus could be playing a role in the survival and proliferation of leukaemic cells in the testis.

GENERAL DISCUSSION

The testis protein extracts that has been used for establishing the immunosuppressive properties of testis extracts or of ISF are crude protein mixtures that are expected to contain both suppressive and stimulatory factors. In various studies testis protein extracts has been shown to contain many of the potent cytokines and growth factors found in the testis, such as, but not limited to, IL-1α, IL-1ra, TGFβ, TNFα and IGF-1 (V, 46, 138). These factors are pleiotropic and might have different activity on different cells and in different concentrations or in different combinations. In assays, differences might therefore be due to relative concentration changes or to the relative production of opposing regulatory factors. Different assay systems will obviously give different results due to the different effector mechanisms at work. This is evident in the present work where testis extracts are stimulatory in the IL-1 bioassay but suppressive in the CCL-64 mink lung epithelial cell line growth inhibition assay. In this context it is interesting to speculate that the sub-maximal IL-1 activity seen in the IL-1 bioassay at high concentration of testis protein extract, that is not seen with recombinant factors in the same concentrations, could be due to inhibitory action of TGFβ, present in the extract.

In order to classify a factor as a paracrine factor, it is necessary to demonstrate that it is locally produced in biologically active form, in amounts that lead to local concentrations that are compatible with biological activity, and blockage or removal of the factor by specific antibodies, antagonists or gene inactivation should influence the activity of the target cells (30). TGFβ fills these requirements for a testicular paracrine factor. There have now been several confirmations by immunohistochemistry of expression of TGFβ1, TGFβ 2 and TGFβ 3 in Sertoli cells, Leydig cells and germ cells. Expression varies with development stage and cell type and is in line with the view that the TGFβs have different actions in different cell types depending on other intra cellular factors (145). It is interesting that there is growing evidence of TGFβ as key autocrine and paracrine factors responsible for maintaining cell cycle arrest in quiescent haematopoietic progenitor cells as well as in some haematopoietic cell lines (146). On the molecular level, several independent mechanisms have been described. In epithelial cells, the major mechanism is a decreased cdk4 kinase activity leading to G1 arrest. This is also the effect in the mink lung epithelial cells used in the TGFβ bioassay used in this paper (V). In haematopoietic cells, including some murine B-cell lines, up regulation of p27Kip1 (a cyclin dependent kinase inhibitor) is the major mechanism leading to G1 arrest (147). There is now mounting evidence that TGFβ might a key factor in regulating Leydig cell proliferation and development (39, 45, 44) as well as steroidogenic activity (39, 119). During postnatal development in rats, TGFβ expression in Leydig cells coincides with low proliferative activity and is absent during the most active phase, to re-emerge in the adult rat (39, 44). It has been suggested that regulation of Leydig cell proliferation by LH is due to regulation of TGFβ expression (44). It is therefore possible that Leydig cell quiescence in boys during pre-puberty is maintained by expression and secretion of TGFβ in the testicular interstitium. At the start of the pre-pubertal development, levels of TGFβ expression would decline under influence of the rising LH stimulation of Leydig cells. It can be speculated that the

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presence of TGFβ in the interstitial tissue of the testis might be inducing quiescence in leukaemic lymphoblasts that circulate through the testis and thus increased resistance to cytotoxic drugs. This would also provide a possible explanation for why testicular relapses are rarely seen in adults.

Both rat and human leukaemic cell proliferation was dose dependently suppressed by rhTGFβ1 in our models. This indication that TGFβ1 is active in growth regulation of leukaemic blasts is in line with mounting evidence that TGFβ1 is a key cytokine for inhibiting cell cycle progression of many types of cells by arresting them in G1 phase (147). In addition, TGFβ1 has been shown to be a key factor in maintaining immature hematologic cells in a quiescent state (148, 149). In several murine B-cell line cells, TGFβ1 has been shown to induce cell cycle arrest through inhibition of the G1 cyclin-Cdk complexes (147). For most of the patients, their cells also showed dose dependant suppression when cultured in combination with rat testis extracts.

On interpretation of the varied result of the testis protein extracts it is worth remembering that these testis extracts are crude protein extracts that are expected to contain all the potent cytokines and growth factors found in the testis, such as, but not limited to, IL-1α, IL-1ra, TGFβ, TNFα and IGF-1 (V, 46, 138). This underscores the importance of specific bioassays or identification techniques when isolating factors with biologic activity.

It is noteworthy that the rat cells were non-responsive to stimulatory cytokines Il-1α, IL-1β and IL-6 and that human cells were non-responsive to IL-6 and several to IL-1α and β. This might be due to malignant haematopoietic cells having a high spontaneous proliferative activity that is not easily stimulated further during short-term culture in standard conditions. The cells might therefore be close to their maximal proliferation rate and that the growth factor and cytokine systems are fully activated by autocrine mechanisms.

In plasma, testosterone is very highly protein bound which significantly reduces the free bioactive fraction. In the testis however, testosterone levels are significantly higher and very high concentrations can probably be produced in the direct vicinity of the androgen producing Leydig cells. We therefore tested very high levels of testosterone on lymphocyte proliferation with no apparent effect. It is not clear what physiologic relevance should be attributed to the absence of positive results, but in summary, the results suggest that testicular TGFβ might have a role to play in regulation of intratesticular growth of leukaemic cells.

From these data, it can be concluded that Leydig cells can exert a suppressive effect on lymphocyte proliferation and could therefore contribute to the immunosuppressive milieu in the testis. The physiological importance of the testicular immunological sanctuary is not known, but an important role might be to suppress antigenic responses toward early spermatogonia carrying cell surface autoantigens residing outside the protection of the Sertoli cell barrier (65). Taken together with the ability of Leydig cells to bind lymphoid cells through non-immune cell recognition, it can be speculated that Leydig cells can cause malignant leukaemic cells to reside in the testis for longer or shorter periods in a state of reduced sensitivity to cytotoxic therapy. Lymphoblasts migrating into the interstitium might even interact with Leydig cells thereby inducing the production of soluble suppressive factor(s).

Inflammatory changes after pharmacological doses of hCG and involvement of Leydig cells have been demonstrated previously in rat testes (49, 150) and found to include an

elevated intratesticular pressure, vascular leakage and an increased apoptotic rate of spermatogonia (50, 51, 52). Inflammatory changes after hCG treatment are also seen in humans (50, 52). In rats, a similar acute inflammatory reaction as that seen in the testis after hCG treatment can be mimicked by intratesticular injections of proinflammatory stimulants including cytokines. Interestingly, IL-1β but not IL-1α induces a vascular response including extravasation of leukocytes into the testis interstitium after local injection (110). However, after proinflammatory stimulation by various agents including endotoxin, testicular macrophages are activated and start production of high concentrations of a multitude of inflammatory cytokines that may act as paracrine mediators of inflammation (62, 111). Therefore, it is possible that the inflammatory effects of hCG may be mediated by cytokines, e.g., IL-1β, released by the activated resident macrophages or by immigrating cells from the blood stream implying that the constitutive production of IL-1α does not contribute to the reaction. This is indeed supported by the report that IL-1α production in Sertoli cells is down regulated after endotoxin activation of testicular macrophages (62) and by the observation that IL-1α failed to induce any inflammatory response when injected intratesticularly (110). These results indicate that Leydig cells can exert some degree of regulatory control over Macrophage function.

SUMMARY AND CONCLUSIONS

There is a complicated paracrine network involved in the regulation of spermatogenesis and steroidogenesis in the testis. This system is involved in the maturational process of the reproductive system as well as regulating the balance of proliferation and steroid production to appropriate levels. The paracrine network involves several pleiotropic factors that have been shown to exert activating and suppressive effects on normal and malignant lymphatic cells and suggests that this system can be of importance for the tendency for leukaemic relapse in the testis.

The present work demonstrated that:
- Stage specific production of immunosuppressive factors by the rat seminiferous epithelium occurs. These factors are produced and released in a bioactive form. The characteristics of this activity indicate that it can be due to bioactive TGFβ.
- Immunosuppressive activity found in whole-testis protein extracts was shown to be identical with bioactive TGFβ. TGFβ was shown to inhibit proliferation of mouse thymocytes stimulated with testicular IL-1α, a constitutive cytokine that is produced in the testis without causing signs of inflammation.
- Leydig cells inhibit IL-1α driven lymphocyte proliferation in addition to inhibiting polyclonal mitogen stimulated lymphocyte proliferation in a dose dependent manner. Leydig cell induced inhibition was apparent from a Leydig cell – lymphocyte ratio of 100:1, indicating that the activity, at least in part, was mediated by a soluble factor. Leydig cells were also shown to be able to induce production and release of inflammatory cytokines from macrophages in response to hCG stimulation.
- Malignant leukaemic rat and human cell proliferation was shown to be responsive to inhibition by TGFβ and stimulation by other testicular cytokines such as IL-1.

Taken together the findings presented in this thesis indicate that factors involved in the intricate paracrine network that tightly regulates testicular function have the potential to be involved in the tendency for testicular relapses of ALL. These functions can be influenced through pituitary hormones. The identification of key factors involved in the regulation of the testicular paracrine network could open the possibility for future pharmacological intervention.

ACKNOWLEDGMENTS

REFERENCES

1 Gustafsson G, Kreuger A, Dohlwitz A. Acta Paediatr Scand 1981 Sep;70(5):609-14 Acute lymphoblastic leukemia in Swedish children 1973-1978.

2 Ritzén EM. Testicular relapse of acute lymphoblastic leukemia (ALL). J Reprod Immunol 1990 Aug;18(1):117-21.

3 Gustafsson G, Schmiegelow K, Forestier E, Clausen N, Glomstein A, Jonmundsson G, Mellander L, Makipernaa A, Nygaard R, Saarinen-Pihkala UM. Improving outcome through two decades in childhood ALL in the Nordic countries: the impact of high-dose methotrexate in the reduction of CNS irradiation. Nordic Society of Pediatric Haematology and Oncology (NOPHO). Leukemia 2000 Dec;14(12):2267-75.

4 Robinson DL Childhood leukemia: Understanding the significance of chromosomal abnormalities J Pediatr Oncol Nurs 2001 May-Jun;18(3):111-23.

5 Greaves M. Childhood leukaemia. BMJ 2002 Feb 2;324(7332):283-7.

6 Stiller CA, Eatock EM Patterns of care and survival for children with acute lymphoblastic leukaemia diagnosed between 1980 and 1994 Arch Dis Child 1999 Sep;81(3):202-8.

7 LeClerc JM, Billett AL, Gelber RD, Dalton V, Tarbell N, Lipton JM, Barr R, Clavell LA, Asselin B, Hurwitz C, Schorin M, Lipshultz SE, Declerck L, Silverman LB, Cohen HJ, Sallan SE Treatment of childhood acute lymphoblastic leukemia: results of Dana-Farber ALL Consortium Protocol 87-01 J Clin Oncol 2002 Jan 1;20(1):237-46.

8 Chessells JM. The management of high-risk lymphoblastic leukaemia in children Br J Haematol 2000 Feb;108(2):204-16.

9 Robison LL, Nesbit ME, Sather NS, Hammond GD, Coccia PF Assessment of the interrelationship of prognostic factors in childhood acute lymphoblastic leukemia: A report from Childrens Cancer Study Group Am J Pediatr Hematol Oncol 1980 Spring 2(1):5-13.

10 Pui CH, Boyett JM, Relling MV, Harrison PL, Rivera GK, Behm FG, Sandlund JT, Ribeiro RC, Rubnitz JE, Gajjar A, Evans WE Sex differences in prognosis for children with acute lymphoblastic leukemia J Clin Oncol 1999 Mar;17(3):818-24.

11 Buchanan GR, Rivera GK, Pollock BH, Boyett JM, Chauvenet AR, Wagner H, Maybee DA, Crist WM, Pinkel D. Alternating drug pairs with or without periodic reinduction in children with acute lymphoblastic leukemia in second bone marrow remission: a Pediatric Oncology Group Study Cancer 2000 Mar 1;88(5):1166-74.

12 Hustu HO, Aur RJ Extramedullary leukaemia. Clin Haematol 1978 Jun;7(2):313-37.

13 van den Berg H, Langeveld NE, Veenhof CH, Behrendt H. Treatment of isolated testicular recurrence of acute lymphoblastic leukemia without radiotherapy. Report from the Dutch Late Effects Study Group Cancer 1997 Jun;79(11):2257-62.

14 R G Grundy, A D Leiper, R Stanhope, J M Chessells Survival and endocrine outcome after testicular relapse in acute lymphoblastic leukaemia Archives of Disease in Childhood 1997;76:190–196.

15 Eden OB, Innes EM, Testicular infiltration in acute lymphoblastic leukaemia. Arch Dis Child. 1978 Oct;53(10):841.

16 Eden OB, Hardisty RM, Innes EM, Kay HEM, Peto J Testicular disease in acute lymphoblastic leukaemia in childhood. Report on behalf of the Medical Research Council's Working Party on leukaemia in childhood. Br Med J 1978 Feb 11;1(6109):334-8.

17 Brecher ML. Treatment of acute lymphoid leukemia in children: current regimens and future prospects. N Y State J Med. 1986 Apr;86(4):188-96.

18 Bowman WP, Aur RJ, Hustu HO, Rivera G. Isolated testicular relapse in acute lymphocytic leukemia of childhood: categories and influence on survival. J Clin Oncol. 1984 Aug;2(8):924-9.

19 Gaynon PS, Qu RP, Chappell RJ, Willoughby ML, Tubergen DG, Steinherz PG, Trigg ME. Cancer 1998 Apr 1;82(7):1387-95 Survival after relapse in childhood acute lymphoblastic leukemia: impact of site and time to first relapse--the Children's Cancer Group Experience.

20 Weinbauer GF, Gromoll J, Simoni M, Nieschlag E. Physiology of testicular function. In: Nieschlag E, Behre HM (eds.) Andrology: Male reproductive health and dysfunction, second edition. Springer Verlag, Heidelberg. 2001, 23-61.

21 Schlatt S, Meinhardt A, Nieschlag E. Paracrine regulation of cellular interactions in the testis: factors in search of a function. Eur J Endocrinol. 1997 Aug;137(2):107-17.

22 Jegou B, Stephan JP, Cudicini C, Gomez E, Bauche F, Piquet-Pellorce C, Touzalin AM. The Sertoli cell-germ cell interactions and the seminiferous tubule interleukin-1 and interleukin-6 system. Results Probl Cell Differ. 2000;28:53-68.

23 Hales DH. Testicular macrophage modulation of Leydig cell steroidogenesis J Reprod Immunol 2002 Oct-Nov; 57: 3-18.

24 Parvinen M, Vihko KK, Toppari J. Cell interactions during the seminiferous epithelial cycle. Int Rev Cytol 1986;104:115-51.

25 Weinbauer GF, Gromoll J, Simoni M, Nieschlag E: Physiology of Testicular Function in: Nieschlag E, Behre HM (eds.) Andrology: Male reproductive health and dysfunction. Springer, Berlin Heidelberg New York, 2001.

26 Setchell BP, Uksila J, Maddocks S, Pollanen P. Testis physiology relevant to immunoregulation. J Reprod Immunol 1990 Aug;18(1):19-32.

27 Sharpe R. Experimental evidence for Sertoli-germ cell and Sertoli-Leydig cell interactions. In: Russel LD, Griswold MD (Eds.). The Sertoli cell, Cache River Press; 1993: 391-418.

28 Niemi M, Sharpe RM, Brown WRA. Macrophages in the interstitial tissue of the rat testis. Cell Tissue Research 1986 243, 337-344.

29 Fawcett DW, Neaves WB, Flores MN. Comparative observations on intertubular lymphatics and the organization of the interstitial tissue of the mammalian testis. Biol Reprod 1973 9, 500-532.

30 Saez JM. Leydig cells: endocrine, paracrine, and autocrine regulation Endocr Rev 1994 Oct;15(5):574-626.

31 Miller SC, Bowman BM, Rowland HG. Structure, cytochemistry, endocytic activity, and immunoglobulin (Fc) receptors of rat testicular interstitial-tissue macrophages. Am J Anat 1983 168, 1-13.

32 Pollanen P, Maddocks S.J Macrophages, lymphocytes and MHC II antigen in the ram and the rat testis. Reprod Fertil 1988 Mar;82(2):437-45.

33 Born W, Wekerle H. Selective, immunologically nonspecific adherence of lymphoid and myeloid cells to Leydig cells. Eur J Cell Biol 1981 Aug;25(1):76-81.

34 Rivenzon A, Rivenzon M, Madden RE. Spontaneous adherence and rosette formation of lymphocytes to Leydig cells: an in vitro technique. Cell Immunol 1974 14, 411-416.

35 Rivenson A, Ohmori T, Hamazaki M, Madden R. Cell surface recognition: spontaneous identification of mouse Leydig cells by lymphocytes, macrophages and eosinophils. Cell Mol Biol. 1981;27(1):49-56.

36 Sharpe RM, Regulation of spermatogenesis. In: Knobil E, Neil S (eds) The Physiology of Reproduction, second edition.. Raven Press, New York, 1994.

37 Kerr JB, Bartlett JM, Donachie K. Acute response of testicular interstitial tissue in rats to the cytotoxic drug ethane dimethanesulphonate. An ultrastructural and hormonal assay study. Cell Tissue Res 1986;243(2):405-14.

38 Ritzén M. Somatisk pubertetsutveckling. In: Fredricsson B, Pousette Å. Andrologi. Almqvist & Wiksell Medicin, Falköping, 1994.

39 Lejeune H, Habert R, Saez JM. Origin, proliferation and differentiation of Leydig cells. J Mol Endocrinol 1998 Feb;20(1):1-25

40 Rouiller-Fabre V, Lecref L, Gautier C, Saez JM, Habert R. Expression and effect of insulin-like growth factor I on rat fetal Leydig cell function and differentiation. Endocrinology 1998 Jun;139(6):2926-34.

41 Ariyaratne HB, Chamindrani Mendis-Handagama S. Changes in the testis interstitium of Sprague Dawley rats from birth to sexual maturity. Biol Reprod. 2000 Mar;62(3):680-90.

42 Prince FP. The triphasic nature of Leydig cell development in humans, and comments on nomenclature. J Endocrinol 2001 Feb;168(2):213-6.

43 Hagenäs L. Normal and delayed puberty--when is androgen therapy justified? Läkartidningen. 1990 Dec 12;87(50):4340-4.

44 Khan SA, Mirsafian M, Howdeshell K, Dorrington JH. Transforming growth factor-beta inhibits DNA synthesis in immature rat Leydig cells in vitro. Mol Cell Endocrinol 1999 Feb 25;148(1-2):21-8.

45 Mendis-Handagama SM, Ariyaratne HB. Differentiation of the adult Leydig cell population in the postnatal testis. Biol Reprod. 2001 Sep;65(3):660-71.

46 Ge RS, Hardy MP. Decreased cyclin A2 and increased cyclin G1 levels coincide with loss of proliferative capacity in rat Leydig cells during pubertal development. Endocrinology 1997 Sep;138(9):3719-26.

42

47 Habert R, Lejeune H, Saez JM. Origin, differentiation and regulation of fetal and adult Leydig cells. Mol Cell Endocrinol 2001 Jun 20;179(1-2):47-74.

48 Ge RS, Hardy DO, Catterall JF, Hardy MP. Opposing changes in 3alpha-hydroxysteroid dehydrogenase oxidative and reductive activities in rat Leydig cells during pubertal development. Biol Reprod. 1999 Apr;60(4):855-60.

49 Setchell BP, Sharpe RM Effect of injected human chorionic gonadotrophin on capillary permeability, extracellular fluid volume and the flow of lymph and blood in the testes of rats. J Endocrinol 1981, 91:245-254.

50 Hjertkvist M, Läckgren G, Plöen L, Bergh A 1993 Does hCG treatment induce inflammation like changes in undescended testes in boys. J Pediatr 103:892-897.

51 Heiskanen P, Billig H, Toppari J, Kaleva M, Arsalo A, Rapola J, Dunkel L 1996 Apoptotic cell death in the normal and cryptorchid human testis: the effect of human chorionic gonadotropin on testicular cell survival. Pediatr Res 40:351-356.

52 Demirbilek S, Atayurt HF, Celik N, Aydin G 1997 Does treatment with human chorionic gonadotropin induce reversible changes in undescended testes in boys? Pediatr Surg Int 12:591-594.

53 Pollanen P, Niemi M. Immunohistochemical identification of macrophages, lymphoid cells and HLA antigens in the human testis. Int J Androl 1987 Feb;10(1):37-42.

54 Gerdprasert O, O'Bryan MK, Muir JA, Caldwell AM, Schlatt S, De Kretser DM, Hedger MP. The response of testicular leukocytes to lipopolysaccharide-induced inflammation: further evidence for heterogeneity of the testicular macrophage population. Cell Tissue Res. 2002 May;308(2):277-85.

55 Meinhardt A, Bacher M, Metz C, Bucala R, Wreford N, Lan H, Atkins R, Hedger M. Local regulation of macrophage subsets in the adult rat testis: examination of the roles of the seminiferous tubules, testosterone, and macrophage-migration inhibitory factor. Biol Reprod 1998 Aug;59(2):371-8.

56 Hedger MP. Macrophages and the immune responsiveness of the testis. J Reprod Immunol 2002 Oct-Nov; 57: 19-34.

57 Bergh A. Effect of cryptorchidism on the morphology of testicular macrophages: evidence for a Leydig cell-macrophage interaction in the rat testis. Int J Androl 1985 Feb;8(1):86-96.

58 Bergh A. Treatment with hCG increases the size of Leydig cells and testicular macrophages in unilaterally cryptorchid rats. Int J Androl 1987 Dec;10(6):765-72.

59 Hutson JC. Secretion of tumor necrosis factor alpha by testicular macrophages. J Reprod Immunol 1993 Jan;23(1):63-72.

60 Afane M, Dubost JJ, Sauvezie B, Issoual D, Dosgilbert A, Grizard G, Boucher D. Modulation of Leydig cell testosterone production by secretory products of macrophages. Andrologia 1998 Mar-Apr;30(2):71-8.

61 Wahab-Wahlgren A, Holst M, Ayele D, Sultana T, Parvinen M, Gustafsson K, Granholm T, Soder O. Constitutive production of interleukin-1alpha mRNA and protein in the developing rat testis Int J Androl 2000 Dec;23(6):360-5.

62 Jonsson CK, Setchell BP, Martinelle N, Svechnikov K, Soder O. Endotoxin-induced interleukin 1 expression in testicular macrophages is accompanied by downregulation of the constitutive expression in Sertoli cells Cytokine 2001 Jun 7;14(5):283-8.

63 Kern S, Robertson SA, Mau VJ, Maddocks S. Cytokine secretion by macrophages in the rat testis. Biol Reprod 1995 Dec;53(6):1407-16.

64 Wang J, Wreford NG, Lan HY, Atkins R, Hedger MP. Leukocyte populations of the adult rat testis following removal of the Leydig cells by treatment with ethane dimethane sulfonate and subcutaneous testosterone implants. Biol Reprod 1994 Sep;51(3):551-61.

65 Yule TD, Montoya GD, Russel LD, Williams TM, Tung KSK. Autoantigenic germ cells exist outside the blood testis barrier. J. Immunol. 1988 141 1161-1167.

66 Alexander NJ, Anderson DJ. Immunology of semen. Fertil Steril 1987 Feb;47(2):192-205

67 Head JR, Neaves WB, Billingham RE. Immune privilege in the testis I. Basic parameters of allograft survival. Transplantation 1983 36, 423-431.

68 Head JR, Billingham RE. Immunologically privileged sites in transplantation immunology and oncology. Persp. Biol. Med. 1985 29, 115-131.

69 Head JR, Neaves WB, Billingham RE. Immune privilege in the testis II. Evaluation of potential local factors. Transplantation 1985 40, 269-275.

70 Whitmore WF, Gittes RF. Intratesticular grafts: the testis as an exceptional immunologically privileged site. Trans. Am. Ass. Gen. Urin. Surg. 1979 70, 76-80.

71 Hedger MP, Eddy EM. The heterogeneity of isolated adult rat Leydig cells separated on Percoll density gradients: an immunological, cytochemical, and functional analysis. Endocrinology 1987 Nov;121(5):1824-38.

72 Pollanen P, von Euler M, Soder O. Testicular immunoregulatory factors. J Reprod Immunol 1990 Aug;18(1):51-76.

73 Hedger MP. The testis: an 'immunologically suppressed' tissue? Reprod Fertil Dev 1989;1(1):75-9.

74 Born W, Wekerle H. Leydig cells nonspecifically suppress lymphoproliferation in vitro: implications for the testis as an immunologically privileged site. Am J Reprod Immunol 1982 Dec;2(6):291-5.

75 Selawry HP, Whittington KB. Prolonged intratesticular islet allograft survival is not dependent on local steroidogenesis. Horm Metab Res 1988 20, 562-565.

76 Cameron DF, Whittington K, Schultz RE, Selawry HP. Successful islet/abdominal testis transplantation does not require Leydig cells. Transplantation 1990 Oct;50(4):649-53.

77 Filippini A, Riccioli A, Padula F, Lauretti P, D'Alessio A, De Cesaris P, Gandini L, Lenzi A, Ziparo E. Control and impairment of immune privilege in the testis and in semen. Hum Reprod Update. 2001 Sep-Oct;7(5):444-9.

78 Eddy EM. Regulation of gene expression during spermatogenesis. Semin Cell Dev Biol. 1998 Aug;9(4):451-7.

79 Hales DB, Diemer T, Hales KH. Role of cytokines in testicular function. Endocrine 1999 Jun;10(3):201-17.

80 Hales DB. Interleukin-1 inhibits Leydig cell steroidogenesis primarily by decreasing 17 alpha-hydroxylase/C17-20 lyase cytochrome P450 expression. Endocrinology 1992 Nov; 131(5):2165-72.

81 Kern S, Robertson SA, Maddocks S. Cellular and molecular regulation of testicular cells. (Abstract). XIIIth North American Testis Workshop 1995; I-36, 46.

82 Cudicini C, Lejeune H, Gomez E, Bosmans E, Ballet F, Saez J, Jegou B. Human Leydig cells and Sertoli cells are producers of interleukins-1 and -6. J Clin Endocrinol Metab 1997 May;82(5):1426-33.

83 Pentikainen V, Erkkila K, Suomalainen L, Otala M, Pentikainen MO, Parvinen M, Dunkel L. TNFalpha down-regulates the Fas ligand and inhibits germ cell apoptosis in the human testis. J Clin Endocrinol Metab 2001 Sep;86(9):4480-8.

84 Boockfor FR, Wang D, Lin T, Nagpal ML, Spangelo BL. Interleukin-6 secretion from rat Leydig cells in culture. Endocrinology 1994 May;134(5):2150-5.

85 Lin T, Wang D, Nagpal ML. Human chorionic gonadotropin induces interleukin-1 gene expression in rat Leydig cells in vivo. Mol Cell Endocrinol 1993 Sep;95(1-2):139-45.

86 Jonsson CK, Zetterstrom RH, Holst M, Parvinen M, Soder O. Constitutive expression of interleukin-1alpha messenger ribonucleic acid in rat Sertoli cells is dependent upon interaction with germ cells Endocrinology 1999 Aug;140(8):3755-61.

87 Petersen C, Boitani C, Froysa B, Soder O Interleukin-1 is a potent growth factor for immature rat Sertoli cells Mol Cell Endocrinol 2002 Jan 15;186(1):37-47.

88 Svechnikov KV, Sultana T, Soder O. Age-dependent stimulation of Leydig cell steroidogenesis by interleukin-1 isoforms Mol Cell Endocrinol 2001 Sep;182(2):193-201.

89 Jonsson C, Setchell BP, Sultana T, Holst M, Parvinen M, Soder O. Constitutive and inducible production of proinflammatory cytokines by the rat testis. Andrologia. 2000 Jan;32(1):63-4.

90 Okano F, Satoh M, Ido T, Yamada K. Cloning of cDNA for canine interleukin-18 and canine interleukin-1beta converting enzyme and expression of canine interleukin-18. J Interferon Cytokine Res 1999 Jan;19(1):27-32.

91 Parvinen M, Hakovirta H, Kaipia A, Penttilä T-L, Toppari J, Syed V, Jegou B, Persson H, Wahab A, Söder O. Local growth factor effects during spermatogenesis. In Bartke A. (ed) Function of somatic cells in the testis. Springer-Verlag, New York, 1994.

92 Strand ML Svechnikov KV Söder O Interleukin-18 is constitutively expressed in rat germ cells and stimulates Leydig cell steroidogenesis in vitro. Abstract. 2002 April; Eur. Testis Workshop, Doorwert, Netherlands.

93 Hutson JC. Secretion of tumor necrosis factor alpha by testicular macrophages. J Reprod Immunol 1993 Jan;23(1):63-72.

94 Schlatt S, Meinhardt A, Nieschlag E. Paracrine regulation of cellular interactions in the testis: factors in search of a function Eur J Endocrinol 1997 Aug;137(2):107-17.

95 Pollanen P, Soder O, Parvinen M. Interleukin-1 alpha stimulation of spermatogonial proliferation in vivo. Reprod Fertil Dev 1989;1(1):85-7.

96 Soder O, Sultana T, Jonsson C, Wahlgren A, Petersen C, Holst M. The interleukin-1 system in the testis Andrologia 2000 Jan;32(1):52-5.

97 Dinarello CA. Interleukin-1. Cytokine Growth Factor Rev 1997 Dec;8(4):253-65.

98 Dinarello CA. The interleukin-1 family: 10 years of discovery. FASEB J 1994 Dec;8(15):1314-25.

99 Tovey MG, Content J, Gresser I, Gugenheim J, Blanchard B, Guymarho J, Poupart P, Gigou M, Shaw A, Fiers W. Genes for IFN-beta-2 (IL-6), tumor necrosis factor, and IL-1 are expressed at high levels in the organs of normal individuals. J Immunol 1988 Nov 1;141(9):3106-10.

100 Khan SA, Soder O, Syed V, Gustafsson K, Lindh M, Ritzén EM. The rat testis produces large amounts of an interleukin-1-like factor. Int J Androl 1987 Apr;10(2):495-503.

101 Granholm T, Söder O. Constitutive production of lymphocyte activating factors by normal tissues in the adult rat. J Cell Biochem 1991 46: 143-151.

102 Dinarello CA. Biology of interleukin 1. FASEB J 1988 Feb;2(2):108-15.

103 Gerard N, Syed V, Bardin W, Genetet N, Jegou B. Sertoli cells are the site of interleukin-1 alpha synthesis in rat testis. Mol Cell Endocrinol 1991 Nov; 82(1):R13-6.

104 Gustafsson K, Söder O, Pöllänen P, Ritzén EM 1988 Isolation and partial characterization of an interleukin-1-like factor from rat testis interstitial fluid. J Reprod Immunol 14:139-150.

105 Syed V., Söder O., Arver S., Lindh M., Khan S. and Ritzén E.M. (1988) Ontogeny and cellular origin of an interleukin-1-like factor in the reproductive tract of the male rat. Int J Androl 11: 437-447.

106 Syed V, Khan SA, Lindh M, Ritzén EM. Ontogeny and cellular origin of a rat seminiferous tubule factor(s) that inhibits LH-dependent testosterone production by interstitial cells in vitro. Int J Androl 1987 Oct;10(5):711-20.

107 Soder O, Syed V, Callard GV, Toppari J, Pollanen P, Parvinen M, Froysa B, Ritzén EM. Production and secretion of an interleukin-1-like factor is stage-dependent and correlates with spermatogonial DNA synthesis in the rat seminiferous epithelium. Int J Androl 1991 Jun;14(3):223-31.

108 Hakovirta H, Penttila TL, Pollanen P, Froysa B, Soder O, Parvinen M. Interleukin-1 bioactivity and DNA synthesis in X-irradiated rat testes Int J Androl 1993 Apr;16(2):159-64.

109 Parvinen M, Soder O, Mali P, Froysa B, Ritzén EM. In vitro stimulation of stage-specific deoxyribonucleic acid synthesis in rat seminiferous tubule segments by interleukin-1 alpha. Endocrinology 1991 Sep;129(3):1614-20.

110 Bergh A, Soder O. Interleukin-1 beta but not interleukin-1 alpha, induces acute inflammation-like changes in the testicular microcirculation of adult rats. J Reprod Immunol. 1990 Apr;17(2):155-65.

111 Jonsson C, Setchell BP, Sultana T, Holst M, Parvinen M, Soder O. Constitutive and inducible production of proinflammatory cytokines by the rat testis. Andrologia. 2000 Jan;32(1):63-4.

112 Massague J, Blain SW, Lo RS TGFbeta signaling in growth control, cancer, and heritable disorders Cell 2000 Oct 13;103(2):295-309.

113 Olaso R, Pairault C, Saez JM, Habert R Transforming growth factor beta3 in the fetal and neonatal rat testis: immunolocalization and effect on fetal Leydig cell function Histochem Cell Biol 1999 Sep;112(3):247-54.

114 Dunker N, Krieglstein K Targeted mutations of transforming growth factor-beta genes reveal important roles in mouse development and adult homeostasis Eur J Biochem 2000 Dec;267(24):6982-8.

115 Itoh S, Itoh F, Goumans MJ, Ten Dijke P. Signalling of transforming growth factor-beta family members through Smad proteins Eur J Biochem 2000 Dec;267(24):6954-67.

116 Massague J Annu Rev Biochem TGF-beta signal transduction 1998;67:753-91

117 Teerds KJ, Dorrington JH Localization of transforming growth factor beta 1 and beta 2 during testicular development in the rat Biol Reprod 1993 Jan;48(1):40-5.

118 Wang RA, Zhao GQ Transforming growth factor beta signal transducer Smad2 is expressed in mouse meiotic germ cells, Sertoli cells, and Leydig cells during spermatogenesis Biol Reprod 1999 Oct;61(4):999-1004.

119 Le Roy C, Leduque P, Yuan Li J, Saez JM, Langlois D Antisense oligonucleotide targeting the transforming growth factor beta1 increases expression of specific genes and functions of Leydig cells Eur J Biochem 1998 Oct 15;257(2):506-14.

120 Kamesaki H, Nishizawa K, Michaud GY, Cossman J, Kiyono T TGF-beta 1 induces the cyclin-dependent kinase inhibitor p27Kip1 mRNA and protein in murine B cells.J Immunol 1998 Jan 15;160(2):770-7.

121 Molenaar R, de Rooij DG, Rommerts FF, Reuvers PJ, van der Molen HJ Specific destruction of Leydig cells in mature rats after in vivo administration of ethane dimethyl sulfonate. Biol Reprod 1985 Dec;33(5):1213-22.

122 Kerr, J.B., Knell, C.M., Abbott, M. and Donachie, K. Ultrastructural analysis of the effect of ethane dimethanesulphonate on the testis of the rat, guinea pig, hamster and mouse. Cell Tissue Res 1987 249, 451-457.

123 Jahnukainen K, Saari T, Morris ID, Salmi TT, Pollanen P. Regulation of testicular infiltration in acute lymphoblastic leukaemia of the rat. Leukemia 1994 Mar;8(3):458-64.

124 Cooke BA, Lindh LM, Janszen FH, van Driel MJ, Bakker CP, van der Plank MP, van der Molen HJ. Biochim Biophys Acta 1979 Mar 22;583(3):320-31 A Leydig cell tumour: a model for the study of lutropin action.

125 Erichsen A, Hansson V. A transplantable rat Leydig cell tumour. Properties of the prostaglandin E receptor. Acta Endocrinol (Copenh) 1989 Apr;120(4):499-504.

126 Erichsen A, Clausen OP, Torjesen P, Dahl E, Wetteland P, Hansson V. A transplantable rat Leydig cell tumour. 6. Effect of castration and hypophysectomy on tumour growth, morphology and plasma levels of pituitary hormones and testosterone. Acta Endocrinol (Copenh) 1988 Oct;119(2):291-300.

127 Erichsen A, Levy FO, Golf S, Hansson V. A transplantable rat Leydig cell tumor--5. Cellular localization of beta-adrenergic and prostaglandin receptors and their effects on testosterone production. J Steroid Biochem 1988 Jul;31(1):41-8.

128 Erichsen AA, Jahnsen T, Attramadal H, Hansson V. A transplantable rat Leydig cell tumor--3. Isoproterenol and prostaglandin E1 (PGE1) responsive adenylyl cyclase (AC). Regulatory effects of nucleotides and magnesium. J Steroid Biochem 1986 Mar;24(3):761-8.

129 Erichsen A, Jahnsen T, Attramadal H, Hansson V. A transplantable rat Leydig cell tumor--2. Adenylyl cyclase activation by prostaglandin E1, isoproterenol and glucagon. J Steroid Biochem 1984 Nov;21(5):545-8.

130 Erichsen A, Jahnsen T, Andersen D, Torjesen P, Hansson V. A transplantable rat Leydig cell tumor--1. LH and prolactin receptors and effects of the endocrine status of the host animal. J Steroid Biochem 1984 Nov;21(5):539-43.

131 Erichsen A, Jahnsen T, Attramadal H, Andersen D, Hansson V. A transplantable rat Leydig cell tumor: binding properties, hormonal responsiveness, and tumor growth. Ann N Y Acad Sci 1984;438:655-8.

132 Granholm T, Froysa B, Lundstrom C, Wahab A, Midtvedt T, Soder O.Cytokine responsiveness in germfree and conventional NMRI mice. Cytokine 1992 Nov;4(6):545-50.

133 Danielpour D, Dart LL, Flanders KC, Roberts AB, Sporn MB. Immunodetection and quantitation of the two forms of transforming growth factor-beta (TGF-beta 1 and TGF-beta 2) secreted by cells in culture. J Cell Physiol 1989 Jan;138(1):79-86.

134 Winqvist O, Gebre-Medhin G, Gustafsson J, Ritzén EM, Lundkvist O, Karlsson FA, Kampe O. Identification of the main gonadal autoantigens in patients with adrenal insufficiency and associated ovarian failure. J Clin Endocrinol Metab. 1995 80:1717-23.

135 Bradford MM.A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976 May 7;72:248-54.

136 Parvinen M, Ruokonen A 1982 Endogenous steroids in rat seminiferous tubules. Comparison of the stages of the epithelial cycle isolated by transillumination-assisted microdissection. J Androl 3:211–220.

137 Skinner MK. Cell-cell interactions in the testis. Endocr Rev 1991 Feb;12(1):45-77.

138 Soder O, Bang P, Wahab A, Parvinen M. Insulin-like growth factors selectively stimulate spermatogonial, but not meiotic, deoxyribonucleic acid synthesis during rat spermatogenesis. Endocrinology 1992 Nov;131(5):2344-50.

139 Kehrl JH, Wakefield LM, Roberts AB, Jakowlew S, Alvarez-Mon M, Derynck R, Sporn MB, Fauci AS. Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. J Exp Med 1986 May 1;163(5):1037-50.

140 Pollanen P, Soder O, Uksila J. Testicular immunosuppressive protein. J Reprod Immunol 1988 Nov;14(2):125-38.

141 Pollanen P, Soder O, Uksila J, Nikula H, Kaipia A, Kangasniemi M, Punnonen J, Huhtaniemi I, Parvinen M. Testicular immunosuppressive peptide. Transplant Proc 1989 Feb;21(1 Pt 1):1144-6.

142 Palladino MA, Morris RE, Starnes HF, Levinson AD. The transforming growth factor-betas. A new family of immunoregulatory molecules. Ann N Y Acad Sci 1990;593:181-7.

143 Kerr JB, Rich KA, de Kretser DM. Effects of experimental cryptorchidism on the ultrastructure and function of the Sertoli cell and peritubular tissue of the rat testis. Biol Reprod. 1979 Nov;21(4):823-38.

144 Hedger MP, Meinhardt A. Local regulation of T cell numbers and lymphocyte-inhibiting activity in the interstitial tissue of the adult rat testis. J Reprod Immunol 2000 Oct-Nov;48(2):69-80.

145 Gautier C, Levacher C, Saez JM, Habert R. Expression and regulation of transforming growth factor beta1 mRNA and protein in rat fetal testis in vitro. Biochem Biophys Res Commun 1997 Jul 9;236(1):135-9.

146 Sainio-Pollanen S, Pollanen P, Setchell BP. Testicular immunosuppressive activity in experimental hypogonadism and cryptorchidism. J Reprod Immunol 1991 May;20(1):59-72.

147 Kamesaki H, Nishizawa K, Michaud GY, Cossman J, Kiyono T TGF-beta 1 induces the cyclin-dependent kinase inhibitor p27Kip1 mRNA and protein in murine B cells. J Immunol 1998 Jan 15;160(2):770-7.

148 Cardoso AA, Li ML, Batard P, Hatzfeld A, Brown EL, Levesque JP, Sookdeo H, Panterne B, Sansilvestri P, Clark SC, et al. Release from quiescence of CD34+ CD38- human umbilical cord blood cells reveals their potentiality to engraft adults. Proc Natl Acad Sci U S A 1993 Sep 15;90(18):8707-11.

149 Dao MA, Hwa J, Nolta JA Molecular mechanism of transforming growth factor beta-mediated cell-cycle modulation in primary human CD34(+) progenitors. Blood 2002 Jan 15;99(2):499-506.

150 Setchell BP, Rommerts FF The importance of the Leydig cells in the vascular response to hCG in the rat testis. Int J Androl 1985, 8:436-440.