Hormonal regulation of apoptosis in the ovary under normal physiological and pathological conditions

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Dissertation Utrecht University, Faculty of Veterinary Medicine

ISBN: 90-393-3929-5

Cover design: Labor Grafimedia BV & Karin Slot
Thesis Layout: Lisanne van der Voort
Printed by: Labor Grafimedia BV, Utrecht

Hormonal regulation of apoptosis in the ovary under normal physiological and pathological conditions

Hormonale regulatie van apoptose in het ovarium onder normale fysiologische en pathologische condities

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen,
ingevolge het besluit van het College voor Promoties in het
openbaar the verdedigen op
donderdag 20 januari des namiddigs te 2.30 uur

door

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geboren op 21 juni 1973 te Borne

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Publication of this thesis was financially supported by the Graduate School of Animal Health, the Department of Biochemistry & Cell Biology (Faculty of Veterinary Medicine, Utrecht University)

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Chapter 1

Introduction to hormonal regulation of apoptosis in the ovary under normal physiological and pathological conditions

1.1 PREFACE

Programmed cell death or apoptosis is an essential component of normal ovarian physiology. Any imbalance between apoptosis and proliferation could lead to pathological conditions. Enhanced apoptosis may attribute to early exhaustion of the oocyte stockpile, whereas diminished apoptosis may promote excessive tissue growth. Hence, a better understanding of programmed cell death in the ovary may help unraveling the endocrine, molecular and cellular bases of ovarian disorders, such as premature ovarian failure, polycystic ovarian syndrome and ovarian cancer. This chapter highlights some recent advances in the hormonal and intra-ovarian mechanisms that control survival and apoptosis of ovarian follicles, corpora lutea and ovarian surface epithelium (paragraph 1.4). Furthermore, this chapter is meant to provide a general overview of fundamental processes associated with reproduction in the rodent ovary (paragraph 1.2.1 –1.2.3) and cancer in the human ovary (paragraph 1.2.4) and of basic apoptotic signaling pathways (paragraph 1.3).

1.2 OVARY

1.2.1 Structure

The ovaries are the primary female reproductive organs. Each ovary has a flattened ovoid shape that is covered on the outside by a single layer of flat-to-cuboidal epithelium, also called germinal epithelium, which is a continuation of the peritoneum (fig. 1). Directly underneath the epithelium there is a fibrous connective tissue layer, known as the tunica albuginea. Like many other organs the ovary is divided into an outer cortex and an inner medulla. The ovarian cortex contains connective tissue, the stroma, in which the ovarian follicles at various stages of development are embedded. The medulla is composed of a loose connective tissue with abundant blood vessels, lymphatic vessels and nerve fibers that enter the ovary at the hilus. In mammals, the ovary is the female gonad responsible for the development and release of mature oocytes ready to be fertilized. Moreover, the ovary is an endocrine organ that produces steroids to allow the development of female secondary sexual characteristics and to support pregnancy.

1.2.2 Follicular development

Morphology

During early fetal development, primordial germ cells migrate into the ovarian cortex where they divide rapidly and differentiate into oogonia. These oogonia become primary

oocytes by starting the first meiotic division, followed by an arrest in the prophase. The primary oocyte has a large nucleus with granular chromatin, a prominent nucleolus and little cytoplasm. When a single layer of flattened follicular cells forms around the naked primary oocyte, the structure is called a *primordial follicle*. The primordial follicle pool forms the resting stockpile of follicles in the ovary. Throughout reproductive life, dormant primordial follicles are continuously recruited from the resting pool to start growing. Figure 1 illustrates several stages of follicular development in the ovary. Following recruitment of primordial follicles, alterations occur in the primary oocyte and the surrounding follicular cells leading to the formation of a *primary follicle*. When recruited, the primary oocyte enlarges and the follicular cells transform from flattened into cuboidal cells, the granulosa cells. A zona pellucida, a thick layer of glycoproteins and acidic proteoglycans presumably secreted by the oocyte, is formed between the oocyte and the granulosa cells. Gap junctions connect the oocyte and surrounding inner layer of granulosa cells and facilitate the transport of amino acids, nucleotides, and lipid precursors from the granulosa cells to the oocyte.

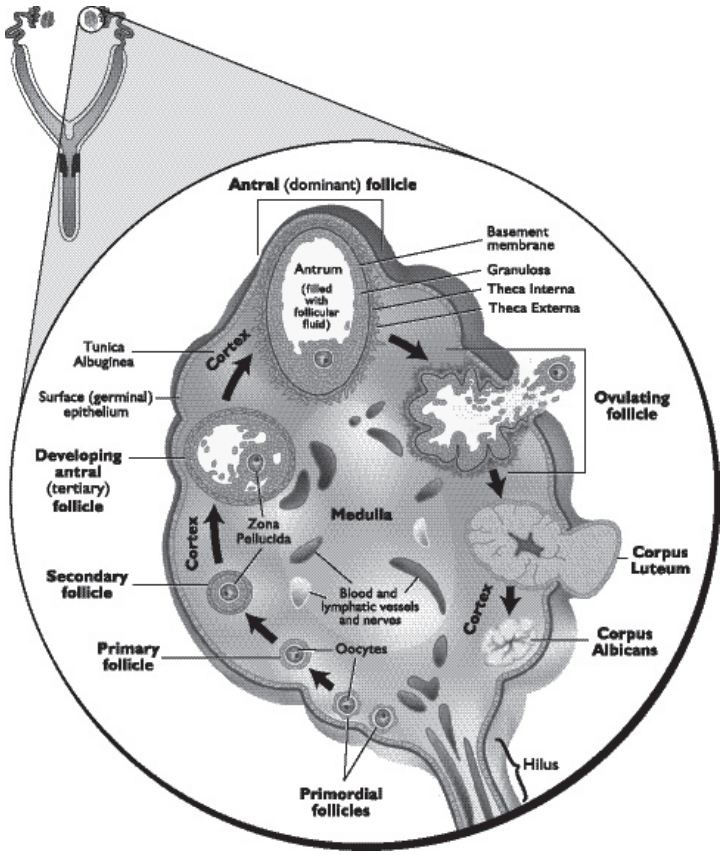

Figure 1. Diagram of ovary showing the various stages of follicles through development, ovulation, corpus luteum formation and regression (1).

Ongoing proliferation of granulosa cells results first in the formation of a double and finally multiple granulosa cell layers surrounding the oocyte. The follicles at this stage are now termed *secondary follicles*. The zona pellucida thickens and the stroma cells surrounding the *secondary follicle* develop into two distinct layers, the theca interna and theca externa. The theca interna is highly vascular and consists of cells with features characteristic of steroid-producing cells, whereas the theca externa, which is continuous with the ovarian stroma, is composed of flattened stroma or fibroblast-like cells. The granulosa cells are separated from the theca interna cells and the ovarian blood supply by a distinct basement membrane. In order to reach the granulosa cells and oocyte, nutrients from the circulation have to pass this barrier by diffusion.

An *antral follicle* is formed as soon as extracellular spaces appear between the granulosa cells that contain fluid secreted by the granulosa cells. These separate spaces enlarge and fuse into a single fluid-filled chamber, the antrum. The *preovulatory follicle*, also called mature or Graafian follicle, represents the final stage of follicular development before ovulation. Such follicles are relatively large and usually extend from the deepest parts of the cortex and expand to the surface of the ovary (fig.1). This type of follicle is characterized by a large antrum. The oocyte is surrounded by specialized granulosa cells, the cumulus cells, and occupies an eccentric position, forming a hillock. The cumulus cells in close contact with the oocyte are known as corona radiata cells, which communicate with the oocyte and with other cumulus cells through gap junctions. The cumulus cells nourish the oocyte and participate in the modulation of oocyte maturation inhibitors. The granulosa cells closest to the basement membrane are known as mural granulosa cells, while those closest to the follicular antrum are known as antral granulosa cells. These subpopulations of granulosa cells differ in their distribution of receptors and steroidogenic characteristics (2). The theca interna is the major source of androgens during follicular development.

Dynamics of follicular growth

Although most of the dormant follicles are maintained in a resting state, primordial follicles are continuously recruited, the so-called initial recruitment, to grow throughout life (fig. 2). Whether replenishment of the primordial follicle pool occurs after birth through adulthood from a oogonial stem cell population is at present a matter of debate (3) Eventually, depletion of the pool of resting follicles leads to ovarian follicle exhaustion and senescence. In rats, primordial follicles are formed already by day 3 after birth, secondary follicles are found by day 7 of age, and follicles of the early antral stage are observed in the third week of life (reviewed by 4). Around day 35 of age, cyclic ovarian function begins, which is the result of the maturation of the gonadotropin releasing hormone (GnRH) pulse generator leading to cyclic changes in circulating follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels during each estrous cycle.

Follicular development in rodents is similar to that in humans; however its duration is much shorter (reviewed by 4). The rodent follicle grows approximately 25-fold in diameter from the primordial (25 µm) to the preovulatory (500–800 µm) stage over a period of approximately 60 days (or about 15 estrous cycles). The time required between the initial recruitment of a primordial follicle and its growth to the secondary stage is approximately 30 days, and the time needed for a secondary follicle to develop further to the early antral stage is about 28 days. Once reaching the early antral stage (200–400 µm in diameter), the follicles are subjected to cyclic recruitment, after which only 2–3 days are needed to grow into preovulatory follicles (fig. 2). During cyclic recruitment, only a limited number of these follicles survive and ovulate, whereas the vast majority of recruited follicles are eliminated from the ovary by programmed cell death. The factors involved in recruitment and elimination are discussed later on in this chapter.

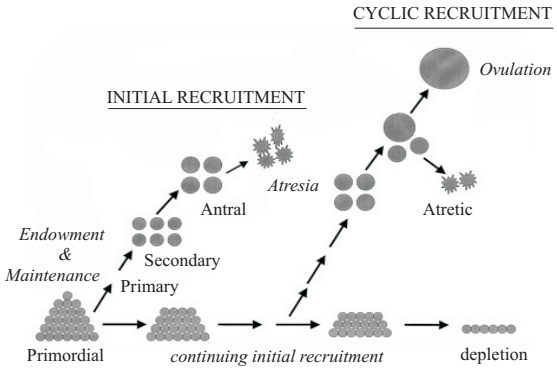

Figure 2. Life history of follicles. Primordial follicles are continuously recruited to grow (initial recruitment) throughout life, while cyclic recruitment starts after pubertal onset and stimulates growth and maturation of follicles beyond the antral stage, which will either ovulate or undergo atresia. During reproductive life, continuous growth of primordial and primary follicles into secondary and larger follicles leads to depletion of the follicle pool. In addition, the primordial follicle pool could also be decreased in size due to apoptosis of resting primordial follicles (adapted from 4).

Prior to puberty, massive loss of oogonia and oocytes occurs. Moreover, the number of primordial follicles declines in rats from 50,000 at birth to 8,000 just before the first ovulation, and eventually more than 92% of these follicles are lost by atresia, a process of follicular degeneration (5). Atresia can occur at all stages of follicle development. In rodents, however, the preantral to early antral transition is most susceptible to atresia (6). The first signs of atresia in antral follicles are manifested by the degeneration of the granulosa cells that loose their aromatase activity and undergo apoptosis (7). Later on, the theca cells undergo hypertrophy and their androgen production decreases. The oocyte is affected only at the very last stage of atresia and is therefore often observed intact in atretic antral follicles surrounded by a few layers of cumulus granulosa cells. In atretic preantral follicles, the oocyte is the first to degenerate with the loss of granulosa cells and massive hypertrophy of the theca cells occurring as a secondary event (7,8). The regulation of follicular development and atresia is a complex process and involves interactions between various hormones and growth factors. The major factors that determine follicular cell fate are discussed in paragraph 1.4.1.

Recruitment and early follicle growth

Resting primordial follicles are thought to be under constant pressure by inhibitory factors to remain dormant. The anti-Müllerian hormone (AMH), that is secreted by the surrounding granulosa cells of pre- and early antral follicles, has been shown to inhibit the recruitment of primordial follicles (9). However, the mechanisms that initiate the primordial follicles to leave the resting pool largely remain elusive. So far, only few factors have been demonstrated to initiate follicular growth *in vitro* using rat ovarian culture systems. One of the factors involved in the primordial to primary follicle transition is the oocyte-derived basic fibroblast growth factor (bFGF), which, presumably by signaling to the surrounding granulosa and stroma cells, may initiate this transition (10). Leukemia inhibitory factor (LIF) also promotes the transition of primordial into primary follicles. Moreover, both LIF and bFGF stimulate the granulosa cell expression of kit ligand (KL) (11). KL promotes the primordial to primary follicle transition possibly by binding to its receptor (c-kit) present on the oocyte, stroma and theca cells (11). Follicular growth beyond the primary stage is absent in KL mutant mice (12). Another factor involved in the stimulation of the primordial to primary follicle transition is insulin [but not insulin-like growth factor I (IGF-I)] (13). Gonadotropins (i.e. LH and FSH) possibly also play a role, since a larger pool of resting follicles has been observed in ovarian sections of hypophysectomized rodents, while elevated serum gonadotropin levels are associated with accelerated initial recruitment (reviewed by 4). However, follicles express functional gonadotropin receptors only from the secondary stage onwards, suggesting that gonadotropins affect initial recruitment in an indirect manner. Possibly, all these factors operate in concert to regulate the process of initial recruitment. So if one of these players becomes dysfunctional, other(s) may compensate for their loss, thereby ensuring constant recruitment.

Compared with the initial recruitment process, substantially more is known about growth regulation of follicles beyond the primary stage. Growth and differentiation factor-9 (GDF-9), a member of the transforming growth factor-ß (TGFß) protein family, plays an important role in preantral follicle development and is highly expressed in oocytes of growing primary and larger follicles but absent in resting primordial follicles (14,15). In GDF-9 deficient mice, follicle development was arrested at the primary stage (16). Conversely, treatment with GDF-9 stimulates progression of primary follicle growth and inhibin production in rat ovarian explants and cultured preantral follicles, respectively (10,17). Again, signaling between oocytes and surrounding somatic cells is essential for normal oocyte growth in early follicles. Immature oocytes separated from granulosa cells of preantral follicles do not grow. This idea is supported further by the fact that incompetent oocytes are observed in mice that lack connexin 37, a gap junction protein that normally is a component of the oocyte-granulosa cell junction of secondary follicles (reviewed by 4).

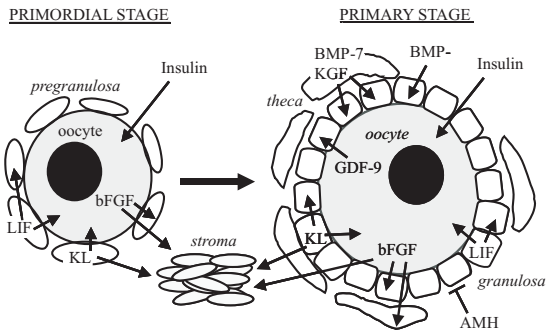

Figure 3. Signaling and cell–cell interactions involved in the primordial to primary follicle transition. In the primordial follicle, insulin and granulosa-secreted KL and LIF act on the oocyte, while oocyte-derived bFGF acts on the granulosa cells to initiate growth. AMH, which receptor is located in granulosa cells of early primary follicles inhibits primordial follicle growth. GDF-9 secreted by oocytes, and the theca-secreted factors BMP-7 and KGF, promote early antral follicle growth by stimulating granulose cell growth and differentiation (adapted from 10).

Granulosa-theca cell interactions also play a role in the development of early follicles. Growth factors secreted by theca cells, i.e. keratinocyte growth factor (KGF) and bone morphogenetic protein-7 (BMP-7), enhance rat preantral follicle growth in culture (18,19). Other factors, like androgen and activin, augment preantral follicle growth in mice (reviewed by 4). Moreover, IGF-I has been suggested to play a role during preantral follicle development, considering the expression of high levels of IGF-I and its receptors in granulosa cells (20). In IGF-I deficient mice, relatively few follicles seem to develop up to the early antral stage (21). IGF-I has been suggested to enhance FSH responsiveness of granulosa cells by augmenting FSH receptor expression (22). FSH promotes rat preantral follicle development *in vitro* and *in vivo*, though FSH receptors are expressed at relatively low levels in preantral follicles. In mice and hamsters, FSH treatment indeed enhances granulosa cell proliferation and antral follicle formation, respectively (reviewed by 4). Moreover, at this follicular stage, thecal cells become responsive to LH, and produce testosterone. This testosterone is transported across the basement membrane and converted into estrogens by the granulosa cells. This step is highly dependent upon FSH suggesting that gonadotropins are involved in preantral follicle growth. Furthermore, FSH both induces proliferation of granulosa cells and increases the number of FSH receptors per granulosa cell during early antral follicle growth, thereby augmenting its own proliferative effects.

Antral follicular growth and selection

Following initial recruitment, most follicles will develop at least until the early antral stage. At that point, follicles either undergo atresia or continue growth, dependent on the level of gonadotropins. Due to increased circulating FSH during the late luteal phase of the estrous cycle, a cohort of antral follicles proceeds to the next developmental stage. The number of follicles in this cyclic recruited cohort is, however, greater than the required number of ovulations so further selection is necessary. Cyclic recruitment of early antral follicles and selection of dominant follicles are best exemplified in monoovulatory species. Nonetheless, the mechanisms behind the selection in rodents

are broadly similar to that of humans except that in these species multiple dominant follicles emerge during each estrous cycle, which is presumably caused by different thresholds for negative feedback signals.

In humans and cattle, just one follicle achieves dominance among the cohort of recruited follicles and continues growth while the remaining subordinate follicles undergo atresia. Although our understanding on the emergence of dominant follicles is still poor, the dominant follicle is thought to indirectly inhibit growth and development of subordinate follicles by releasing high levels of estrogens and inhibins, which in turn exert a negative feedback upon the release of FSH by the anterior pituitary. Less-developed subordinate follicles do not survive such a decline in FSH concentrations (23). Dominant follicles, however, are tolerant against diminished trophic support and proceed to the preovulatory stage, presumably because they become more sensitive to FSH (reviewed by 24). The continued growth of dominant follicles is partly a consequence of increased exposure to locally produced growth factors. Increased production of basic fibroblast growth factor (bFGF), for instance, leads to an enhanced vascularized theca allowing a higher uptake of LH and FSH from the circulation. Moreover, an enhanced bioavailability of IGF-I further augments FSH responsiveness (22,25). Such increased responsiveness of dominant follicles to FSH in turn stimulates the expression of both FSH and LH receptors in the granulosa cells (26). In addition, it has been suggested that dominant follicles produce atretogenic factors, which directly affect neighbouring subordinate follicles, resulting in their degeneration (reviewed by 24).

Finally, selection and follicular growth are also highly dependent on the metabolic state of animals, and metabolic hormones, like leptin, growth hormone (GH) and thyroid hormone (T_3/T_4). These factors act in tight conjunction with gonadotropins to control ovarian function (27,28). For instance, GH can act directly on the ovary by binding to GH-receptors on theca, granulosa, and luteal cells or in an indirect manner via ovarian and/or hepatic IGF-1, affecting gametogenesis, ovarian steroidogenesis, follicular growth, development and atresia (reviewed by (29)). Thyroid hormone has also been shown to influence ovarian function by interacting with the hypothalamic-pituitary-ovarian axis, thereby modulating the secretion of GnRH, gonadotropins, prolactin, androgens and estrogens (30). However, it is not yet clear whether impaired follicular development in hypothyroidism is a result of increased prolactin secretion, which blocks the production and action of gonadotropins, or is due to a direct effect of the decreased thyroid hormone levels on the ovary. The relevance for changes in GH or thyroid hormone levels and reproductive state is demonstrated by epidemiological studies (reviewed by 30,31). Moreover, GH or thyroid hormone replacement therapy improves fertility in some infertile women. The mechanisms and regulation of antral follicle growth and selection is, however, much more complicated than indicated here. Obviously, ovulation

requires the interplay of numerous survival factors during various stages of follicular development of which the most important factors are discussed in paragraph 1.4.1.

Ovulation and luteal development
Ovulation requires the rupture of healthy tissue at the surface of the ovary. The total duration of the periovulatory interval, i.e the onset of the LH surge until follicular rupture is about 14 hours in the rat (32). The LH surge initiates the luteinization process of the theca interna and granulosa cells of the mature follicles, resulting in decreased P450c17 (17α-hydroxylase) and P450aromatase activity, as well as a significant decrease in ovarian androgen and estrogen secretion. Simultaneously, P450 side chain cleavage (P450scc) activity is enhanced and progesterone synthesis is significantly increased. Granulosa cells from periovulatory follicles loose their proliferative activity and differentiate further into a luteal phenotype. Survival of granulosa cells at this stage is thought to be dependent upon LH, while progesterone has been suggested to play a role in granulosa cell cycle arrest (reviewed by 33).

The LH surge also initiates primary oocytes in the preovulatory follicle to complete meiosis I, and to start the second meiotic division. The resulting secondary oocyte does not complete meiosis II, but stops at metaphase II and remains arrested until fertilization. Ovulation may occur when oocytes reach metaphase II. The preovulatory follicle swells, bringing the follicle close to the ovarian surface, and a thin transparent stigma is formed at the apex of the follicle. Proteolytic activity of serine proteases and metalloproteinases within the theca interna and tunica albuginea, partly stimulated by progesterone, facilitate the rupture of the follicular stigma (34). The surface epithelial cells surrounding the apex of the follicle die and the follicle ruptures. The secondary oocyte, zona pellucida, corona radiata and some layers of cumulus cells are released into the peritoneal cavity near the entrance of the fallopian tube. Upon fertilization, when a sperm passes through the corona radiata and zona pellucida and enters the cytoplasm of the secondary oocyte, meiosis II is resumed resulting in the formation of a mature ovum and a second polar body. The first polar body also usually divides, producing two additional polar bodies. When no fertilization occurs, the second meiotic division will not be completed and the secondary oocyte will degenerate within the next few days.

At the time of ovulation, the wall of the ruptured follicle is converted into the corpus luteum, a process that is accompanied by a series of changes. The granulosa cells proliferate, enlarge and are converted into highly steroidogenic luteal cells that synthesize and secrete high levels of progesterone. Moreover, the granulosa layer becomes highly vascularized, presumably to aid the progestational activity essential for pregnancy. This angiogenic process includes the dissolution of the basement membrane between the granulosa and inner theca cell layers and expansion of the theca capillaries into the newly formed corpus

luteum (reviewed by 35). LH and to a lesser extent progesterone, are major stimulators of angiogenesis causing increased expression of vascular endothelial growth factor A (VEGF-A) and angiopoietin (Ang-1) thus promoting vessel formation and maturation, respectively (35). The blood clot that fills the remains of the antrum after ovulation gradually reduces in size due to phagocytosis. The corpus luteum continues to enlarge followed by an involution stage, in which its hormone output ceases. If fertilization takes place, the corpus luteum persists and continues its hormone producing functions (i.e. production of progesterone and estrogen) until the placenta is developed sufficiently to produce the necessary hormones. Ultimately, the corpus luteum degenerates.

1.2.3 Control of estrous cycle

In both humans and other mammals, follicular recruitment is a cyclic process. In primates this involves a menstrual cycle, while in non-primates an estrous cycle is apparent. In this paragraph the control of the estrous cycle is discussed. Small rodents, like rats, mice and hamsters are non-seasonal, spontaneously ovulating polyestrous animals, which have their first ovulation between 35 to 45 days after birth. Rats have a short, incomplete estrous cycle throughout the year of approximately 4 to 5 days, i.e. metestrus (6-8h), diestrus (55-57h), proestrus (12-14h) and estrus (25-27h). The estrous cycles of rats continue until around 10 to 12 months of age; thereafter the cycles become prolonged and irregular. By the age of 12 to 15 months, rats enter persistent estrus, followed by persistent diestrus and ultimately anestrus. Rodents lack a long luteal phase (11 to 14 days) as presented in most mammals (36). Instead, they have short-lived nonfunctional corpora lutea, which secrete progesterone for only 1 to 2 days after ovulation. Upon mating, the luteal phase of these incompletely cycling rodents is, however, extended, thereby resembling a typical mammalian luteal phase. In rats, such corpora lutea are allowed to persist for 12 to 14 days, in the case of pseudopregnancy (when the mating is infertile or artificial), or 20 to 22 days, as during pregnancy. This rescue of the corpora lutea is caused by enhanced pituitary prolactin secretion, which is responsible for continued progesterone secretion (37). The increase in prolactin secretion leads to an increased number of LH receptors in luteal cells thereby enhancing the steroidogenic responsiveness of corpora lutea to LH (36).

The endocrine control of the estrous cycle represents a complex interplay of positive and negative feedback mechanisms by ovarian steroids acting at both the hypothalamic and the anterior pituitary level (reviewed by 36) (a simplified mechanism is presented in fig. 4). The key neuropeptide controlling reproductive function in all vertebrate species is gonadotropin-releasing hormone (GnRH) that is synthesized by the hypothalamus and released into the hypophyseal-portal circulation. GnRH drives the synthesis and secretion of LH and FSH into the systemic circulation.

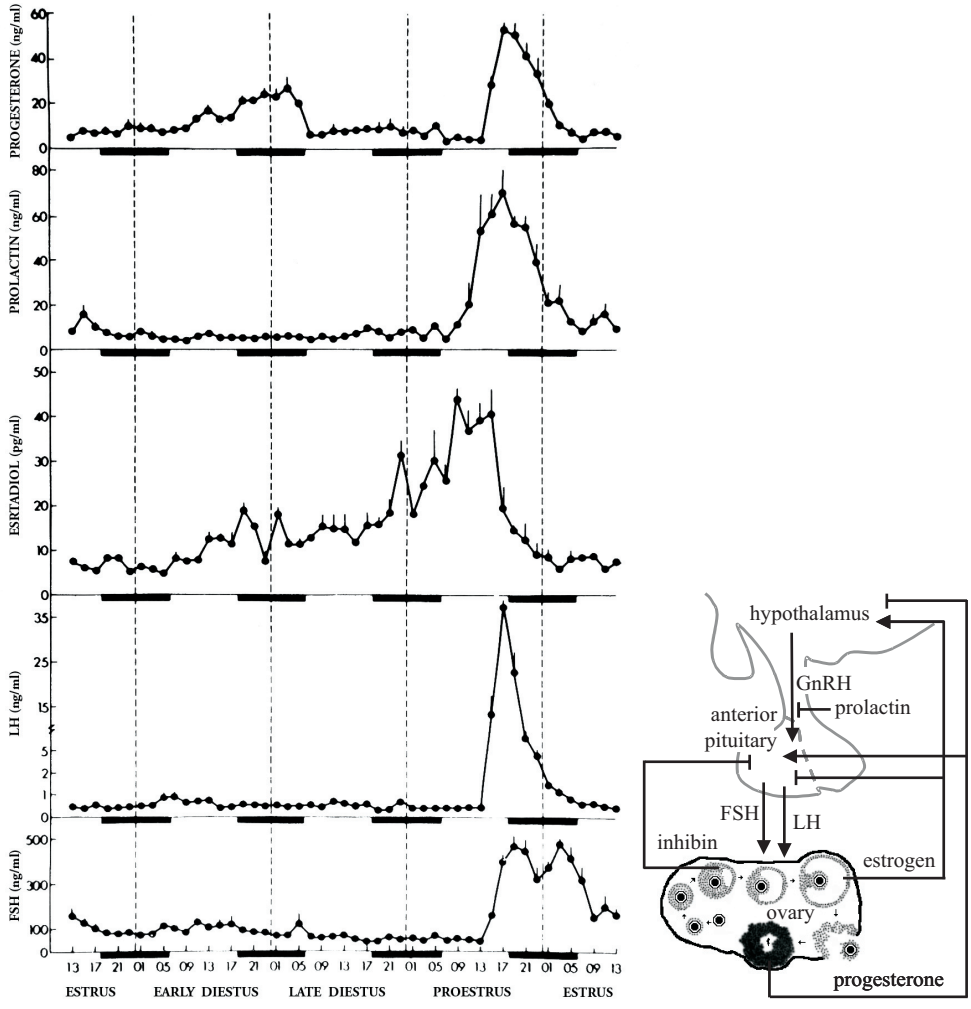

Figure 4. Hormone plasma levels and its regulation throughout the rat estrous cycle. The estrous cycle is driven by alterations in GnRH pulsation from the hypothalamus, which differentially favors the production of gonadotropins. Feedback stimulation (↑) and/or inhibition (⊤) from ovarian steroids acting on both the hypothalamus and the anterior pituitary are essential components for a dynamic gonadotropin release during the estrous cycle. The estrous cycle of rodents is divided into a "luteal phase" (i.e. early and late diestrus) and a follicular phase (i.e proestrus and estrous). The follicular phase starts at proestrus, when the dominant preovulatory follicles produce high levels of estradiol (and later progesterone as well), which stimulate a LH surge to induce ovulation at estrous. The short "luteal phase" of the estrous cycle starts when the postovulatory follicles differentiate into corpora lutea and is ended when progesterone secretion stops 1 to 2 days after ovulation (adapted from 37).

In the rat, the start of the follicular phase is characterized by a relatively high FSH/LH ratio, due to increased FSH levels, which is thought to be the result of a low GnRH pulsatile frequency. This period of FSH drive is critical for the growth of recruited ovarian follicles. FSH acts on granulosa cells to stimulate first the expression of the

enzyme aromatase in late preantral follicles and later on the expression of LH receptors in antral follicles, which are both essential for the synthesis of estradiol. Through the follicular phase, LH pulse frequency increases resulting in enhanced LH levels and subsequent increased release of androgens followed by estradiol. The increase in estradiol and most importantly inhibin production by dominant follicles exerts negative feedback on FSH release, thereby possibly limiting the growth of subordinate follicles. Note that the pattern of androgen secretion throughout the estrous cycle is quite similar to that of estrogen. The increasing amounts of estradiol secreted by the dominant preovulatory follicles reach a peak near the end of proestrus, and appear to be the primary trigger for lordosis and the LH surge, leading to ovulation. Progesterone, the other major secretion product of preovulatory follicles, further augments the release of LH. In association with the LH surge, inhibin levels decrease at proestrus, releasing the negative feedback on the pituitary, ensuring the subsequent secondary FSH surge. The secondary FSH surge on estrus acts on large preantral follicles by initiating their growth and development into early antral follicles, the pool from which the follicles are selected that ovulate at the next estrus (4 to 5 days later).

After the LH surge, estradiol en progesterone levels decline rapidly, and return to basal levels by the start of estrus. When ovulation has occurred, luteinization of the follicular remnants results in increasing production of progesterone. This second peak in progesterone during early diestrus plays a critical role in decreasing GnRH pulse frequency and thereby reduces LH release. In addition to progesterone, the corpus luteum also releases high levels of inhibin, which reduces FSH release and prevents follicle recruitment. The subsequent fall in progesterone production at late diestrus during the estrous cycle results in an increase of the GnRH pulse frequency. Together with the fall of inhibin secretion this leads to a rise in FSH secretion, resulting in the primary FSH peak at proestrus, follicular growth and subsequent new ovulations shortly thereafter (fig. 4).

1.2.4 Ovarian cancer

During fertile life or after entering menopause, the ovarian composition can become disturbed and ovarian cancer may develop. Ovarian cancer represents the fourth most frequent type of cancer among women and is the leading cause of gynecological cancer deaths in the Western world, affecting approximately 1.5% of all women. In the Netherlands 1500 new cases are reported each year (Dutch Cancer Registration). The probability of ovarian cancer is relatively rare in women younger than 30 years, and increases with age, especially after menopause (reviewed by 38). This type of cancer is characterized by an insidious onset and lack of early specific symptoms. When diagnosed,

about two-thirds of the patients with ovarian cancer already suffer from an advanced disease stage (FIGO stage III), having widespread tumor distribution in the abdominal cavity. The prognosis of these patients remains poor, with a 5-year survival rate of less than 20% (39). Management of these patients consists mainly of surgery followed by platinum-based chemotherapy (39). Despite a primary response rate of up to 70%, the majority of patients will suffer from relapse (40). In this thesis, the term ovarian cancer is used to denote the ovarian surface epithelial (OSE) tumor types, which constitutes more than 85% of all human ovarian cancers. Germ cell, stromal and other kinds of tumors also occur but are not the main focus here.

There are many difficulties associated with studying early events in ovarian carcinogenesis. First of all, there are no good animal models. Most experimental animals do not develop OSE cancers, but instead obtain non-epithelial (such as germ cell or granulosa cell) ovarian tumors, of which the underlying biology is different from that of OSE cancers. Secondly, it is often difficult to attain human OSE cells because they are particularly fragile, easily removed as a consequence of surgical handling of the ovary, and thus often absent in pathological samples. Moreover, the availability of early OSE cancer tissue samples is unfortunately limited as most patients present at an advanced disease stage. Thirdly, OSE cells are of an uncommitted phenotype. During neoplastic transformation, OSE cell may differentiate into a phenotypic variety of Müllerian-like epithelial cells (i.e. serous and mucinous cells), which display different patterns of tumor-suppressor genes loss and/or oncogene activation.

Within ovarian cancer tissues, numerous genetic changes have been observed, which are too numerous to be reviewed in detail here. An inherited mutation in the BRCA 1 and/or BRCA2 gene, have been implicated in 5-10% of all cases of ovarian cancer (41). However, in the majority of cases the etiology of ovarian cancer remains poorly understood, in particular those early events that lead to neoplasia.

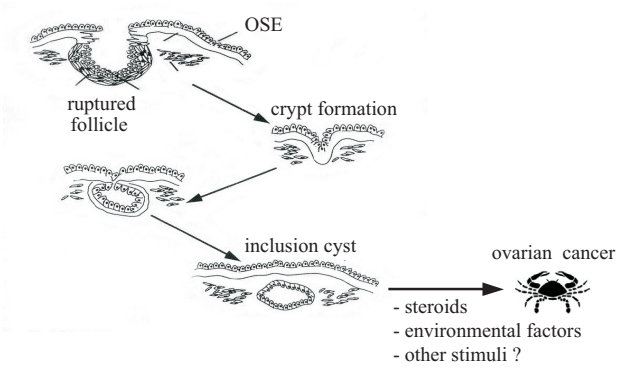

Figure 5. Etiology of ovarian cancer. The incessant ovulation and gonadotropin hypotheses postulates that the chronically repeated formation of crypts and inclusion cysts following ovulation, and the excessive exposure to gonadotropins stimulate the proliferation and subsequent malignant transformation of sequestered OSE cells (adapted from 42).

The proliferative behavior of the OSE cells following ovulation has been suggested to play a role in the etiology of ovarian cancer (43). After ovulation, OSE cells proliferate at the edges of the ovulatory wound, and have an increased tendency to form clefts that extrude into the cortical stroma. Frequently such clefts are sealed off, and as a consequence inclusion cysts may develop (fig. 5). The inclusion cysts may either remain in the stroma for longer periods of time or regress and disappear. Clefts and inclusion cysts are common features of ordinary human ovaries. Ovarian cancers are thought to arise from such sequestered OSE cells. The mechanisms, however, by which these OSE cells become carcinogenic are not well understood (38). Epidemiological studies have demonstrated that the risk of epithelial ovarian cancer development increases with the number of lifetime ovulations (44). Accordingly, the incessant ovulation (45) and gonadotropin hypothesis (46) were proposed to explain these epidemiological data. The incessant ovulation hypothesis postulates that the subsequent repeated rupture and repair of OSE at the ovulation site may result in the accumulation of mutations in OSE cells that promote tumor formation (45). This theory was supported by in vitro experiments in which spontaneous transformation of rat OSE cells was observed following prolonged subculturing (47). The gonadotropin hypothesis postulates that the surges of pituitary gonadotropins, which initiate each ovulation and persist at high levels for years following menopause, enhance the exposure of sequestered OSE cells to gonadotropins, stroma-derived growth factors and/or steroids. This leads to increased proliferation and thus may support malignant transformation of these cells (46). This theory is consistent with the known protective effects against ovarian cancer development of oral contraceptives, pregnancy and breastfeeding, all factors that lower basal and peak gonadotropin levels (46,48). Furthermore, cases have been reported of OSE cancers arising in infertile women during or after prolonged treatment with gonadotropins (49,50). *In vitro*, gonadotropins (FSH and/or LH/hCG) stimulate cell survival of some but not all primary ovarian cancer cells and cell lines, in a dose- and time-dependent manner (51,52), although the mechanism involved remains to be elucidated. It has been suggested that estradiol production is of importance, since hCG, but not FSH, stimulated estradiol synthesis in primary OSE cancer cell cultures (52). Further studies showed that the combined treatment with hCG and estradiol stimulated the growth response through the IGF-I and EGF pathways (53). Despite these observations, controversy exists whether gonadotropins affect OSE cancer cell growth *in vitro* (further discussed below and in paragraph 1.4.4 of this chapter). Inflammation of the OSE was suggested as another mechanism by which gonadotropin stimulation and ovulation play a role in the pathology of ovarian cancer (54,55). Ovulation has many characteristics of an inflammatory reaction, such as local elevation of levels of pro-inflammatory cytokines, prostaglandins and leukotrienes. Conditions that enhance local inflammation (ovarian endometriosis, pelvic inflammatory disease, talc and asbestos exposure) are also associated with an increased risk of ovarian cancer (54).

While the incessant ovulation, gonadotropin stimulation and inflammation individually or all together may play a role in ovarian cancer development, new convincing data have indicated that additional hormonal factors are also involved. In the past, breast and endometrial cancers were above all representing the classical hormone-responsive type of cancers in women. At present, however, ovarian cancers also are thought to be sensitive to hormones and sex steroids (reviewed by 56,57), since the majority of ovarian cancers express receptors to a number of hormones (GnRH, FSH, LH, estrogen, progesterone, androgens, activin and corticosteroids) as well as to various growth factors (EGF, TGFα, TGFβ, TNFα, HGF, KGF and PDGF). Furthermore, OSE cancer cells can secrete hormones like GnRH, inhibin, hCG and estrogens (reviewed by 38). With regard to estrogens, although older studies were inconclusive (58,59), recent studies have consistently demonstrated that the risk of developing ovarian cancer increases with the duration of postmenopausal estrogen replacement therapy (60,61). Conversely, breastfeeding, which is associated with reduced serum estradiol concentrations, protects against ovarian cancer. *In vitro*, estrogens, both estradiol-17β and estrone, stimulate estrogen receptor-mediated growth of OSE (cancer) cells (51). These mitogenic effects of estrogens are mediated through activation of the IL-6/STAT-3 signaling pathway (62). On the other hand, estrogens have been suggested to cause genomic damage to ovarian cancer cells, as is the case in breast cancer cells (63). In an ovarian cancer cell line, estrogens were found to regulate mutations in the BRCA1 gene, the genetic alteration that is strongly associated with inherited ovarian cancer (64,65). Moreover, estrogens have also been shown to activate the early growth response genes c-myc and c-fos (64,66).

Estrogens, however, may not be the most relevant etiological factor, since raised serum estrogen levels due to the oral-estrogen-based contraceptives or pregnancy reduce the risk of ovarian cancer in humans (reviewed by 57). Given the fact that contraceptives are comprised of progestins with extremely high progestational potency, the protective effects of such contraceptives may, like for pregnancy, be attributed to high progesterone levels (67). Indeed, evidence exists that progesterone offers protection against ovarian cancer development. Loss of heterozygosity at 11q23.3-24.3, which harbors the progesterone receptor gene locus, is observed in 75% of ovarian cancer patients and this genetic alteration is associated with poor prognosis (68). Moreover, ovarian cancer risk is increased among women with progesterone deficiency. *In vitro*, progesterone showed inhibitory actions on ovarian cancer cell growth (reviewed by 56) (for more details see paragraph 1.4.4). The putative protective effect of progesterone has raised the issue whether combined estrogen and progestin replacement therapy in the postmenopausal years is a safer alternative than estrogen replacement therapy (ERT) with respect to ovarian cancer.

Appreciable evidence implicates a role for androgens in the pathogenesis of ovarian cancer. In particular 5α-dihydrotestosterone (5α-DHT), but also testosterone, stimulate proliferation of ovarian cancer cells *in vitro* (69,70) and in an animal model (71). Moreover, high androgen serum levels, which are generally observed in women with polycystic ovary (PCO) syndrome (72) and in obese postmenopausal women with a high waist-to-hip ratio (73), are associated with an increased risk of ovarian cancer. Alternatively, such effects of androgens may also reflect changes in the patterns of pituitary gonadotropin secretion. The most important argument is that the incidence of ovarian cancer peaks in the years after menopause when gonadotropin levels are elevated, while ovarian production of progesterone and estrogen has ceased (except in obese women where the latter production is essentially limited to skin and adipose tissue). Moreover, case studies have reported development of ovarian cancers in women undergoing fertility treatment with gonadotropins and an increased risk has been suggested in association with the use of fertility drugs in large population studies (49,50), again supporting the hypothesis that gonadotropins may play a major role in the etiology of ovarian cancer.

In conclusion, the cause of ovarian cancer remains obscure. Hypotheses relating to incessant ovulation, excessive gonadotropin secretion, inflammation, estrogen/progestin imbalance and apoptosis (see also paragraph 1.4.4) have been evoked as etiological explanations. All these hypotheses are supported by epidemiological studies. Both pituitary and sex hormones affect ovarian cancer cell growth *in vitro.* However, hardly any information is available whether and how such hormones modulate the early events of ovarian cancer development.

1.3 APOPTOSIS

1.3.1 Significance of apoptosis

Cell death has long been recognized as an integral and necessary phenomenon of the normal life of multicellular organisms, in particular during embryonic development and metamorphosis. The term programmed cell death was launched in 1964, proposing that cell death was not an accidental occurring process, but a coordinated one, with sequence of controlled a events eventually leading to self-destruction (74). In 1972, Kerr introduced the term apoptosis (which in Greek has the meaning "falling off" in the sense of leaves falling of trees") in order to describe the active programmed form of cell death in which cells trigger an intrinsic suicide program in order to eliminate themselves when damaged or unwanted (75). Nowadays, it has been recognized that apoptosis is of importance in a variety of biological processes, not only in the development of multicellular organisms but also in maintaining tissue homeostasis, architecture and

function. Moreover, the apoptotic machinery is ubiquitously present in metazoans and similar cell death mechanisms have even been observed in plants, yeast, eukaryotes and some prokaryotes (76).

During (embryonic) development, cells are produced in excess, many of which eventually undergo apoptosis, thereby contributing to the shaping and rebuilding of developing tissues. Prominent examples of apoptosis during development include the regression of the tail in tadpoles during metamorphosis and the formation of separate digits in the primitive limb during human embryogenesis. Another example is the regression of the Müllerian duct during development of the male reproductive organs. In adult organisms, cells may also undergo apoptosis, which is often linked to cell proliferation in order to maintain tissue homeostasis. Tissue homeostasis is tightly regulated by the interplay of various trophic factors, cell-cycle regulators and apoptotic stimuli that may affect either apoptosis or cell proliferation, or both. The major endocrine and paracrine factors that determine cell fate in the ovary are reviewed in paragraph 1.4 of this chapter.

Alterations in the cell death control mechanisms may contribute to the pathogenesis of a variety of human diseases (77). Diseases that are linked to excessive apoptosis include ischemic diseases and neurodegenerative disorders, while cancer, autoimmune disorders and spreading of viral infections are generally associated with moderate or deficient apoptosis. In many cases, however, it is unclear whether modifications in the apoptotic program are causal or merely a consequence of the disease process. The mechanisms by which hormonal factors possibly affect apoptosis during ovarian cancer development and chemotherapeutical treatment are reviewed in paragraph 1.4.4.

1.3.2 Morphological features of apoptosis

Apoptosis is in most situations characterized by uniform morphological changes; the apoptotic cell shrinks, distorts and looses contact with its neighboring cells (78). Its chromatin condenses and aggregates on the nuclear membrane, while the plasma membrane protrudes and shapes into discernable membrane blebs. Finally, the cell is fragmented into compact membrane-enveloped structures, called apoptotic bodies, which contain cytosol, condensed chromatin and organelles (fig. 6). The apoptotic bodies are engulfed and digested either by neighboring cells or by macrophages and thus removed from the tissue without causing an inflammatory response. Recognition and phagocytosis of apoptotic bodies is primarily mediated by exposure of phospatidylserine at the outer cell membrane. The morphological changes in apoptotic cells are a consequence of characteristic molecular and biochemical events, such as the activation of proteolytic enzymes, which eventually mediate the cleavage of DNA into

oligonucleosomal fragments (of 180 kilo base pair fragments) as well as the cleavage of a multitude of specific protein substrates which usually determine the integrity and shape of the cytoplasm and organelles (the basic apoptotic signaling pathways are discussed below).

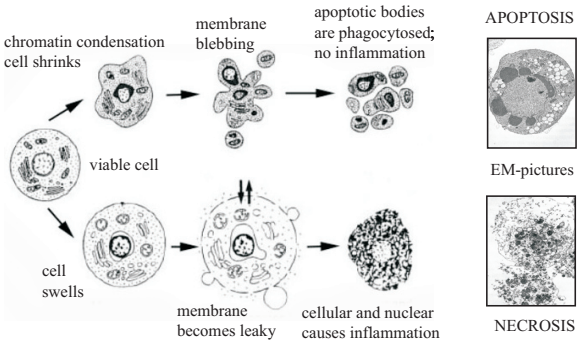

Figure 6. Hallmarks of apoptotic and necrotic cell death. Apoptosis includes cellular shrinkage, chromatin condensation, and the formation of apoptotic bodies that are phagocytosed without triggering inflammation. In contrast, necrosis is accompanied by an inflammatory response resulting from cell swelling, loss of membrane integrity and leakage of intracellular contents into the surrounding environment (modified from 79).

Apoptosis is distinguished from necrosis, or accidental cell death, which is characterized by nuclear autolysis and cell disintegration. Necrosis results from a major insult, causing loss of membrane integrity and disruption of the cell. During necrosis, cellular DNA may be degraded nonspecifically and the cellular content leaks into the surrounding tissue, resulting in damage of surrounding cells and inflammation. One has to keep in mind that typical physiological tissue responses to damaging stimuli usually involve both apoptosis and necrosis, though apoptosis is the most frequent form of physiological cell death (79,80).

1.3.3 Apoptotic signaling pathways

Caspases

The initiation, execution and regulation of apoptosis involves various biochemical factors and pathways. It is widely accepted that caspases play a central role in the apoptosis-signaling network. Caspases are members of the highly conserved family of cysteine proteases with aspartate specificity. Caspases have an uniform nomenclature; caspase-1 through caspase-14 (81,82). Within the cell, caspases are constitutively and ubiquitously expressed as catalytically inactive zymogens, the so-called procaspases. Caspase activation requires proteolytical cleavage of the proenzyme resulting in the removal of the N-terminal prodomain and the formation of a heterodimer containing one small and one large subunit. Two joined heterodimers then form the active caspase (fig. 7). Once caspases have been activated, they can proteolytically activate downstream procaspases, which subsequently cleave a specific set of protein substrates, including other procaspases, resulting in the amplification of the death signal and eventually in the execution of cell death (81,82).

The caspases can be divided into a group of initiator caspases (i.e. procaspase-2, -8, -9 and -10) and into a group of executioner caspases (i.e procaspase-3, -6 and -7). Initiator caspases possess long prodomains containing either death effector motifs (DED), as is the case for procaspase-8 and –10, or caspase recruitment domains (CARD), as is the case for procaspase-2. Via their specific prodomains, the initiator caspases with DED motifs are recruited to death receptor signaling complexes in response to ligation of the death receptor (for more detail see below), while the CARD-containing caspases correspond closely to the proteolytic activation of effector caspases (see also below). In contrast, executioner caspases posses a small prodomain containing a motif (DXXD or VEXD) that associates with the cleavage sites in the vast majority of apoptotic target substrates, including the DNA repair enzymes PARP and DNA-PK and structural proteins within the nuclear envelope (e.g. lamins) and cytoskeleton (e.g. actin).

Death receptor signaling pathways

Cells activate their apoptotic machinery either in response to environmental stress, internal damage, lack of survival signals, or via instruction of specific signals via death receptors (extrinsic apoptotic pathways) (fig. 9). These death receptors are exposed on the cell surface and transmit apoptotic signals after ligation with cognate death ligands. Death receptors belong to the tumor necrosis factor (TNF) receptor superfamily, and include TNFR-1, Fas/CD95, and the TRAIL receptors DR-4 and DR-5. Association of death ligands with their cognate receptors results in activation of the signaling pathway as a consequence of receptor trimerization (fig. 7).

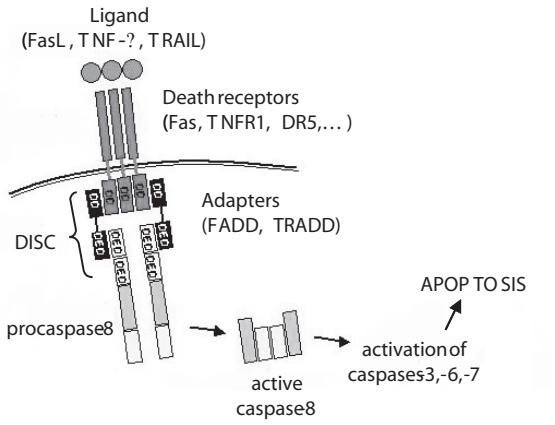

Figure 7. The DISC and death receptor mediated caspase activation. Binding of death ligands to their cognate receptors leads to assembly of the DISC. The key components of this structure include protein interaction of the DD-bearing adapter proteins (e.g. FADD) with the DED-containing initiator procaspase (procaspase-8), an event and leads to the proteolytical cleavage of caspase-8 and consecutive downstream caspases that ultimately results in the execution of apoptosis (adapted from 83).

Subsequent apoptotic signaling is mediated by the cytoplasmic domain of the death receptor, which contains a region termed the death domain (DD). Adapter molecules like Fas- or TRAIL-associated death domains (FADD or TRADD, respectively) bind through protein DD-DD interaction domains to the activated death receptor, thereby forming the so-called death inducing signaling complex (DISC). In addition to its DD,

the adaptors FADD and TRADD also contain another protein-protein interaction domain termed the death effector domain (DED), which through protein DED-DED interaction assemble procaspase-8 to the DISC (fig. 7). When bound to the DISC, several procaspase-8 molecules are in close proximity to each other resulting in cross activation by (auto)proteolysis (for more details see below). A number of signaling proteins may bind directly to the DISC and interfere with the recruitment of caspases. For instance, c-FLIP$_L$ (Fas-associated death domain-like ICE inhibitory proteins), an anti-apoptotic protein that is overexpressed in human melanomas, competes with caspase-8 binding at the DISC and thereby inhibits death receptor mediated apoptosis (84).

Following activation in the DISC, caspase-8 can initiate the apoptotic program. Two pathways of Fas signaling, which depend on the release of active caspase-8, have been described (85) (see also fig. 1 of chapter 2). In 'type I cells', a high concentration of caspase-8 at the DISC can process downstream effector caspase-3 directly, resulting in its activation and definitive death of the cell. In 'type II cells', however, a small caspase-8 signal is generated and the signal requires an amplification loop via a mitochondria-dependent apoptotic pathway. The link between the death receptor activated caspase-8 and mitochondrial cytochrome c release is created by Bid. Bid is cleaved by caspase-8 to truncated Bid (tBID) and transferred to the mitochondria where it acts in concert with the pro-apoptotic Bcl-2 family members Bax and Bak to induce the release of cytochrome c (for more details see below), which eventually results in downstream activation of effector caspases and demise of the cell (fig. 8).

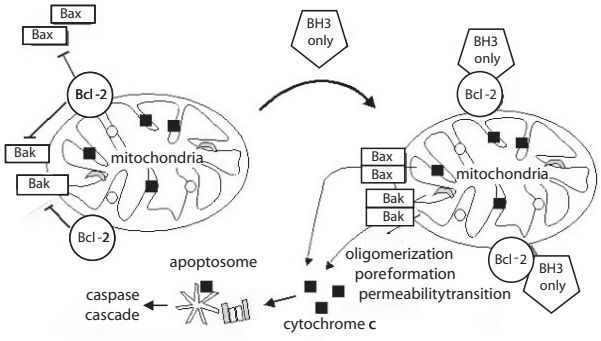

Figure 8. Regulation of apoptosis by the Bcl-2 protein family. Many death signals converge onto mitochondria and are mediated through members of the Bcl-2 protein family called BH3-only proteins (Bid and Bad). As a result, Bax and Bak proteins are activated forming homodimers resulting in cytochrome c release. Once in the cytosol, cytochrome c activates caspase-9 by binding to the Apaf-1 forming an apoptosome (adapted from 83).

Mitochondria and Bcl-2 family proteins

Mitochondria are not only important for amplifying extrinsic apoptotic pathways but also for transmitting death signals caused by oxidative stress, starvation and DNA damage. Mitochondrial integrity and the release of cytochrome c into the cytosol are primarily under control of members of the Bcl-2 family. The Bcl-2 family of proteins can be divided into three categories by the presence of conserved sequence motifs known as Bcl-2 homology domains (BH1 to BH4). Anti-apoptotic members, such as

Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and Boo, contain all four conserved regions (BH1, BH2, BH3, and BH4), while pro-apoptotic members either possess the domains BH1, BH2, and BH3 (like Bax, Bak, Bok and Diva) or have only the BH3 domain (Bid, Bad, Bim, Bik, Bmf, Hrk, Noxa, Puma, Blk, Bnip3 and Spike). The BH4 domain is required for anti-apoptotic activity while BH3 is an interaction domain that is both necessary and sufficient for pro-apoptotic action (86,87).

The formation of either homo- or heterodimers between pro-apoptotic and anti-apoptotic members enables them to regulate apoptosis. In general, however, only members of the BH3-only subfamily are thought to interfere with the fine-tuned balance of homo- or hetero-oligomerization, since they are the ones that are activated during apoptosis following cellular stress conditions, such as DNA damage and growth factor deprivation. In response, pro-apoptotic family members such as Bax and Bak, undergo a conformational change, oligomerize and translocate to the outer mitochondrial membrane, a process that is followed rapidly by cytochrome c release (fig. 8). Although the mechanism is still controversial, Bax proteins are thought to contribute to the permeabilization of the outer mitochondrial membrane, either by forming channels by themselves or by interacting with components of the mitochondrial membrane pores (86). In contrast, anti-apoptotic Bcl-2 members inhibit Bax or Bak activation and mitochondrial pro-apoptotic events by binding to the BH3 domains of the pro-apoptotic Bcl-2 members.

Released cytochrome c may assemble with the adaptor protein APAF-1 and procaspase-9 to form a large protein complex, the apoptosome (fig. 8). This results in activation of caspase-9, which then processes and activates other downstream effector caspases to orchestre the execution of the cell. In addition to cytochrome c, several other proteins are released from mitochondria undergoing apoptosis. Among them is Smac/Diablo, which binds to and inactivates inhibitors of apoptosis proteins (IAPs). IAPs act by binding to both pro- and activated caspases, thereby blocking the processing of caspase activation (fig. 9).

p53

The p53 tumor suppressor gene is functionally inactivated in 70% of human tumors. The 53 protein functions as a transcription factor in response to DNA damage, inducing either growth arrest or apoptosis. The induction of growth arrest depends on its activity and on the target genes, i.e. p21, Bax, Apaf-1 and Fas, and on repression of the expression of anti-apoptotic proteins, such as Bcl-2, Bcl-x_L or survivin (fig. 9). In non-stressed, undamaged cells p53 therefore must be kept at low cellular concentrations in the cytosol to prevent translocation to the nucleus. Central to p53 regulation is the oncogene Mdm2, which binds to and thereby inhibits p53. Mdm2 also targets p53 for

ubiquitination-mediated degradation guaranteeing safe low p53 levels in normal healthy cells. In response to cellular stress (such as DNA damage) p53 is phosphorylated, which prevents the Mdm2-p53 interaction, and thus p53 is stabilized and activated. P53 activation may then induce cell death. Therefore, in many instances an oncogenic insult resulting in increased proliferation and eventually malignant transformation can occur only when p53 inhibitors such as mdm2 are inactivated.

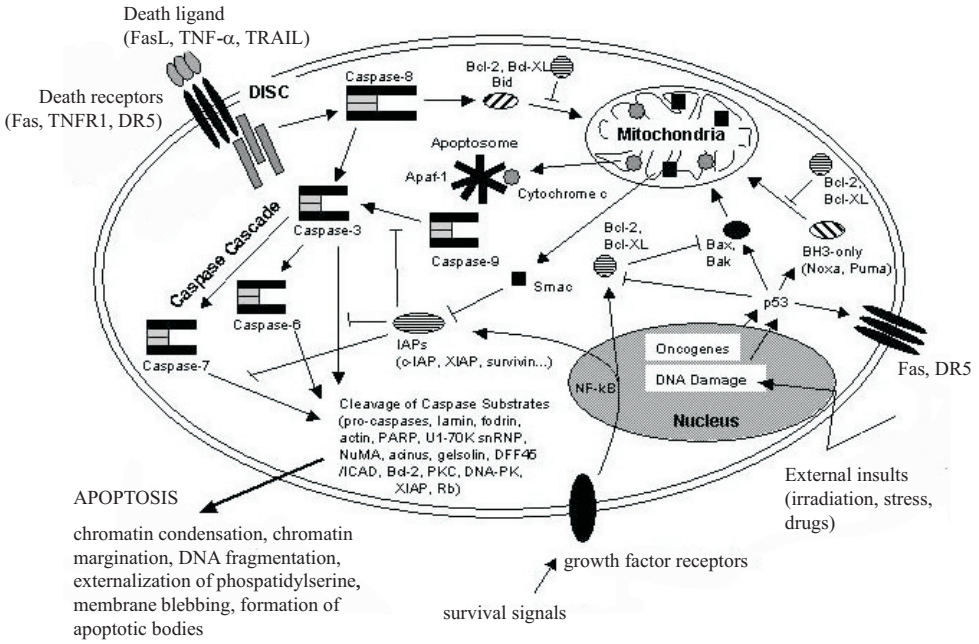

Figure 9. Schematic overview of the major apoptotic pathways. Simplified scheme of how various apoptotic signals, including specific signals via cell surface death receptors, environmental stress, internal damage or a lack of survival signals may activate the multifaceted intracellular apoptotic machinery (adapted from 83).

1.4. HORMONAL REGULATION OF APOPTOSIS IN THE OVARY

1.4.1 Regulation of follicular cell death

Follicular atresia is a hormonally regulated process with differential hormonal responsiveness of follicles at different stages of follicular development. Nowadays, it is generally accepted that atresia is the default pathway of follicles, which occurs when cells fail to receive sufficient signals from regulatory hormones required to suppress the apoptotic pathway and/or when atretogenic factors promote follicular cell death. The main physiological regulatory hormones of follicular survival are the gonadotropins. Suppression of serum gonadotropins due to hypophysectomy leads to massive apoptosis of granulosa cells in developing follicles resulting in atresia, whereas gonadotropin

treatment of early antral and preovulatory follicles prevents the spontaneous onset of follicular atresia in culture (88). In preantral follicle cultures, FSH, but not LH or hCG, inhibited granulosa cell apoptosis (89), suggesting that gonadotropins are probably less important survival factors during preantral follicular development compared to antral follicular development.

The mechanisms by which gonadotropins ensure follicle survival are not yet clear. Possibly, suppression of the death receptor-signaling pathway present in 'type II cells' is, in part, responsible for endurance. A shortage of FSH exposure leads to apoptosis in granulosa cells at the early antral stage of follicular development which is associated with increased levels of Fas and Fas ligand in the rat ovary (90). Similarly, in rats following gonadotropin withdrawal, Fas and Fas ligand are highly expressed in granulosa cells culminating in caspase-mediated apoptosis of follicles (90). Moreover, granulosa cells isolated before the LH surge are susceptible to apoptosis induced by soluble Fas ligand (91), while cells isolated after the LH surge are resistant to apoptosis (92). Such increased resistance may, however, not be associated with a change in Fas levels (92), but with an increase in anti-apoptotic Bcl-x_L expression, thus inhibiting apoptosis as has been demonstrated in hens (fig. 9)(93). In contrast, increased apoptosis in preovulatory follicles following gonadotropin suppression (by treatment with GnRH analogues) in rats, is accompanied by reduced expression of Bcl-x_L protein (94). Increased and reduced Bax mRNA levels were observed in response to respectively withdrawal and enhanced exposure to gonadotropins and accompanied with relatively constitutive levels of Bcl-x_L and Bcl-2 expression (93,95). Bcl-2 family member proteins are thought to play a major role in atresia, since follicle apoptosis is decreased in ovaries of both Bcl-2 overexpressing (96) and Bax knockout mice (97). In conclusion, these studies suggest that the Fas death receptor acts in concert with a balance of pro- and anti-apoptotic Bcl-2 family members to facilitate gonadotropin mediated follicular survival.

Important regulators of follicular cell survival and apoptosis include sex steroids. Progesterone and estrogens have anti-apoptotic properties while androgens act as atretogenic factors. Progesterone inhibits granulosa cell apoptosis through binding to the progesterone receptor (98). Estrogens reduce the incidence of atretic follicles in ovaries when administered to hypophysectomized rats (99). Not much is known about the anti-apoptotic action of estrogens. However, it has been suggested that granulosa cell secreted estrogens stimulate theca cell expression of various growth factors including TGF-α, which in turn inhibits apoptosis by up-regulating the expression of intracellular anti-apoptotic proteins like XIAP and FLIP (100). Follicular estrogen production is dependent upon both LH stimulation of androgen production by theca cells and FSH stimulation of aromatase activity in granulosa cells, suggesting that gonadotropins are indirectly responsible for the anti-apoptotic action of estrogens. In contrast to estrogens, treatment

with androgens is atretogenic to ovarian follicles. In hypophysectomized rats, the anti-apoptototic effect of estrogen treatment is blocked following additional treatment with testosterone (99), while androgen receptor blockers and testosterone antibodies inhibit follicular atresia (101). An increased androgen to estrogen ratio is found in follicular fluid of atretic follicles (102,103). In general, changes in steroidogenesis are observed prior to the appearance of morphological signs of atresia (104).

In addition to gonadotropins and sex steroids, GH (105) and local growth factors including IGF-I, EGF and TGFα (88) also affect the onset of apoptosis in serum-free cultures of preovulatory follicles. IGF-I is, however, ineffective in preventing spontaneous apoptosis in cultured granulosa cells. The anti-apoptotic action of IGF-I, however, is regulated, in part, via the action of growth hormone and gonadotropins on the ovary. Moreover, IGF-I synergizes with gonadotropins to promote granulosa cell differentiation and local granulosa cell IGF-I production. Secreted IGF-I acts on theca cells to stimulate the production of EGF and TGFα, which, in turn, diffuse back to the granulosa cells to prevent apoptosis. Additional information on the hormonal regulation of apoptotic regulators in follicular cells can be found in the chapters 2, 3 and 4, whereas chapter 7 aims to provide a better understanding of the mechanisms of follicle cell demise.

1.4.2 Regulation of luteal cell death

Regression of the corpus luteum is a process that is associated with loss of progesterone production capacity and apoptosis of luteal cells. The level of apoptosis in the corpus luteum varies with the phase of the estrous cycle and the highest degree of luteal cell death has been observed during the follicular phase. This is accompanied by an increased expression of various components of the Fas signaling pathway, such as Fas, Fas ligand, Bax and caspase-3 (106-109), which individually or in concert may contribute to luteal cell death. Further evidence has come from animal studies, which have demonstrated that the incidence of luteal cell death was delayed markedly in mice lacking functional Fas, Fas ligand (108), Bax (97), or caspase-3 (110). Thus, the Fas system may control structural involution of the corpus luteum, however, the physiological trigger for this extrinsic apoptotic pathway remains to be identified.

Many endocrine glands involute after removal of trophic hormone support and/or by activation by negative stimuli that promote apoptosis. Several *in vitro* studies have shown that luteothropic factors, like progesterone and human chorionic gonadotropin (hCG) suppressed apoptosis in luteal cells by lowering Fas, Fas ligand, Bax and/or p53 expression (109,111,112). It has been suggested that when luteal cells loose their ability

to secrete progesterone, the extrinsic apoptotic pathway blockade is released, resulting in increased expression of Fas on their surface. On the other hand, luteolytic factors, such as prolactin (113), TNFα (112) and $PGF_{2\alpha}$ (110,112) induce luteal cell death, without any clear effect on the expression of Fas signaling proteins (112). This suggests that other factors may also play a role. A few explanations by which mechanisms luteolytic factors affect luteal cell death have been put forward (fig. 10).

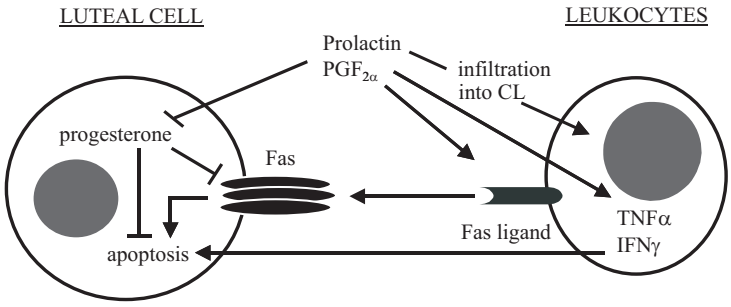

Figure 10. Signaling and cell–cell interactions involved in luteal cell death. Progesterone suppresses expression of various components of the Fas pathway and apoptosis of luteal cells. Luteolytic factors like prolactin and $PGF_{2\alpha}$ act directly or indirectly on luteal cells to suppress the synthesis of progesterone. Prolactin and $PGF_{2\alpha}$ also stimulate the infiltration of leukocytes in the corpus luteum. Moreover, they act by stimulating the secretion of TNFα IFNγ, and expression of Fas ligand on leukocytes which upon interaction may result in luteal cell death (modified from 114).

Prolactin binds to its cognate receptor on leukocytes present in the corpus luteum thereby stimulating the expression of Fas ligand on their surface. Subsequently, Fas ligand expressing leukocytes in the corpus luteum interact with Fas-expressing luteal cells resulting in luteal cell death (114). Although, prolactin can elevate the number of leukocytes and/or apoptosis in these regressing corpora lutea, blockade of the proestrous prolactin surge *in vivo* does not result in complete reversal (115), suggesting that prolactin is not solely responsible for the induction of this process. Moreover, it has been suggested that progesterone counteracts the deleterious effects of prolactin. This assumption is based on the observation that the prolactin surge in cycling rats or the administration of exogenous prolactin *in vitro*, induces apoptosis in luteal cells isolated on the days of proestrus and estrus (115), but not in cells isolated during metestrus or early diestrus and also not in luteal cells isolated from (pseudo)pregnant rats, under circumstances when progesterone levels were high. Incidentally, if corpora lutea at all stages of development were always responsive to prolactin-induced apoptosis, they would be completely eliminated during the first week of pregnancy by the two prolactin surges induced by mating (116).

In rodents and cattle, however, the role for prolactin in luteal function is secondary. The major factor that initiates luteolysis is uterine-derived prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). $PGF_{2\alpha}$ *in vivo* reduces the blood flow to the corpus luteum thereby depriving the gland of

essential substrates for steroidogenesis resulting in a decline in progesterone production and ultimately in luteal cell death. $PGF_{2\alpha}$ also decreases progesterone production in luteal cells *in vitro*, however, it does not affect cell numbers or viability, suggesting that either other endocrine factors or interaction between endothelial cells and steroidogenic luteal cells is required for $PGF_{2\alpha}$ to kill luteal cells. Indeed, $PGF_{2\alpha}$ stimulates the infiltration of Fas ligand expressing leukocytes into the regressing corpus luteum, thereby promoting Fas-induced apoptosis of luteal cells (111). Moreover, increased infiltration of macrophages and lymphocytes capable of secreting TNFα and IFNγ occurs during natural regression of the corpus luteum and could also potentially contribute to activation of the Fas pathway (117) (fig. 10).

1.4.3 Regulation of ovarian surface epithelial cell death

Ovarian surface epithelial cells have been suggested to undergo cycles of proliferation and degeneration that are associated with the ovulatory process. In ewes prior to ovulation, OSE cells surrounding the follicles destined to rupture, are exposed to both inflammatory agents and reactive oxidants, a condition which is accompanied by increased apoptosis and upregulation of p53 (118). Concomitantly, OSE cells located along the margins of ruptured follicles showed enhanced survival and expression of anti-apoptotic Bcl-2, which probably functions to mend the insult following ovulation (118). The Fas system has been proposed to play a role in ovulation-induced OSE cell death. Fas signaling proteins are present in OSE cells (118-120) and cultured mouse OSE cells undergo apoptosis in response to Fas activation when pretreated with IFNγ (121). However, not much is known about the hormonal control mechanisms that influence OSE cell survival associated with ovulation.

Progestins in oral contraceptives promote apoptosis in OSE cells in primates, a process that is probably mediated by TGF-β (122,123). *In vitro*, progesterone affects OSE cell survival differentially; a growth promoting effect of progesterone was observed at low concentrations (below 10^{-8} M), whereas progesterone at higher concentrations had either an anti-proliferative and/or pro-apoptotic effect (51). High progesterone doses, in the range experienced after the LH-surge, enhances p53 expression (119) and induces apoptosis via enhanced Fas/FasL signaling and caspase-8 activation (70). The high levels of estrogens released in the follicular fluid at the time of ovulation, may play a role in the survival of undamaged OSE cells next to the rupture site (119). High concentrations of estrogens have been shown to increase proliferation and to suppress apoptosis in OSE cells *in vitro* (51,119). As a final point, the above-mentioned hormonal factors modulate the survival of ovarian epithelial cells that have become neoplastic (for more details see below).

1.4.4 Regulation of ovarian cancer cell death

The concept that ovarian cancer progression might involve alterations in cell death control is strongly supported by the observation that apoptosis and the expression of individual apoptotic proteins in ovarian cancers is correlated with type and grade of the tumor (124-127). The level of apoptosis increases during the course of ovarian tumor development (124,125), but is outnumbered by proliferatively active OSE cells, which clearly demonstrates a disturbed balance between tumor OSE cell growth and loss. Furthermore, mutations in the p53 tumor suppressor gene are a common aspect in serous OSE tumors (124,128,129). Such mutations are associated with a loss of p53 function and thereby, in response to DNA damage, may inhibit cell cycle arrest and apoptosis. When apoptosis is impaired, mutated cells might accumulate leading to propagation of mutations and an increased risk of transformation, tumor formation and progression. However, p53 mutations are likely to be involved late in ovarian carcinogenesis, as it has been suggested that inhibited apoptosis, caused via p53 mutation may not be a contributing factor in the onset of OSE cancer (42).

It is also likely that hormonal factors influence OSE tumor growth and survival through modulating apoptosis. Indeed, as mentioned above, high levels of progesterone induce apoptosis in normal OSE cells but also in ovarian cancer cells *in vitro* (51), involving enhanced Fas/FasL signaling and caspase-8 activation (70). In contrast to the pro-apoptotic action of progesterone, androgens and estrogens generally exert anti-apoptotic effects in OSE cancer cells. Estrogens and androgens individually suppressed ovarian cancer cell death *in vitro* (69). Conversely, anti-estrogens (e.g. taximofen) and anti-androgens (e.g. flutamide) have been shown to stimulate apoptosis or inhibited cell growth, respectively in a dose-dependent manner in OSE cancer cell lines (130,131).

A growing body of evidence indicates that hormones also interfere with the mechanisms of chemotherapy resistance. Such resistance is thought to be caused, in part, by deregulation of apoptotic pathways. Several key apoptotic regulators, including p53, the IAP family, the Akt family and the death receptor family, modulate the sensitivity to death-inducing cancer agents in ovarian cancer cells (reviewed by 132). Cisplatin, for instance, is a frequently used chemotherapeutic agent for treatment of ovarian cancer, and upregulates Fas and Fas ligand resulting in activation of caspases and induction of apoptosis in ovarian cancer cells (133,134). In a chemoresistant ovarian cancer cell line, cisplatin failed to induce Fas ligand upregulation and apoptosis. Such resistance was suggested to be caused by interactions of X-linked inhibitor of apoptosis proteins (XIAPs) with the phosphoinositide-3 kinase (PI3K)/Akt cell survival pathway, which either inhibited caspase-3 directly or indirectly via modulation of the mitochondrial apoptotic pathway (for more details about the apoptotic signaling pathways see paragraph 1.3.3) (132).

Due to the pro-apoptotic properties of progesterone (see above), the effects of progestational agents combined with chemotherapy have been studied extensively. Generally, the prognosis of advanced ovarian cancers was improved by the combination of chemotherapeutic drugs and progestins, although the different studies were difficult to compare due to inconsistent doses of drugs used (135). Anti-estrogens (primarily taximofen) delay the development of resistance to cisplatin in ovarian cancer, though considerable variation among patient cases has been observed (136). In vitro, estrogens attenuate paclitaxel-induced apoptosis in ovarian cancer cells (137). Hormone therapies using anti-androgens have suggested that blockade of androgen action or synthesis may have therapeutically beneficial effects in ovarian cancer (reviewed by 135). Usage of gonadotropin releasing hormone (GnRH) analogs in combination with chemotherapeutic regimes also resulted in a longer survival in subgroups of recurrent ovarian cancer patients in some but not all studies (reviewed by 138). An anti-proliferative effect of GnRH was also observed in an animal model of ovarian cancer, but this effect was latent and transient (139). Moreover, GnRH analogs are thought to lower gonadotropin levels. *In vitro*, hCG lowers the chemosensitivity to cisplatin in ovarian cancer by inhibiting apoptosis (140). The inconsistency in the variety of doses and of the different potencies of the preparations used, particularly in the case of GnRH analogues, may explain the poor evaluation of hormonal therapeutic effects in ovarian cancer. Further studies are definitely needed to resolve whether hormones have therapeutical value in treatment of ovarian cancer (see for more information chapter 6 and 7).

1.5 AIM AND OUTLINE OF THIS THESIS

As described in the previous sections of this chapter, apoptosis plays an important role in normal reproductive function. Since apoptosis attributes to the exhaustion of the oocyte/follicle reserve, either directly through germ cell death or indirectly through follicular atresia, this process has been proposed to be the major mechanism that determines the female reproductive lifespan. Moreover, an imbalance between proliferation and apoptosis in the ovary may promote unwanted tissue growth, resulting in ovarian cancer development. A better understanding of programmed cell death in the ovary may help to develop novel therapies to treat females with ovarian disorders, such as premature ovarian failure and ovarian cancer. In this thesis, the function of several hormones on the process of apoptosis in the ovary is investigated. Furthermore, the expression of several apoptotic regulators has been investigated in a variety of ovarian cell types under physiological and pathological conditions.

To gain more insight in the proteins involved in ovarian apoptosis under normal physiological conditions, we have investigated the localization and distribution of the

Fas system and its related proteins in the ovary throughout the estrous cycle in the rat. The results of this study are described and discussed in chapter 2. In chapter 3 we examined the effect of loss of growth hormone (GH) signaling on follicular recruitment, development and atresia by using GH receptor null mice and investigated whether IGF-1 administration could antagonize the absence of GH actions. In chapter 4, the effect of hypothyroidism on follicular development and atresia was investigated in the rat ovary.

The ovarian surface epithelium, which covers the ovary, is the source of a frequent and often lethal form of cancer in females. To gain more insight in the proteins that determine the fate of OSE cells and their relation with ovarian cancer, we examined the expression of components of the Fas signaling pathway in relation to apoptosis in human OSE cells at the human ovarian surface, in inclusion cysts, borderline tumors and carcinomas (chapter 5). As described above, gonadotropins, including luteinizing hormone (LH), have been suggested to play an important role in the etiology of epithelial ovarian cancers. Therefore, we examined the effect of LH on the occurrence of Fas-induced apoptosis in human OSE cancer cell lines and determined whether signaling occurs through the activation of protein kinase A (PKA) and/or protein kinase C (PKC) (chapter 6).

In the summarizing discussion, the mechanisms by which hormones regulate apoptosis in the ovary are discussed. In addition, the potential means of manipulating apoptosis and its clinical relevance are discussed (chapter 7).

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Chapter 2

Estrous cycle dependent changes in expression and distribution of Fas, Fas ligand, Bcl-2, Bax and pro- and active caspase-3 in the rat ovary

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Abstract

We studied the expression of proteins involved in ovarian apoptosis throughout the estrous cycle in the presence of fluctuating hormone levels. For Fas, Fas ligand, Bcl-2, Bax and caspase-3, both mRNA and protein expression was detected in all ovarian tissue extracts, though the amount of protein varied with the phase of the estrous cycle. Fas, Bax and caspase-3 protein levels were highest at diestrus and decreased thereafter towards metestrus. In contrast, Fas ligand and Bcl-2 protein expression remained relatively constant. Immunohistochemistry revealed that the expression of the anti-apoptotic protein Bcl-2 was higher in healthy (pre)antral follicles than in atretic follicles. In contrast, the pro-apoptotic proteins Fas, Fas ligand, Bax and active caspase-3 were more predominantly present in atretic follicles. In the ovarian surface epithelium (OSE), Fas, procaspase-3 and Bcl-2 immunostaining appeared independent of the phase of the estrous cycle. Fas ligand and Bax staining was detected particularly during (pro)estrus in OSE cells surrounding the ovulatory follicles, while active caspase-3 was observed only in OSE cells at the ovulatory site. The proportion of luteal cells that stained positively for Fas, Bax, and caspase-3 increased with the age of the corpus luteum, while Fas ligand and Bcl-2 immunostaining was highest in newly formed corpora lutea and decreased thereafter. In conclusion, the components of the Fas signaling pathway were differentially expressed throughout the estrous cycle in a variety of ovarian cell types, which may correspond to hormone dependent survival mechanisms.

Introduction

Programmed cell death or apoptosis is an essential component of normal reproductive function and development in the ovary. Most importantly, apoptosis attributes to the exhaustion of the oocyte reserve, either directly through germ cell death or indirectly through follicular atresia (1). In this regard, apoptosis has been proposed to be the major mechanism that determines female reproductive life span (2). Indeed, recent studies have shown that inhibition of apoptosis by selectively disrupting Bax function in female mice was accompanied by an increased primordial follicle pool and a prolonged ovarian life span (3). Hence, a better understanding of programmed cell death in the ovary may help to develop novel therapies to treat females with ovarian disorders such as premature ovarian failure.

Throughout life, dormant primordial follicles are continuously recruited from the resting pool to start growing. Until reaching puberty, these follicles do not survive beyond the early antral stage. In (post)pubertal females, however, some early antral follicles may become selected and continue growth under the influence of elevated FSH levels during each estrous cycle (4). Among this cohort, depending on the species, usually one or a few large antral follicles will reach the preovulatory stage and ovulate following the LH surge, whereas the remaining antral follicles that have been recruited during this growth wave will degenerate as a result of the process of follicular atresia (1). Atresia, which involves apoptosis of granulosa cells, oocytes and eventually theca cells, can occur during any stage of follicular development, although early antral follicles are most vulnerable to undergo follicular degeneration (1,5). During each estrous cycle, other ovarian cell types in addition to follicular cells may also undergo apoptosis. The ovarian surface epithelial (OSE) cells that cover the protruding preovulatory follicles degenerate from the ovarian surface by apoptosis just prior to ovulation to facilitate oocyte release (6). Moreover, corpora luteal cells arising from the ovulatory follicles undergo apoptosis during luteal regression, when ovulation is not associated with a fertile mating and implantation of the conceptus (7,8).

The role of regulatory proteins and genes involved in apoptosis in the ovary is relatively well understood. However, the hormonal factors that trigger apoptosis in the various ovarian cells types during the estrous cycle are not yet clearly defined. The major players of apoptosis in the ovary are assumed to be the Fas system and the Bcl-2 family members (9-13). Recently, it has been suggested that interaction of the Fas system and Bcl-2 family members may define the rate of apoptosis (14). Fas receptor (Fas/CD95) is a member of the tumor necrosis factor/nerve growth factor family, which is activated upon binding Fas ligand, leading to receptor aggregation and formation of a death-inducing signaling complex (DISC) (14). Once Fas has been activated, two distinct intracellular apoptotic

pathways can be executed, depending on the level of released active caspase-8 (fig. 1)(15). In type I cells, high levels of caspase-8 at the DISC directly initiate cleavage of other downstream effector caspases, such as caspase-3, thereby initiating the execution phase of apoptosis. In type II cells, however, a small caspase-8 signal is generated which in order to induce apoptosis requires an amplification loop via the mitochondria involving cleavage of the pro-apoptotic Bcl-2 family member Bid (15). It has been proposed that cleaved Bid stimulates dimeric binding of other pro-apoptotic Bcl-2 family members (e.g. Bax and Bak) to the mitochondria at the expense of anti-apoptotic Bcl-2 family members (e.g. Bcl-2, Bcl-w, Bcl-x_L). This triggers cytochrome c release, which eventually results in downstream activation of effector caspases and cell death (14).

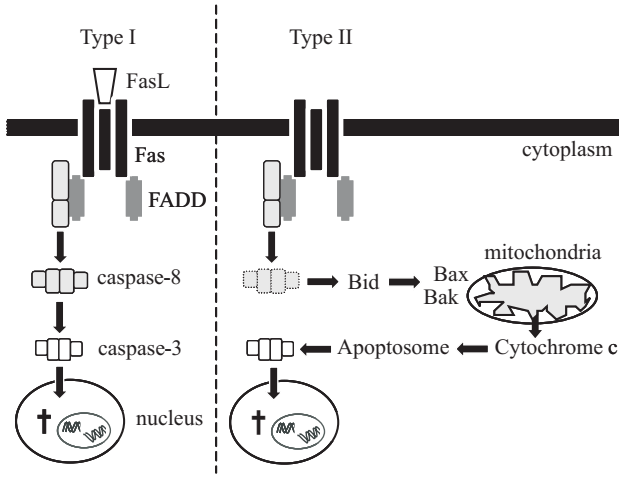

Figure 1. Schematic overview of Fas-induced apoptosis in type I and II cells. Fas is activated upon ligation to Fas ligand, resulting in receptor trimerization and the recruitment of adapter proteins. When procaspase-8 becomes associated, it is autoproteolytically cleaved into active subunits that are released into the cytoplasm. In type I cells, active caspase-8 might then act directly to cleave other effector caspases, such as caspase-3, thereby initiating the execution phase of apoptosis. In type II cells, however, active caspase-8 cleaves Bid, a proapoptotic member of the Bcl-2 family. Cleaved Bid stimulates the binding of proapaptotic Bcl-2 proteins (Bax, Bak) to the mitochondria and inhibits association of anti-apoptotic Bcl-2 proteins (e.g. Bcl-2, Bcl-w, Bcl-x_L). This causes leakage of cytochrome c from the mitochondria into the cytosol, which in turn promotes formation of the "apoptosome" and triggers caspase-3. Activation of caspase-3 is considered to be the final executioner of apoptosis responsible for cleavage of key substrates, such as DNA repair enzymes and cytoskeletal and nuclear scaffold proteins. Picture adapted from Scadiffi et al (15).

Several studies have demonstrated Fas and Fas ligand expression during follicular atresia (9-11,16-18) and luteal regression (8,13). However, controversy exists about the distribution of these proteins between follicular cells during follicular development. The differences between studies may in part be explained by the different experimental models for the induction of apoptosis. In these models apoptosis is often induced artificially, for instance by gonadotropin withdrawal or treatment with prostaglandin $F_{2\alpha}$. This type of induced degeneration may differ in key aspects from normal spontaneous atresia

or luteal regression. In fact, little is known regarding expression of either Fas or other components associated with Fas signaling under normal physiological conditions during the estrous cycle. Furthermore, the simultaneous assessment of relevant apoptotic proteins in the whole ovary throughout the estrous cycle has not been reported so far. We, therefore, investigated the localization and distribution of the Fas system and its related proteins, i.e. Fas, Fas ligand, Bcl-2, Bax, pro- and active caspase-3, during normal follicular development, atresia, ovulation and luteolysis in the rat throughout the estrous cycle. Furthermore, the corresponding mRNA and protein expression profiles in whole rat ovaries were determined.

Materials & Methods

Reagents

Antibodies against Fas receptor (sc-715), Fas ligand (sc-956), procaspase-3 (sc-1226), Bcl-2 (sc-1226), Bax (sc-526), and actin (sc-1616) were purchased from Santa Cruz Biotechnology (SanverTech, Heerhugowaard, the Netherlands). The antibody against active caspase-3 (AF835) that detects only cleaved caspase-3 was obtained from R&D systems (ITK Diagnostics, Uithoorn, The Netherlands), while the antibody against caspase-3 used for Western blot analysis, which detects both the caspase-3 precursor of 32 kDa and the cleaved active caspase-3 fragment of 20 kDa, was obtained from Cell Signaling Technology (Westburg BV, Leusden, The Netherlands). Secondary biotinylated goat anti-rabbit, rabbit-anti-goat or goat-anti-mouse IgG's were purchased from Vector Laboratories (Vectastain kit Elite, Vector Laboratories, Burlingame, CA, USA), while horseradish peroxidase-conjugated goat-anti-rabbit IgG was obtained from Nordic Immunological Laboratories (Tilburg, The Netherlands). TRIzol reagent, acetylated BSA and 3,3'-diaminobenzidine tetrachloride were purchased from Gibco BRL (Life technologies, The Netherlands), Aurion (Wageningen, The Netherlands) and Sigma (St. Louis, MO, USA), respectively. The ABC-peroxidase complex staining kit Elite was purchased from Vector Laboratories (Burlingame, CA, USA), the *in situ* cell death detection kit was bought from Roche Diagnostics GmbH (Mannheim, Germany), while the Coomassie® Plus protein assay reagent kit and supersignal chemiluminescent substrate kit (ECL) were bought from Pierce (Tattenhall, Cheshire, UK). The ImProm-II™ reverse transcription system kit, oligo dT primer, dNTPs and RQ1 Rnase-Free Dnase were obtained from Promega Corporation (Madison, USA).

Animals

Female outbred Wistar rats (Harlan, Horst, The Netherlands) were housed in pairs and kept in a temperature- and light-controlled room (LD 12:12) with free access to food and water. Estrous cycles were tracked by daily taking of vaginal smears, and only

the rats that showed two consecutive 4-day cycles were used for the experiments. The 16 to 19-week old animals were sacrificed at four different stages of the estrous cycle, i.e. diestrus (n=6), proestrus (n=6), estrus (n=6) and metestrus (n=5). The ovaries were carefully excised to avoid damage to the ovarian surface epithelium. After excision the ovaries were snap frozen in liquid nitrogen and stored at -80 C until use for RT-PCR and immunoblot analysis (n=8). For immunohistochemistry, the ovarian tissues were fixed for 24 hours in 4% buffered formalin, embedded in paraffin and stored at room temperature until immunohistochemical processing (n=23). The experiments described in the present study have been approved by the ethical committee for laboratory animal welfare of Wageningen University.

Reverse transcription-polymerase chain reaction

The TRIzol method was used to extract total RNA from snap-frozen rat ovaries according to the manufacturer's instructions. RNA integrity was confirmed by running 2 µg RNA on a 1% agarose denaturing gel. The amount of RNA was determined by spectophotometry at 260 nm. Prior to RT-PCR, RNA samples were treated with RQ1 RNase-Free DNase, then purified by phenol:chloroform extraction followed by precipitation with ethanol. RT-PCR reverse transcriptase was performed in a volume of 20 µl reaction mixture containing 1 µg total RNA, 0.5 µg oligo dT primer, 0.5 mM dNTPs, 5 mM $MgCl_2$, 4 µl ImProm-II™ 5x reaction buffer and 1 µl ImProm-II™ reverse transcriptase at 42 C for 60 min. Then, reverse transcriptase was inactivated by heating at 75 C for 15 min. The synthesized complementary DNA was stored at -80 C until use. The amplification was carried out in a volume of 50 µl reaction mixture prepared on ice which contained 5 µl 10x thermophilic reaction buffer, 1U Taq polymerase, 2 mM $MgCl_2$, 0.2 mM dNTP, PCR buffer, 0.5 µM upstream primer, 0.5 µM downstream primer and 1 µl reverse transcription mixture (cDNA). The PCR primers used for amplification were synthesized by Isogen Bioscience on the basis of the earlier described rat DNA sequences for Fas (19), Fas ligand (20), caspase-3 (21), GAPDH (22), Bcl-2 and Bax (23,24). The PCR conditions were 94 C for 5 min followed by 40 cycles of 94 C for 45 sec, 55 C for 60 sec, and 72 C for 45 sec, respectively and then 72 C for 5 min. After amplification, 10 µl of the PCR products were separated by electrophoresis on 1.5 % agarose gels containing 0.002% ethidium bromide, visualized by UV light and photographed. The loading of the samples was demonstrated by RT-PCR analysis of the housekeeping gene GAPDH. Negative controls (reactions without reverse transcriptase and reactions without template) were performed to verify the absence of (DNA) template contamination. No products were observed in these control reactions.

Western blot analysis

Protein samples were prepared from snap-frozen rat ovaries using TRIzol reagent according to the manufacturer's instructions. Protein concentrations were determined

by the Bradford assay with the Coomassie® Plus protein assay reagent kit and resolved in SDS-sample buffer (62.5mM Tris, 2% SDS, 10% glycerol, 1% β-mercaptoethanol and 0.003% Bromophenol Blue, pH6.8) in a final concentration of 500 µg/ml. After boiling the samples for 10 minutes, the proteins (10 µg/lane) were separated by 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in TTBS (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween-20) for 1 hour, and incubated overnight at 4 C with a rabbit anti-human polycloncal antibody against Fas and Fas ligand (diluted 1:500); caspase-3 (which reacts with both the 32 kDa pro-enzyme and the spliced 20 kDa active form of caspase-3), Bcl-2 and Bax (diluted 1:250), or with a goat anti-human polyclonal antibody against actin (diluted 1:1000) in blocking buffer. After washing three times with TTBS, the membranes were incubated for 1 hour with horseradish peroxidase-conjugated goat-anti-rabbit IgG (Fas, FasL, caspase-3, Bcl-2 and Bax) diluted 1:15000 or rabbit-anti goat (actin) diluted 1:5000 in blocking buffer. The presence of antibody-protein complexes was detected by enhanced chemiluminescence.

Immunohistochemistry

Immunohistochemistry was performed as has been described by Teerds et al (25). Briefly, 5 µm thick paraffin embedded ovarian sections were deparaffinized and treated with 1% H_2O_2 in methanol for 30 min to block endogenous peroxidase activity. The slides were subsequently washed in 0.01 M Tris-buffered saline (TBS pH 7.4), micro-waved in buffered citrate for 10 min (only in case of procaspase-3), incubated with 0.1 M glycine in TBS for 30 min, and rinsed with TBS. Sections were blocked for 30 min with 10% normal goat or rabbit serum, and incubated at 4 C overnight with rabbit anti-human polycloncal antibodies against Fas, Fas ligand, Bcl-2, Bax (all diluted 1:200), active caspase-3 (concentration 10 µg/ml), or with a goat anti-human polyclonal antibody against procaspase-3 (diluted 1:100), respectively. All antibodies were diluted in TBS containing 0.05% acetylated BSA. Slides were again washed in TBS and incubated for 60 min with the corresponding biotinylated goat anti-rabbit or rabbit anti-goat IgGs respectively, diluted 1:200 in TBS containing 0.05% acetylated BSA. Sections were subsequently washed in TBS and incubated for at least 60 min with the components avidin (A) and biotin (B) of the ABC staining kit Elite. Both components (A and B) were diluted 1:1000 and the solution was prepared at least 15 min before use. Slides were washed in TBS, rinsed in 0.05 M Tris-HCl (pH 7.5) and finally bound antibody was visualized after the addition of a 0.6 mg/ml solution of 3,3'-diaminobenzidine tetrachloride (DAB) in Tris-HCl to which 0.03% H_2O_2 was added. The slides were subsequently counterstained with Mayer's hematoxylin. Control sections, in which the primary antibody was replaced by either normal rabbit or goat serum, were similarly processed. No staining was observed in these controls (fig. 5G). For each primary antibody the intensity of immunostaining was determined by estimating the percentage

of positively labelled cells. The intenstity of immunostaining was assigned as being absent (-), faint (-/+), moderate (+) and high (++) when respectively <1, 1-10, 10-50, 50-100 percent of the cells in the follicular or corpus luteum cross section stained positively. All OSE cells covering the surface of the ovary were counted.

In situ DNA labeling for apoptosis

The laboratory protocol for TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) was used according to the manufacturer's instructions with some minor modifications adapted from Negoescu et al. (26). Briefly, paraffin embedded ovarian sections were deparaffinized, micro-waved in buffered citrate for 5 min, cooled rapidly and rinsed in PBS, pH 7.4. The slides were covered with the TUNEL reaction mixture (1:10 dilution of enzyme solution containing calf thymus terminal deoxynucleotidyl transferase and label solution containing a nucleotide mixture of digoxigenin-11-2'-dUTP) for 60 min at 37 C. Then, slides were blocked for 30 min with 5% non-fat dry milk and 10% normal horse serum in PBS. The slides were subsequently washed in PBS and incubated with a 1:4 dilution of peroxidase-labeled digoxigenin sheep Fab antibody for 30 min at 37 C. After washing the sections in PBS, the DNA strand breaks were visualized with a DAB color reaction, and counterstained with Mayer's hematoxylin, as described above. Negative controls, by omitting terminal deoxynucleotidyl transferase TdT, were similarly processed. No staining was observed in these negative controls (data not shown).

Results

Expression of the Fas system in whole rat ovaries throughout the estrous cycle

To examine the expression levels of Fas receptor and signaling molecules throughout the estrous cycle, we determined their expression in whole rat ovarian tissue extracts at diestrus, proestrus, estrus and metestrus. Fas, Fas ligand, caspase-3, Bcl-2 and Bax mRNA and protein were detected by RT-PCR and Western blot analysis respectively (fig. 2). As shown in figure 2A, mRNAs of all Fas signaling molecules studied were detected at all stages during the estrous cycle. The amplified PCR products were of the expected size (fig 2A), i.e. Fas (282 bp), Fas ligand (491 bp), caspase-3 (282 bp), Bcl-2 (349 bp), Bax (301 bp) and GAPDH (318 bp). The negative controls (cDNA synthesis performed without RT or without template) revealed no expression for any of the investigated target sequences (data not shown). Western blot analysis revealed variations in the amount of Fas (45 kDa), Fas ligand (40 kDa), caspase-3 (32 kDa), Bcl-2 (26 kDa) and Bax (22 kDa) protein during the estrous cycle (fig. 2B). Fas, caspase-3 and Bax protein levels were maximal at diestrus and decreased towards metestrus. In contrast, Fas ligand and Bcl-2 protein expression remained relatively constant.

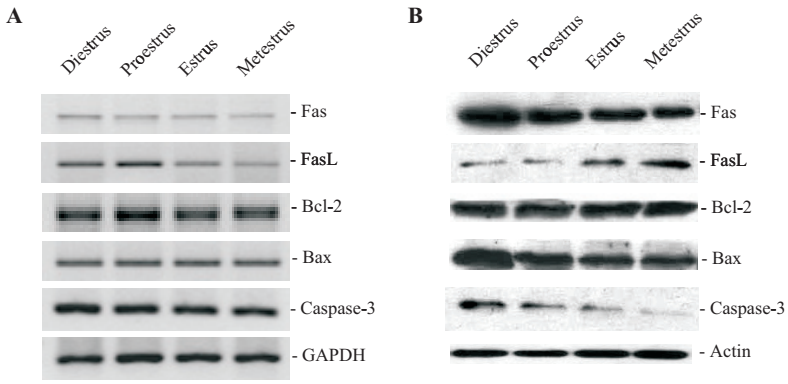

Figure 2. Expression of Fas, Fas ligand, Bcl-2, Bax and caspase-3 mRNA and protein in whole rat ovaries throughout the estrous cycle. Ovaries were obtained from rats sacrificed at four different stages of the estrous cycle and Fas, Fas ligand, Bcl-2, Bax and caspase-3 mRNA and protein expression were determined by RT-PCR (A) and Western blotting (B), as described in *Materials and Methods*. Positive (loading) controls represent a GAPDH mRNA amplification and an actin protein immunoblot staining. Representative figures from three independent experiments are shown.

Immunolocaliztion of the Fas system during follicular development

To study the cellular distribution of Fas receptor and signaling molecules, immunohistochemistry was performed on ovarian sections. The localization and the intensity of the immunostaining for various proteins of the Fas system in the ovary have been summarized in Table 1.

TABLE 1. Localization of various apoptotic proteins in the rat ovary. Immunostaining intensity; absent (-), faint (-/+), moderate (+) or high (++).

			Fas	*Fas ligand*	*Bcl-2*	*Bax*	*pro-caspase-3*	*active caspase-3*
Preantral follicles	healthy	granulosa	-	-	+	-/+	++	-
		theca	-/+	+	+	+	++	-
	atretic	granulosa	-/+	-/+	-/+	-/+	++	+
		theca	++	++	-/+	++	++	-/+
Antral follicles	healthy	granulosa	-/+	-	+	-/+	++	-
		theca	+	+	+	+	++	-
	atretic	granulosa	+	-/+	-/+	-/+	++	+
		theca	++	++	-/+	++	++	-/+
OSE	surface		+	-/+	+	-/+	++	-
	at postovulatory site		+	+	+	+	+	+
Corpora lutea	1st and 2nd generation		+	++	+	-/+	+	-/+
	3rd generation		++	-/+	-/+	++	++	++

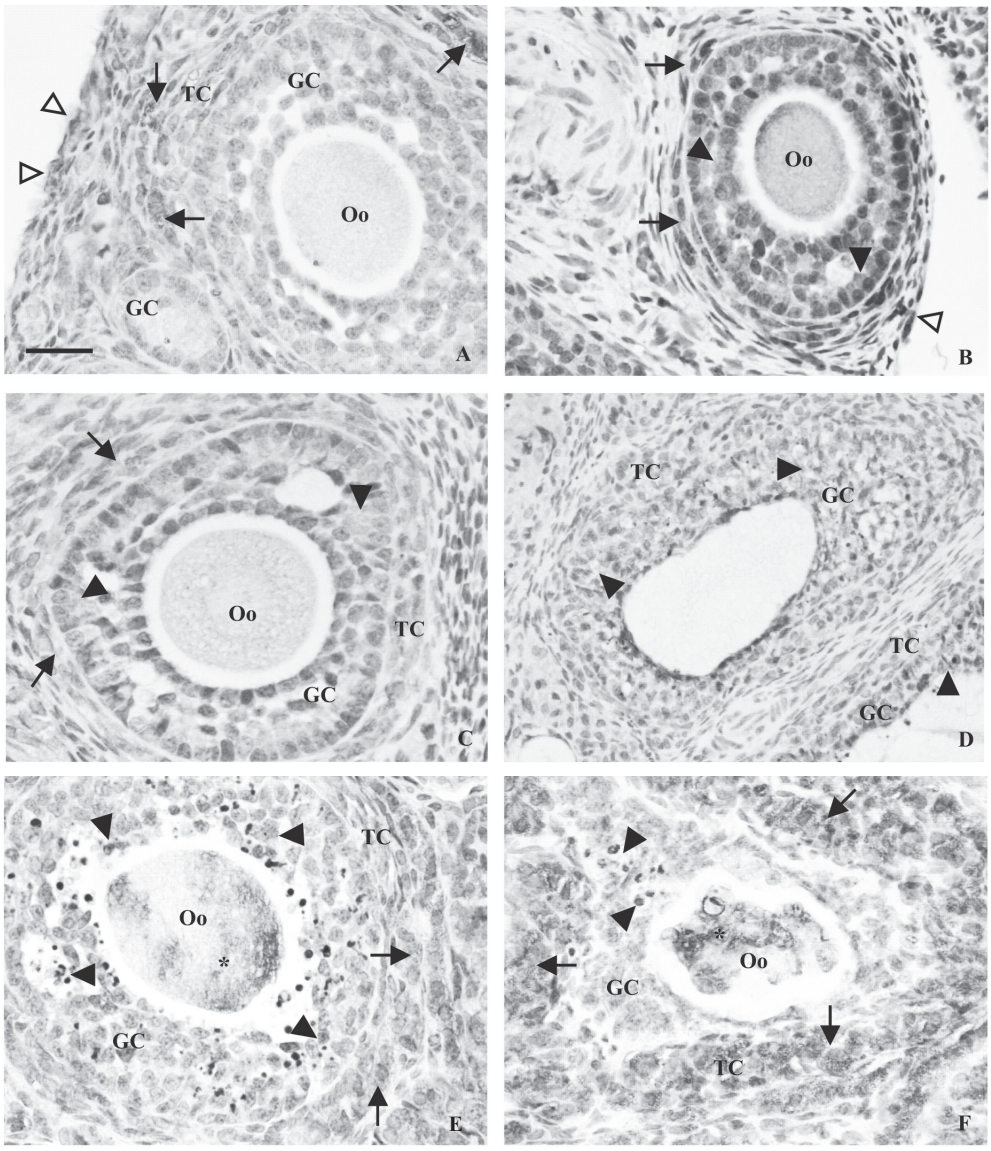

Figure 3. Immunohistochemical localization of various apoptotic proteins in preantral follicles of cycling rats. Positive staining is shown in brown and indicated by arrow(head)s. A-B) Healthy preantral follicles; Fas (A) immunostaining was found in theca cells but not in granulosa cells, whereas procaspase-3 (B) and Bcl-2 (C) were observed in both granulosa and theca cells. D-F) In atretic preantral follicles; less Bcl-2 immunopositive cells were observed (D), while Bax (E) and Fas ligand (F) immunostaining was now also present in some granulosa cells of both moderately (E) and severely (F) atretic follicles. The immunohistochemical labeling experiments were repeated at least three times with similar results. Theca cells (TC; arrows), granulosa cells (GC; arrowheads), oocytes (Oo and degenerating oocytes; asterisk), ovarian surface epithelium (open arrowheads). Bar = 20 µm (A-C,E,F) or 40 µm (D).

Differences in immunostaining patterns were observed in healthy follicles at various stages of follicular growth (fig. 3 and 4). In healthy follicles, immunostaining for Fas was detected in theca cells of secondary follicles onwards (fig. 3A), whereas faint and inconsistent Fas staining appeared in granulosa cells at the early antral stage of follicular development (data not shown). Fas ligand immunoreactivity was present in theca cells, but absent in granulosa cells of healthy follicles throughout most of follicular development (fig. 4A), though at proestrus some Fas ligand staining was observed in preovulatory follicles in the mural granulosa cells close to the basal membrane (fig. 4A, insert). Bax immunostaining was also predominantly found in theca cells. In granulosa cells, Bax immunoreactivity was faint and restricted to secondary and antral follicles (not shown). Bcl-2 (fig. 3C and 4B) and procaspase-3 (fig 3B) immunostaining was present in granulosa and theca cells of healthy follicles at all stages of development, though more frequently expressed in dominant preovulatory follicles compared to smaller antral follicle. The spliced active form of caspase-3 and TUNEL activity were never found in healthy follicles (not shown).

In atretic follicles, however, TUNEL labeling and immunoreactivity for active caspase-3 were often observed, predominantly in the apoptotic granulosa cells around the antral region but also in some scattered apoptotic theca cells (fig. 4E and 4F). The increase in active caspase-3 expression and TUNEL labeling, occurring after the onset of atresia, was accompanied by increased staining intensity for Fas and Fas ligand in granulosa cells (fig. 3F). Fas and Fas ligand staining was even more intense in theca cells of atretic follicles, which was accompanied by enhanced Bax staining (fig. 3E). Moreover, the immunostaining for Bax (in theca cells only), Fas and Fas ligand (in particular in theca cells, but also in granulosa cells), and active caspase-3 and TUNEL labeling (mainly in granulosa cells) appeared to be more intense and widespread in severely atretic follicles (fig. 3F and 4F) compared to moderately atretic follicles (fig. 3E). In contrast, the intensity of the anti-apoptotic protein Bcl-2 declined through the progressive stages of atresia in both granulosa and theca cells (fig. 3D). Immunostaining for procaspase-3 was uniform throughout follicular development (fig. 3B) and its relatively high level in both granulosa and theca cells was not influenced by advancing atresia (fig 4D). There were no differences in the distribution or intensity of immunostaining patterns of above studied apoptotic proteins, among atretic follicles at various stages of follicular development (fig 3D-F and fig. 4C-E).

Immunolocalization of the Fas system during ovulation

In ovarian surface epithelial (OSE) cells, Fas (fig. 3A and 5A), Bcl-2 (fig. 5C) and procaspase-3 (fig. 5E) immunostaining was generally observed throughout the entire estrous cycle (Table 1). In contrast, active caspase-3 immunopositive OSE cells were only observed during estrus in some OSE cells at the ovulatory site (fig. 5F).

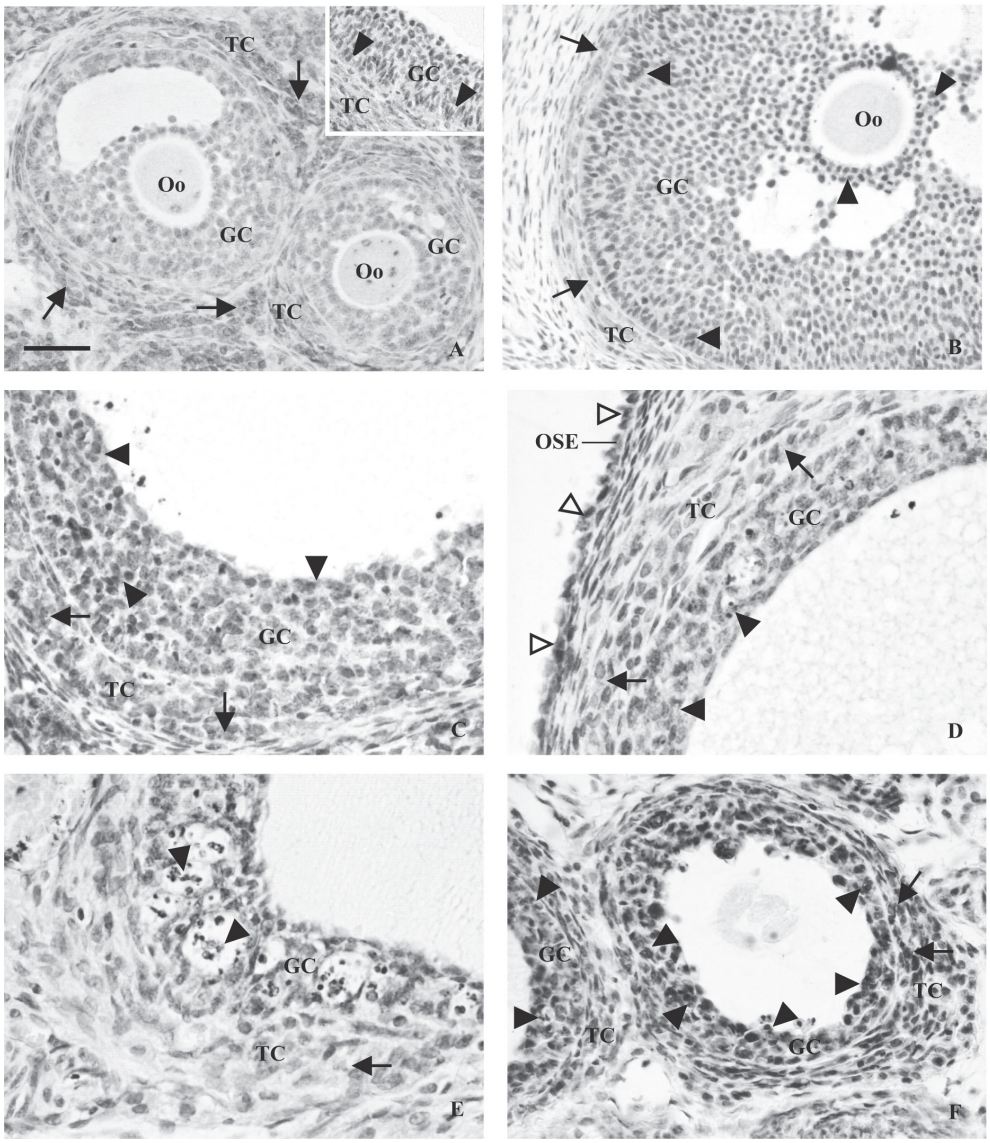

Figure 4. Immunohistochemical localization of various apoptotic proteins in antral follicles of cycling rats. Positive staining is shown in brown and indicated by arrow(head)s. A-B) Healthy antral follicles; Fas ligand (A) immunostaining was observed in theca cells only, though at proestrus some Fas ligand was observed in the mural granulosa cells of preovulatory follicles close to the basal membrane (insert), whereas Bcl-2 (B) was present in both granulosa and theca cells. C-F) Atretic antral follicles; Fas (C) and procaspase-3 (D) immunostaining are present in both granulosa and theca cells; staining for active caspase-3 (E) and TUNEL labeling (F) is often observed in granulosa with condensed nuclei but also in some apoptotic theca cells. The immunohistochemical labeling experiments were repeated at least three times with similar results. Theca cells (TC; arrows), granulosa cells (GC; arrowheads), oocytes (Oo), ovarian surface epithelium (OSE; open arrowheads). Bar = 20 μm (C-E) or 40 μm (A,B,F).

Immunoreactivity for Fas ligand (fig. 5B) and Bax (fig. 5D) was heterogeneous, but in general, more frequently observed in OSE cells that lined the ovulatory site during (pro)estrus compared to OSE lining other areas of the ovary.

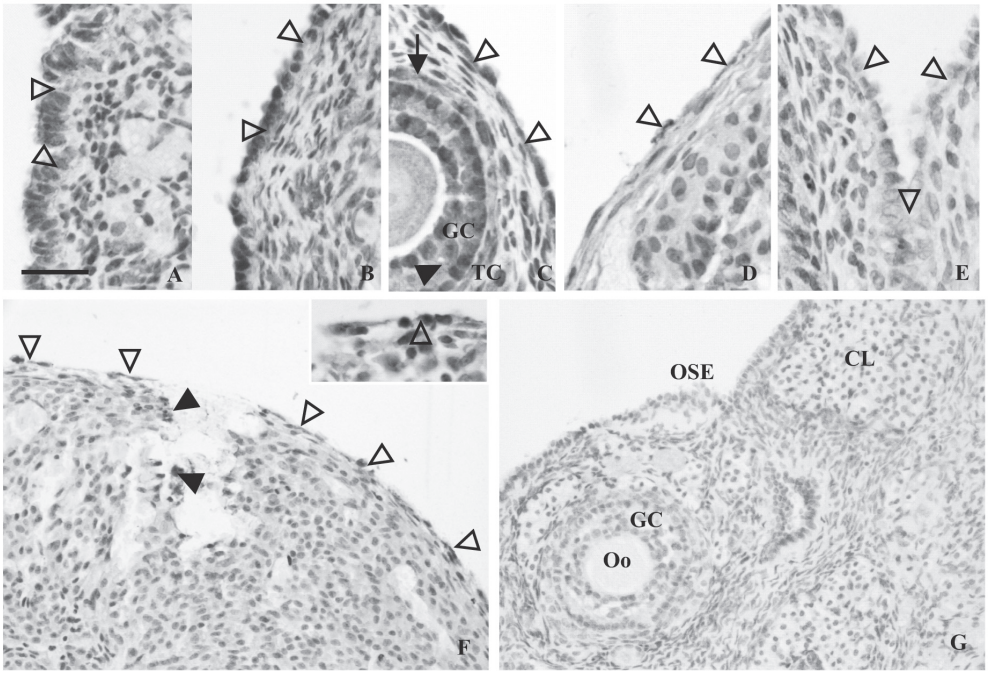

Figure 5. Immunohistochemical localization of various apoptotic proteins in ovarian surface epithelial cells (OSE) of cycling rats. Positive staining is shown in brown and indicated by arrow(head)s. A-E) On the ovarian surface; flat-to-columnar OSE cells show a clear positive immunostaining for Fas (A), Fas ligand (B), Bcl-2 (C) and procaspase-3 (E), while immunoreactivity for Bax (D) was mainly restricted to OSE cells at the ovulatory site. F) At the postovulatory site; some OSE cells have now become immunopositive for active caspase-3 (see also insert) as well. G) Control ovarian section incubated with normal rabbit serum instead of primary antibody. The immunohistochemical labeling experiments were repeated at least three times with similar results. Theca cells (TC; arrows), granulosa cells (GC; arrowheads), oocytes (Oo), ovarian surface epithelium (OSE; open arrowheads), corpora lutea (CL). Bar = 10 µm (A-E and insert of F), 40 µm (F) or 100 µm (G).

Immunolocaliztion of the Fas system during luteolysis

The age of the corpora lutea was identified on basis of their morphology. Corpora lutea derived from the most recent ovulation, which had a maximal size and consisted mainly of large luteal cells with a minor number of fibroblasts and apoptotic cells, were classified as new corpora lutea of the first generation. The regressing corpora lutea of the previous estrous cycles were assigned as corpora lutea of the second and third generation. Corpora lutea of the second and, in particular, the third generation were smaller in size and consisted not only of luteal cells, but also of a significant number of fibroblasts, leukocytes and apoptotic cells (27). Immunoreactivity for Fas, Fas ligand, Bcl-2 and Bax, pro- and active caspase-3 protein was always present in all corpora lutea, independent

of the stage of development. However, their immunostaining patterns changed during luteal development. In the new corpora lutea of the first generation, observed at metestrus, many luteal cells stained positively for both Fas ligand (fig. 6A) and Bcl-2, while in the corpora lutea of older generations, less luteal cells were positive for Bcl- 2 and Fas ligand. Additionally, we observed a change in histological localization of Fas ligand during luteal regression. In corpora lutea of the first generation, Fas ligand was predominantly localized in granulosa and theca lutein cells, whereas in the older corpora lutea, Fas ligand was mainly observed in the leukocytes and to a much lesser extent in the lutein cells. The number of luteal cells that showed Bax (fig. 6B), Fas (fig. 6C-D), pro- and active caspase-3 (fig. 6E) immunostaining increased with the age of the corpora lutea. Furthermore, apoptotic cells, i.e. cells with condensed and/or fragmented nuclei, were only occasionally observed in corpora lutea of the first and second generation while they were present in larger numbers in regressing corpora lutea of the third generation.

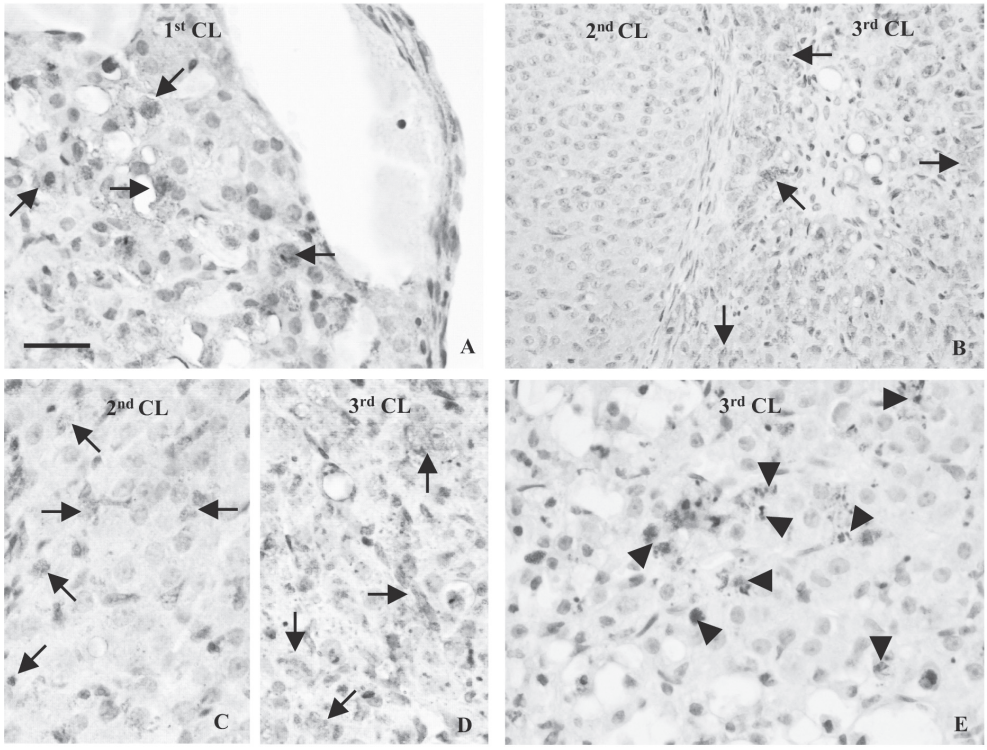

Figure 6. Immunohistochemical localization of various apoptotic proteins in corpora lutea (CL) of cycling rats. Positive staining is shown in brown and indicated by arrow(head)s. A) Early corpus luteum of the first generation (1st CL) at metestrus; clear immunostaining for Fas ligand is present. B-D) CL of different age; immunostaining for Bax (B) and Fas (C-D) was faint in the CL of the first and second generation (2nd CL) while high in CL of the third generation (3rd CL). E) CL of the third generation (3rd CL); active caspase-3 immunopositive cells often had condensed and fragmented nuclei (arrowheads) that are indicative for apoptosis. The immunohistochemical labeling experiments were repeated al least three times with similar results. Bar = 20 µm (A,C-E) or 40 µm (B).

Discussion

This is the first study in which the expression and localization of various components of the Fas apoptotic machinery has been examined by immunohistochemistry, Western blot analysis and RT-PCR throughout the estrous cycle in the rat ovary. Fas, Fas ligand, caspase-3, Bcl-2 and Bax mRNA and protein expression were detected in all ovarian tissue extracts, though the amount of protein varied with the phase of the estrous cycle. Fas, caspase-3 and Bax protein levels reached a maximum around diestrus and steadily decreased thereafter towards metestrus. In contrast, Fas ligand and Bcl-2 immunostaining remained stably expressed. We speculate that the reduced expression levels of the proapoptotic proteins Fas, Bax and caspase-3, and stable expression of anti-apoptotic Bcl-2 and Fas ligand in the rat ovary around the time of ovulation may reflect physiological survival mechanisms to ensure ovulation of the dominant follicles and subsequent corpus luteum formation. Indeed, immunohistochemistry revealed that Bcl-2 and Fas ligand were more frequently expressed in dominant preovulatory follicles and newly formed corpora lutea around estrus, when compared to older corpora lutea. In addition, the prominent presence of corpora lutea during late diestrus and the relatively high expression of the proapoptotic proteins, Fas, Bax and caspase-3, in corpora lutea of the third generation, may explain that their levels peaked around late diestrus.

In follicles, Fas ligand expression in granulosa cells was limited to some mural granulosa cells at proestrus, whereas this factor was predominantly localized in theca cells throughout most stages of follicular development. Theoretically, Fas ligand expressing theca cells can mediate apoptosis through binding to Fas receptor either on leukocytes thereby withstanding leukocyte attack (28), or to adjacent Fas-expressing theca cells. Concerning the latter, rat thecal/interstitial cells can undergo Fas-induced apoptosis *in vitro* (29). Nevertheless, we as well as some other groups (30) observed only a few apoptotic theca cells in atretic follicles, despite abundant Fas expression, suggesting that theca cells are moderately resistant to Fas-induced apoptosis. Currently, it is not known by which mechanism theca cells resist apoptosis *in vivo*. This is probably not due to the absence of the executioner apoptotic enzyme, caspase-3, because theca cells of both healthy and atretic follicles displayed intense positive immunostaining for procaspase-3. In atretic follicles, some theca cells (that showed morphological signs of apoptosis, e.g. fragmented nuclei) stained also positively for active caspase-3, which is in line with observations for humans (31), but not for mice (32). The induction of apoptosis in theca cells was confirmed by the presence of a limited number of TUNEL positive cells in atretic follicles. Possibly, an additional signal downstream of Fas activation is responsible for theca cell apoptosis and activation of caspase-3.

The majority of active caspase-3 staining was restricted to apoptotic granulosa cells in atretic follicles consistent with earlier observations (33). The number of TUNEL positive granulosa cells in atretic follicles was much higher compared to the theca cells, which is in agreement with the higher incidence of activated caspase-3 positive granulosa cells in these follicles. Furthermore, the low intensity of Fas staining in granulosa cells of healthy follicles was significantly increased in atretic follicles. Fas ligand expression and Bax staining, however, remained faint in granulosa cells of atretic follicles. The physiological significance of this puzzling observation is not known and emphasizes our limited understanding of the factors and mechanism that may cause apoptosis in granulosa cells in follicles at the onset of atresia. Nonetheless, as Fas ligand also exists in a soluble form that can move freely among cells, it is possible that a yet unknown atretic factor may trigger a switch from membrane-bound Fas ligand to the soluble form in theca cells, which in turn may diffuse to the granulosa cells in atretic follicles. At the same time, Fas expression is enhanced in granulosa cells of atretic follicles, and binding of soluble Fas ligand could trigger apoptosis in these cells. Other cellular control mechanisms, however, may also be involved. Although apoptosis in granulosa cells was not accompanied by clearly increased Bax levels, our observation that Bcl-2 protein staining was less intense in atretic follicles compared to healthy follicles, may suggest that apoptosis of granulosa cells depends on the balance between anti- and pro-apoptotic Bcl-2 family proteins.

In the ovary, the loss of secretion of crucial hormonal survival factors is thought to stimulate atresia. For example, the gonadotropins FSH and LH have been shown to inhibit apoptosis in cultured preovulatory follicles (34). The mechanisms by which gonadotropins ensure follicle survival are not yet clear. Possibly, suppression of the death receptor-signaling pathway may, in part, be responsible for survival. Treatment of preovulatory follicles with FSH and LH has been demonstrated to suppress Fas expression (35). Moreover, reduced FSH exposure leads to caspase-mediated apoptosis in granulosa cells at the early antral stage of follicular development, which was associated with increased levels of Fas and Fas ligand in the rat ovary (28). Both observations are confirmed by our immunohistochemical data showing that Fas and Fas ligand expression were faint to absent in granulosa cells of healthy antral follicles present during the follicular phase of the estrous cycle when gonadotrophins levels were high. Furthermore, moderate Fas staining was observed in granulosa cells of atretic follicles at the early antral stage, which was accompanied with respectively slightly higher Fas ligand and lower Bcl-2 staining intensity, while Bax staining intensity remained unchanged.

Important regulators of follicular survival include sex steroids and growth factors. In cultured preovulatory follicles, estrogens and the growth factors EGF, TGF-α, bFGF and IGF-I have been shown to inhibit apoptosis (34). Not much is known about the mechanism by which these factors inhibit apoptosis. It has been suggested that granulosa cell secreted estrogens may stimulate theca cells to express a variety of growth factors,

including TGF-α. TGF-α, in turn, may appear in granulosa cells of large healthy follicles (25), where it may inhibit apoptosis by up-regulating the expression of intracellular anti-apoptotic proteins like XIAP and FLIP (36). This assumption is supported further by the observation that TGF-α was not observed in granulosa cells of large atretic antral follicles (25). Besides regulating TGF-α secretion, estrogen has also been proposed to regulate Fas ligand expression; estrogen can enhance Fas ligand expression, a process that could be antagonized by the estrogen receptor inhibitor, tamoxifen (37).The present study shows that the expression of Fas ligand in ovarian tissue extracts peaked during estrogen dominance prior to ovulation. Immunohistochemical analysis revealed that Fas ligand and the anti-apoptotic protein Bcl-2 were more frequently expressed in dominant preovulatory follicles compared to smaller antral follicles.

As indicated above, the staining intensity of the anti-apoptotic protein Bcl-2 declined through the progressive stages of atresia in both granulosa and theca cells. In contrast, a higher degree of expression of pro-apoptotic proteins, i.e. Fas and Fas ligand (in particular in theca cells, but also in granulosa cells), Bax (only in theca cells) active caspase-3 and TUNEL labeling (mainly in granulosa cells) was observed in atretic follicles versus healthy follicles. Such labeling also appeared to be more intense and widespread with advancing atresia. The latter was accompanied by an increased number of, in particular, apoptotic granulosa cells, but also of theca cells, while no changes were observed among preantral, early antral or preovulatory atretic follicles. These findings suggest that the expression of Fas-related proteins is increased as an integral part of the apoptotic process and is not due to follicular growth, since a similar apoptotic executioner program occurs among all stages of follicular atresia. These observations also suggest that the Fas death receptor in granulosa cells of atretic follicles may act along with the Bcl-2 family proteins to elicit an apoptotic response, characteristic for type II cells. We can, however, not completely exclude, due to the low levels of Bax staining, the possibility that granulosa cells are type I cells (14). Theca cells are presumably type II cells, as Bax, Fas and Fas ligand expression altogether is enhanced in atretic follicles. On the other hand, theca cells do not undergo massive apoptosis, suggesting that a regulatory mechanism determines theca cell susceptibility for Fas signaling activation and cell death.

The localization of Fas and Fas ligand in this study is in agreement with observations in immature rats treated with equine chorion gonadotropin (eCG) (18,28) except that under these conditions Fas was moderately expressed in granulosa of healthy follicles (10,28) and Fas ligand was virtually absent in theca cells of atretic follicles (10). In our study the Bcl-2 family proteins, Bcl-2 and Bax, were found in both granulosa and theca cells throughout all stages of follicular development. In the primate and human ovary, Bcl-2 and Bax expression has been observed in granulosa cells as well, but not in theca cells (38,39). This suggest that there may be differences between species concerning

the presence and function of Bcl-2 family members in the ovary, and that one must be cautious in extrapolating the results obtained in rat to other species. On the other hand, differences between studies may also be explained by different experimental conditions. Indeed, we used a very sensitive enzyme-based antigen detection method to localize the expression patterns of the six different apoptotic proteins altogether in follicular cells under normal physiological conditions throughout follicular development.

Little is known about the role of the Fas system in OSE cells. OSE cells have been suggested to undergo cycles of proliferation and degeneration that are associated with the ovulatory process. In ewes, it has been demonstrated that prior to ovulation, OSE cells surrounding the follicles destined to rupture, are highly exposed to both inflammatory agents and reactive oxidants, a condition which is accompanied by increased apoptosis and enhanced expression of p53 (40,41). In contrast, OSE cells located along the margins of ruptured follicles survive and express anti-apoptotic Bcl-2, which probably functions to compensate the insult following ovulation (40). A novel observation in the present study is that Bcl-2, Fas and procaspase-3 are moderately expressed in OSE cells throughout the estrous cycle at locations where only few apoptotic cells are present. In addition, Fas ligand and Bax appeared to be more frequently expressed in OSE cells that lined the follicles destined to ovulate than in OSE cells covering the remaining part of the ovary. OSE cells expressing active caspase-3 were observed only at the ovulatory site. *In vitro* studies have shown that cultured mouse OSE cells undergo apoptosis in response to Fas activation when pretreated with IFNγ (42). Hence, we would like to hypothesize that activation of the Fas death receptor pathway is blocked in OSE cells, until ovulation. The appearance of Fas ligand and Bax in OSE cells that surround the preovulatory follicles prior to ovulation, may in turn elicit the execution of apoptosis, indicating that the Fas-induced signaling in apoptotic OSE cells is characteristic of type II cells (14). Based on our findings, we hypothesize the following model for OSE cell survival associated with ovulation. Proteolytic factors, released prior to ovulation, may facilitate the rupture of the follicular stigma and induce apoptosis in OSE cells surrounding the apex of the follicle. The LH surge-initiated rise in progesterone may attribute to apoptosis of these damaged OSE cells (40,41). Indeed, progesterone has been shown to enhance p53 expression in sheep OSE cells (41) and to induce *in vivo* (43) and *in vitro* OSE cell death via enhanced Fas/FasL signaling (44). On the other hand, the high levels of estrogens, released with the follicular fluid at the time of ovulation, may play a role in the survival of undamaged OSE cells next to the rupture site (41). High concentrations of estrogens increase proliferation and suppress apoptosis in OSE cells *in vitro* (41,45).

In copora lutea, the immunostaining intensity of various components of the Fas signalling pathway, i.e. Fas, Bax, pro- and active caspase-3, increased in luteal cells as the corpora lutea advanced with age, which is in line with previous observations in various species

(8,11,13,46). All these observations strongly suggest that the Fas death receptor acts in concert with a balance of pro- and anti-apoptotic Bcl-2 family members in luteal cells, and therefore these cells are presumably type II cells. On the other hand, in humans, Bcl-2 and Bax have been reported to be constantly expressed throughout the luteal phase (47,48). However, comparisons may be difficult to interpret as in humans, in contrast to rats, single ovulations take place alternating from one to the other ovary (49). It has been well established that the Fas system may control structural involution of the corpora lutea. The incidence of luteal cell death was markedly delayed in mice lacking functional Fas, Fas ligand (8), Bax (3) or caspase-3 (50), whereas *in vitro* Fas ligand or agonistic-Fas antibodies induced luteal cell death (51,52). Fas signaling proteins may individually or together contribute to luteal cell death. However, the physiological trigger for this extrinsic apoptotic pathway remains to be identified.

Many endocrine glands involute after removal of trophic hormone support and/or by activation of negative stimuli that promote apoptosis. *In vitro* studies have demonstrated that luteothropic factors, like progesterone and human chorionic gonadotropin (hCG), suppressed apoptosis in luteal cells by lowering Fas, Fas ligand, Bax and/or p53 expression (46,53). It has been proposed that when luteal cells loose their ability to secrete progesterone, the extrinsic apoptotic pathway blockade is released, resulting in increased expression of Fas on their surface. The finding that the degree of luteal cell death was highest in regressing corpora lutea during the follicular phase when progesterone levels were low, supports this hypothesis (54). Our observation that the massive luteal cell death in regressing corpora lutea of especially the third generation at proestrus was preceded by an enhanced expression of various components of Fas signaling pathway, such as Bax, on late diestrus, provides additional *in vivo* support for this hypothesis.

Increased levels of luteolytic factors, such as prolactin (55) and $PGF_{2\alpha}$, have been shown to stimulate infiltration of Fas ligand expressing leukocytes into the regressing corpora lutea, which in turn could induce apoptosis in Fas-expressing luteal cells (56). Consistent with this assumption, we observed Fas ligand expression in infiltrating leukocytes in the regressing corpora lutea of the third generation. Additionally, we found a change in histological localization of Fas ligand during luteal regression, which has not been reported before. In newly formed corpora lutea, Fas ligand was predominantly localized in granulosa and theca lutein cells, while in the older corpora lutea of the third generation, expression was diminished in these cell types and switched to leukocytes. We speculate that the appearance of Fas ligand in luteinized cells in newly formed corpora lutea may function to protect these cells against programmed cell death by resisting the leukocyte invasion that accompanies ovulation (56).

In conclusion, apoptosis in the reproductive tissues of both males and females is thought to occur after withdrawal of crucial hormonal support. Unlike the prostate and testis, however, apoptosis in the ovary and the uterus is linked to the estrous cycle driven variations in hormonal secretions (57). Such fluctuations in hormone levels may possibly regulate the expression levels of components of the Fas apoptotic pathway in the ovary throughout the estrous cycle. In fact, the reduced expression levels of the proapoptotic proteins Fas, Bax and caspase-3, and the stable expression of anti-apoptotic Bcl-2 and Fas ligand in the rat ovary around the time of ovulation may reflect physiological survival mechanisms to ensure ovulation of the dominant follicles, corpus luteum formation and OSE cell repair. Hence, apoptosis of the various ovarian cell types seems to depend on hormonal support as well as on the presence of the Fas system and Bcl-2 family members.

Acknowledgments

We would like to thank Dr. D. Duijsings and E. Verbraak (Department of Biochemistry & Cell Biology, Utrecht University) for their valuable comments in the PCR experimentation, and Prof. W. Stoorvogel and Prof. W. den Otter (Department of Biochemistry & Cell Biology, Utrecht University) for reviewing this paper.

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Chapter 3

Reduced recruitment and survival of primordial and growing follicles in growth hormone receptor deficient mice

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Abstract

Growth hormone (GH) influences female fertility. The goal of the present study is to obtain more insight in the effect of loss of GH signaling, as observed in humans suffering from Laron syndrome, on ovarian function. Therefore, ovaries of untreated and IGF-I treated female GH receptor knock out (GHR/GHBP-KO) mice were examined to determine the follicular reserve and percentage of follicular atresia. Our observations demonstrate that the amount of primordial follicles was significantly elevated in GHR/GHBP-KO mice, while the numbers of primary, preantral and antral follicles were lower compared to wild-type values. The reduced number of healthy growing follicles in GHR/GHBP-KO mice was accompanied by a significant increase in the percentage of atretic follicles. IGF-I treatment of GHR/GHBP-KO mice for 14 days resulted in a reduced number of primordial follicles, an increased number of healthy antral follicles, and a decreased percentage of atretic follicles. The results of the present study suggest that GH has, besides its facilitatory role in follicular selection and atresia, an important role either directly or indirectly via IGF-I, in the recruitment of primordial follicles into the growing pool.

Introduction

In the ovary, a fixed number of dormant primordial follicles is endowed during early life. From the resting pool, follicles are recruited to grow and depending on the species, only 1 to 10 % of these growing follicles reach the preovulatory stage and ovulate during female reproductive life. The remaining follicles degenerate as a result of follicular atresia, a process with apoptosis as the underlying mechanism (1). The fate of follicles is controlled by peptide and steroid hormones as well as intraovarian growth factors. Gonadotropins are the most important survival factors of follicular development around and beyond the antral stage. Preantral follicular development has generally been considered to be largely gonadotropin independent; the mechanisms that control the initiation of primordial follicle growth and the subsequent exhaustion of the dormant primordial follicle pool at the end of the fertile life, are still unclear (2). Metabolic hormones, such as growth hormone (GH) have, however, been suggested to play a role (3).

Until now, it is unclear whether GH acts directly on the ovary or via an indirect endocrine route by stimulating the release of insulin-like growth factor I (IGF-I) by the liver (4). Both GH and IGF-I affect numerous processes associated with ovarian function, like gonadotropin release, ovarian steroidgenesis, as well as follicular growth, development and atresia (5-11). GH receptors, IGF-I receptors and IGF-I are widely expressed in the ovary together with receptor derived soluble binding proteins, GHBP and IGFBPs, that regulate the bioavailability and action of GH and IGF-I, respectively (3,12,13). Animal studies have shown that IGF-I is absolutely required for reproduction; female IGF-I deficient mice are sterile as they fail to ovulate (14). In contrast, female GH receptor null mice (GHR/GHBP-KO mice) are fertile, though their litter size is significantly reduced (15,16). In addition, women suffering from GH resistance or insensitivity (the so-called Laron syndrome) commonly require assisted reproductive treatment to induce ovulation, suggesting deficits in reproductive function (3). Indeed, ovarian dysfunction in some women has been associated with partial GH-deficiency, as GH replacement therapy has been demonstrated to improve fertility in these infertile women (3).

GHR/GHBP knockout (KO) mice have characteristics mimicking the Laron phenotype in humans (17,18) and display severe postnatal growth retardation, elevated GH and reduced IGF-I, IGFBP-3 and estradiol levels in serum (8,15,18). As indicated above, most female GHR/GHBP-KO mice are fertile, but puberty is delayed and the estrous cycle is often irregular and/or prolonged. The number of preovulatory follicles and corpora lutea, as well as the ovulation and embryo implantation rate are significantly reduced, resulting in smaller litter sizes compared to wild-type mice (15,16). Early follicular development in these GHR/GHBP-KO mice has, however, not yet been studied in detail. Moreover, it is unclear whether the effect of the absence of GH signaling is directly or indirectly a

result of reduced IGF-I signaling. Therefore, we investigated whether supplementation with IGF-I could antagonize the effects caused by deficient GH signaling on follicular development and atresia in the kock out mice.

Materials & Methods

Animals

GHR/GHBP-KO mice were produced as described previously (18) and were subsequently established on a pure Sv129Ola background. Adult GHR/GHBP-KO females were mated with either GHR/GHBP-KO or wild-type males. Mice were housed in a room with a photoperiod of 12 h of light/d (lights on from 0700–1900 h) and a temperature of 20 C. Mice were given free access to a nutritionally balanced diet and tap water. GHR/GHBP-KO mice were treated with recombinant human IGF-I (Genentech, Inc., South San Francisco, CA) by using micropumps (Alzet, Palo Alto, CA) releasing 6 mg/kg/d for 14 d. Micropumps were inserted dorsally at 7 wk of age (15). Three groups of 9-week-old mice, i.e. wild-type mice (n=5), GHR/GHBP-KO mice (n=8) and GHR/GHBP-KO mice treated with IGF-I for 14 days (n=4), were sacrificed and the ovaries were excised. All experimental designs and procedures were in agreement with the guidelines of the animal ethics committee of the French Ministère de l'Agriculture.

Histological evaluation of follicle numbers

The ovaries were fixed in Bouins fluid for 24 h, embedded in paraffin and serially sectioned at a thickness of 7 µm. A stratified sample consisting of every fifth section was mounted on glass stained with Periodic Acid Schiff (PAS) and Mayers haematoxylin. Sections were examined by light microscopy in order to determine the total number of healthy primordial, primary, preantral, and antral follicles per ovary according to the method of Flaws et al. (19). The selected sections from at least one ovary (29-39 sections per ovary) were randomized and the number of healthy follicles was counted in each of these sections. Follicles were identified as healthy when they contained an intact oocyte, organized granulosa and theca cell layers, and less than 5% of the granulosa cells showed signs of apoptosis. Only follicles with an oocyte containing a visible nucleus with a nucleolus were counted to avoid double counting of the same follicle. Follicles were scored as primordial when they contained an intact oocyte with a healthy nucleus surrounded by a single layer of flattened pregranulosa cells. Follicles were classified as primary when they consisted of an intact, enlarged oocyte with a healthy nucleus and a single layer of cuboidal granulosa cells. Follicles were scored as preantral if they contained an oocyte with a healthy nucleus, more than one layer of granulosa cells and a developing theca layer. Follicles were scored as antral if they contained a healthy oocyte, a minimum of two antral spaces of which the diameter had at least the size of the oocyte. To obtain

an estimate of the total number of follicles per ovary, the number of primordial, primary, preantral and antral follicles present in the marked sections was multiplied by five to account for the fact that every fifth section was used in the analysis (19).

Histological evaluation of atresia

The percentage of atretic follicles per ovary was determined according to the method of Dijkstra et al. (20). Based on morphological criteria, follicles were classified as healthy or atretic as described previously (21). Healthy follicles showed frequent mitosis of granulosa cells while the percentage of apoptotic granulosa cells was less than 5%. In atretic preantral follicles, the oocyte had degenerated and was surrounded by either a disorganized granulosa layer with signs of apoptosis or a few granulosa cells, and/or a hypertrophied theca layer. In atretic antral follicles the oocyte was usually intact, whereas the layer of granulosa cells contained more than 5% of apoptotic cells and as atresia proceeded, the granulosa cells were lost. The theca layer showed signs of hypertrophy (22). In three sections of the ovary (at a quarter, half and three-quarters of the ovary), all preantral and antral healthy and atretic follicles were counted. Because the counted numbers reflected only part of the total follicle population in an ovary, the mean percentage of non-atretic and atretic follicles was calculated in each group of mice and analyzed statistically. Primordial and primary follicles were often arranged in small or large clusters. The number of follicles in these clusters varied considerably. Therefore, primordial and primary follicles were excluded from these calculations. Atresia of these follicles was determined separately. The primordial and primary healthy and apoptotic-like follicles were counted in all sections. Primordial and primary follicles that showed signs of atresia were identified by the presence of a reduced oocyte size, condensation of the nuclear chromatin (41) and sometimes extensive PAS staining of the cytoplasm of the oocyte.

Statistical analysis

Statistics were performed by a one-way analysis of variance (ANOVA), unless otherwise mentioned. Differences between group variances were determined by Tukey's multiply comparison test. Values were considered to be statistically significant when $P<0.05$

Results

Effect of growth hormone receptor deficiency on follicle development

To obtain more insight into the physiological role of GH on ovarian function, the effect of absence of GH signaling on follicular development was tested in GHR/GHBP-KO mice by determining the number of healthy and atretic follicles in the ovaries. Moreover, we investigated whether IGF-I supplementation could antagonize the effects caused by deficient GH signaling on follicular development and atresia.

The pool of resting follicles from 9-week old control ovaries of wild type mice contained approximately 2600 primordial follicles per ovary (fig. 1A). In contrast, ovaries of GHR/GHBP-KO females contained approximately 42% more primordial follicles per ovary compared to wild type mice (P<0.01). Treatment of GHR/GHBP-KO mice with IGF-I for 14 days resulted in a significant reduction in the number of primordial follicles per ovary (P<0.01) to levels similar as observed in wild type mice (fig. 1A).
The number of growing follicles per ovary in GHR/GHBP-KO mice also differed significantly from wild type mice (fig. 1A). The percentages of primary, preantral and antral follicles were reduced by 35, 52 and 84%, respectively in GHR/GHBP-KO mice compared to wild type mice (P<0.05). IGF-1 treatment of GHR/GHBP-KO mice for 14 days resulted in an increase in the number of antral follicles to levels similar as observed in wild-type mice (P<0.05). The number of primary and preantral follicles per ovary, however, remained low upon IGF-treatment. Remarkably, the total follicle count per ovary was 20% lower in IGF-I treated GHR/GHBP-KO mice compared to untreated GHR/GHBP-KO and wild type mice (fig. 1B).

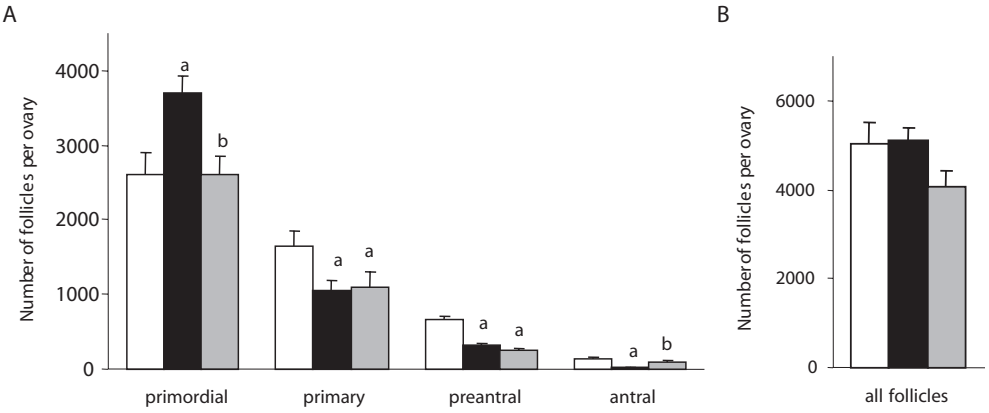

Figure 1. Effect of IGF-I on follicle number per ovary in GHR/GHBP-KO mice. A. The total numbers of primordial, primary, preantral and antral follicles per ovary in wild-type (open bars; n=5), GHR/GHBP-KO (filled bars; n=8) and GHR/GHBP-KO mice treated with IGF-I (gray bars; n=4) were determined using the criteria as described in *Materials and Methods*. B. The sum of the total number of follicles per ovary. Values represent mean ± SEM. [a] Significantly different from wild-type mice. [b] Significantly different from GHR-KO mice (P<0.05).

Effect of growth hormone receptor deficiency on follicular atresia

To investigate whether deficient GH signaling affects follicular atresia, we determined the percentage of atretic follicles in the ovaries, which were identified as described in the *Materials and Methods* section. Representative examples are depicted in Figure 2.
The reduced number of healthy growing follicles in GHR/GHBP-KO mice (fig. 1A) was accompanied by a significant increase in the percentage of atretic follicles from the primary stage onwards compared to wild type mice (fig. 3A-B, P<0.01). A considerable

number of follicles in GHR/GHBP-KO mice contained two or more oocytes (fig. 4A-C), which was almost never observed in wild type mice. These multi-oocyte follicles, which have been demonstrated in other knockout models as well, were identified at the primary, preantral and early antral stage of development but not in large antral follicles.

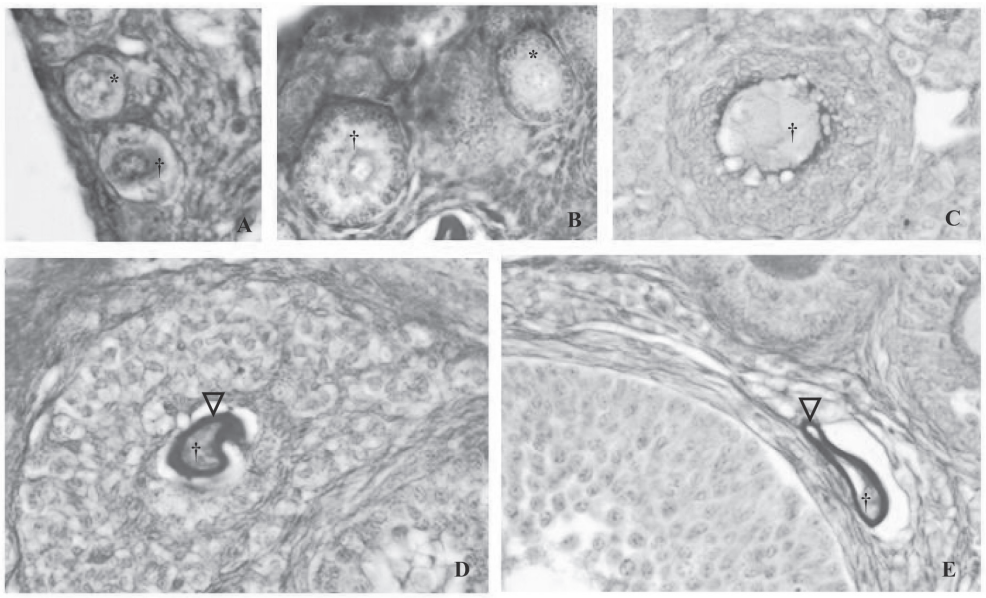

Figure 2. Representative photomicrographs of atretic follicles in ovaries of wild-type and/or GHR/BP-KO mice. Atretic follicles were defined as desribed in *Materials and Methods*. A. Atretic (crucifix) and healthy (asterisk) primordial follicle. B. Atretic (crucifix) and healthy (asterisk) primary follicle. C. Early atretic preantral follicle displaying a degenerating oocyte (crucifix). D. Moderate atretic (pre)antral follicle (crucifix) in which granulosa cells have disappeared, while the zona pellucida (arrowhead) is still present. E. Late atretic (pre)antral follicle (crucifix), in which most theca cells have degenerated, while the zona pellucida (arrowhead) is still present. Magnification 40x.

In IGF-1 treated GHR/GHBP-KO mice, the increased number of healthy antral growing follicles (fig. 1A) was accompanied by a significant decrease in the percentage of atretic follicles from the preantral stage onwards compared to untreated GHR/GHBP-KO mice (fig. 3B, $P<0.05$), suggesting increased follicular survival.

Our data suggest that a growth hormone receptor deficiency in mice is accompanied by reduced follicular recruitment and survival, of which the latter is probably the result of both decreased growth and increased atresia of follicles from the primary stage onwards.

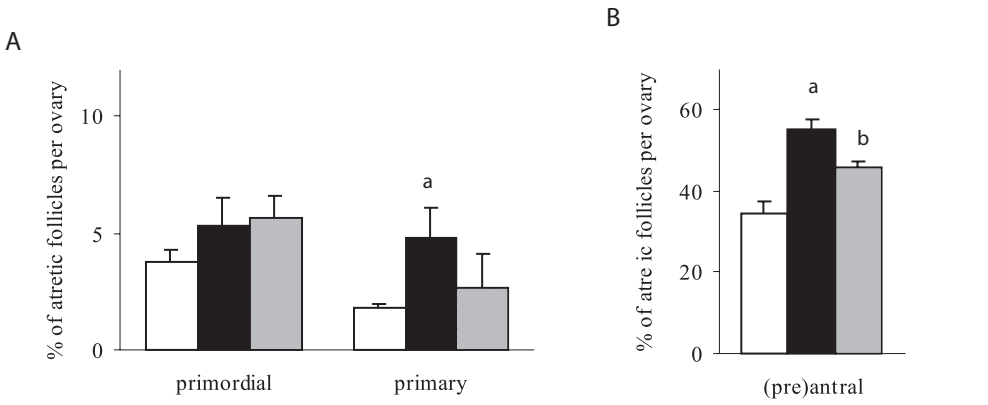

Figure 3. Effect of IGF-I on the percentage of atretic follicles per ovary in wild-type (open bars; n=5), GHR/GHBP-KO (filled bars; n=8), and GHR/GHBP-KO mice treated with IGF-I (gray bars; n=4). A. Percentage of apoptotic primordial and primary follicles. B. Percentage of (pre)antral atretic follicles per ovary in wild-type. The percentages of atresia were estimated as described in *Materials and Methods*. Values represent mean ± SEM. A student's T-test was used to compare the mean percentage of atresia. [a] Significantly different from wild-type mice. [b] Significantly different from GHR-KO mice ($P<0.05$).

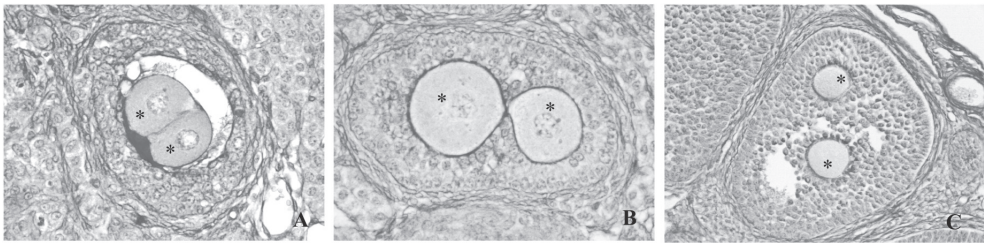

Figure 4. Abnormal follicles in GHR/GHBP-KO mice. A-B. Preantral follicles with two oocytes (asterisks). Magnification 40x. C. Early antral follicles follicle with 2 oocytes. Magnification 20x..

Discussion

The results of this study indicate that adult GHR/GHBP-KO mice contained higher numbers of primordial follicles, lower numbers of healthy growing primary, preantral and antral follicles and had an increased percentage of atretic follicles compared to wild-type animals. Our results extend data from an earlier report on GHR/GHBP-KO mice that showed a markedly reduced number of healthy growing antral follicles (15,16). The present study is the first to show that GH is important for the recruitment of primordial follicles into the growing pool.

The reduction in the primordial follicle pool in GHR/GHBP-KO after postnatal IGF-I treatment suggests that GH may indirectly, via IGF-I, play a role in the recruitment of

primordial follicle pool into the growing pool. The reduced numbers of growing follicles in GHR/GHBP-KO mice seems to be in agreement with data from IGF-I null mice (14), though serial section follicular counts in IGF-I null mice are required to establish whether primordial and primary follicular counts are indeed similar in IGF-I and GHR/GHBP deficient mice. *In vitro* studies using a rat ovarian culture system, on the other hand, have shown that IGF-I was unable to affect recruitment of primordial follicles (23). This suggests that there may be differences between mice and rats concerning the intraovarian IGF-I system (12). On the other hand, the difference between these studies may also be explained by different experimental conditions. It is possible that the actions of GH and IGF-I *in vivo* may be through (in)direct stimulation of other regulatory factors, such as insulin, that are involved in primordial follicle recruitment, which are absent under culture conditions (23). Insulin levels are greatly reduced in GHR/GHBP-KO animals, making insulin a potential candidate responsible for the reduced primordial to primary follicle transition in ovaries of GHR/GHBP-KO females (24). In addition, IGF-I binds, though with low affinity, to the insulin receptor (25). The micropumps in the IGF-I treated GHR/GHBP-KO mice released large amounts of IGF-I on a daily basis. The observation that smaller numbers of primordial follicles were observed upon IGF-I treatment in GHR/GHBP-KO mice, may therefore be due to high levels of IGF-I that act on the insulin receptor, resulting in follicular recruitment. Nevertheless, the reduced number of primordial follicles in IGF-I treated mature GHR/GHBP-KO mice was not accompanied by increased primary and preantral healthy follicle numbers, nor by increased degeneration of primordial and primary follicles. Due to the fact that we have only investigated the effects of IGF-I 14 days after the initiation of treatment, we can not exclude that loss of primordial and/or primary follicles from the stockpile occurred at an earlier stage of IGF-I treatment. The manifestations of apoptosis in primordial and primary follicles are thought to be of short duration and therefore difficult to detect histologically (26). It is possible that the continuous high levels of IGF-I in our experimental setup may have inhibited further stimulatory effects by IGF-I, due to, for instance, receptor down regulation or feedback mechanisms. Of interest is to investigate whether there exists an interaction between GH/IGF-I and other factors that affect primordial follicle growth, like stem cell factor (SCF), GDF9, kit ligand (KL), basic fibroblast growth factor (bFGF), nerve growth factor (NGF), insulin (23,27,28) and anti-Müllerian hormone (AMH) (29). Hence, further experiments with IGF-I treated wild type mice are required to elucidate whether IGF-I treatment has detrimental effects on the primordial/primary follicle pool.

Besides the role of GH in the recruitment of follicles, our data also suggest that GH is important for both preantral and antral follicular survival. Deficient GH signaling in mice resulted in a reduced number of healthy growing follicles and an increase in the percentage of atretic follicles from the primary stage onwards. *In vitro* experiments have suggested that the effect of GH on preantral follicular growth in immature mice

is independent of IGF-I (30-32). Our results are in agreement with these data, since IGF-treatment did not affect preantral follicular growth in GHR/GHBP-KO mice. The mechanism by which GH regulates growth in ovarian follicles is yet not exactly known. *In vitro* studies have shown that the stimulatory effect of GH on preantral follicular growth could be antagonized by follistatin (32). In addition, follistatin binds and inactivates activin, a potent stimulator of preantral follicle growth in vitro (32). *In vivo*, GH administration increased the number of small preantral follicles in cattle (33,34) and horses (35). Moreover, GH-binding activity was highest in granulosa cells of preantral follicles compared to large antral follicles in porcine and piscine ovaries (36,37), suggesting that GH is important in preantral follicular growth, possibly through increasing ovarian activin production (32), and not through the action of IGF-I.

Our observations on follicular development beyond the antral stage in GHR/GHBP-KO mice are in line with earlier data obtained in GHR/GHBP-KO mice, which showed a markedly reduced number of healthy growing follicles (15,16) and an increased percentage of atresia in follicles from 200 µm (antral stage) onwards (15). That atresia was not increased in another study (16) might be due to the underestimation of atresia. These authors used TUNEL labeling of granulosa cells as a marker of follicular atresia. However, in advanced preantral and antral atretic follicles granulosa cells have disappeared while hypertrophied theca cells remain present, the latter displaying a limited number of TUNEL positive cells (chapter 2), and therefore TUNEL labeling may not be detected (38). Moreover, in GH overexpressing transgenic mice the incidence of apoptosis in preovulatory follicles was significantly reduced (9,32). Since treatment with IGF-I also suppresses apoptotic DNA fragmentation in preovulatory follicles, this stimulatory effect of GH may reflect indirect actions mediated through the local production of IGF-I. Indeed, we observed increased follicular development accompanied by reduced atresia beyond the preantral stage in ovaries of GHR/GHBP-KO mice upon IGF-I treatment. It is possible that GH, indirectly trough IGF-I enhances FSH responsiveness by augmenting FSH receptor expression (11), thereby allowing antral follicles to escape atresia. GH may also enhance follicular survival and cell proliferation by potentiating LH action, since GH deficiency is associated with decreased LH receptor gene expression and LH responsiveness in rats (32,39). Exogenous GH *in vivo* corrects both effects (40), and increases the number of large follicles in GH-deficient dwarf rats (9).

In conclusion, the ovaries of adult GHR/GHBP-KO female mice contained more primordial follicles compared to wild type females, suggesting that GH affects the size of the primordial follicle pool. The reduction in the primordial follicle pool in GHR/GHBP-KO after postnatal IGF-I treatment suggests that GH may indirectly, possibly via IGF-I, play a role in the recruitment of primordial follicle pool.

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Chapter 4

Mild secondary hypothyroidism in the rat reduces antral follicular development and induces atresia

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Abstract

Hypothyroidism is known to impair female reproductive function. However, the effects of mild hypothyroidism on ovarian function largely remain elusive. The objective of this study was to examine the effects of mild hypothyroidism on the estrous cycle, gestation length, litter size and the number of healthy and atretic follicles in adult female rats. Mild hypothyroidism was accomplished by feeding the animals an iodide-free diet supplemented with perchlorate to deplete the endogenous iodide stores. Hypothyroidism resulted in slightly reduced body weights, increased serum TSH levels, irregular estrous cycles and reduced litter sizes. The ovaries of hypothyroid animals contained similar number of follicles compared to control rats, however, the ratio of antral follicles versus preantral follicles was decreased, possible due to an increase in atretic follicles. These data suggest that mild hypothyroidism affected ovarian function by reducing ovarian follicular survival.

Introduction

Thyroid hormone is obligatory for growth and development of many tissues and organs. It has also a function in reproductive tissues, since both hypothyroidism and hyperthyroidism impair female fertility. The most common thyroid hormone disorder is hypothyroidism, which is associated with delayed puberty, menstrual disturbances, infertility and spontaneous abortions (1,2). Thyroid hormone affects ovarian function by interacting with the hypothalamic-pituitary-ovarian axis through modulation of the secretion of gonadotropic releasing hormone (GnRH), gonadotropins, prolactin, growth hormone, androgens and estrogens (1). It is not clear yet whether impaired follicular development in hypothyroidism is a result of increased prolactin secretion, which then blocks the production and action of gonadotropins, or is due to a direct effect of the decreased thyroid hormone levels on the ovary (3). A direct effect of thyroid hormone could be mediated by the granulosa cells, which express thyroid hormone receptors (4-6).

The notion that hypothyroidism impairs female reproduction is supported by animal studies; both female hypothyroid *rdw* rats, which have a mutation in the thyroglobulin gene (7), and thyroid hormone receptor deficient (*hyt*) mice, are infertile (8,9). The effects of hypothyroidism on female reproduction have furthermore been studied in rats through the induction of hypothyroidism either by propylthiouracyl (PTU) treatment or by thyroidectomy (3,10-12). PTU-treated female rats show irregular estrous cycles, spontaneous pseudopregnancies and smaller litter sizes, accompanied by depressed GH and estrogen levels, as well as elevated prolactin levels at the end of pregnancy (11). Thyroidectomized rats have irregular estrous cycles and a decreased ovulation rate (13), possibly due to an altered ovarian sensitivity to gonadotropins (14). Within a few weeks, these conditions lead to anoestrus. Controversy exists on the effects of hypothyroidism on gonadotropin levels since both decreased (15) as well as increased (16) secretion of LH has been reported at the time of the proestrus surge. It has been suggested that hypothyroid animals have impaired follicular maturation and development of corpora lutea (3). Follicular development and atresia have not been studied yet in much detail.

In general, in the animal models described above, the conditions of severe hypothyroidism generally resulted in anoestrus. In hypothyroid women, however irregular menstrual cycles are observed, suggesting that relative mild forms of hypothyroidism may differ in key aspects from severe hypothyroidism as the case in the indicated animal models. The objective of the present study was, therefore, to investigate the effects of mild hypothyroidism on ovarian function in adult female rats. Mild hypothyroidism was induced in these animals by placing them on an iodide-free diet, supplemented

with perchlorate to deplete the endogenous iodide stores. We investigated the effects of this form of hypothyroidism on the estrous cycle, gestation length and litter size. Furthermore, we determined the effects on the development of healthy preantral and antral follicles, as well as atretic follicles.

Materials & Methods

Animals

Female outbred Wistar rats (Harlan, Horst, The Netherlands), 10 weeks of age on arrival, were individually housed and kept in a temperature- and light-controlled room (lights on from 03:00 to 17.00h) with free access to food and tap water. All female rats (n=12) were randomly assigned into one of the two treatment groups. Hypothyroidism was induced in six animals (hypothyroid group) by depleting the animals of iodide, which was accomplished by feeding them a diet consisting of rat chow (American Institute of Nutrition, Research Diet Services, Wijk bij Duurstede, The Netherlands) mixed (1:1) with iodide free demi-water and supplemented with 0.5% sodium perchlorate. Perchlorate, which is a competitive inhibitor of the cellular iodide pump, prevents the uptake of iodide by the thyroid gland (2). Control rats (n=6) received a control feed, which also consisted of the same rat chow pap, supplemented with potassium iodide (70 µg/KI/kg rat chow). All experiments described in the present study have been approved by the Wageningen University ethical committee for laboratory animal welfare.

Experimental design

Estrous cycles were checked by daily taking of vaginal smears. After approximately 4-5 weeks on iodide-free or control diet, the rats were rendered pregnant by housing them overnight on proestrus with a fertile Wistar male (fig.1). If spermatozoa were detected in the vaginal smear the next day, this day was designated as day 1 of pregnancy. The mild hypothyroid treatment was continued during gestation (circa 3 weeks) and the lactation/postweaning period (6 weeks). Thyroid hormone statuses of the mother animals were checked by taking blood samples by the tail-nick technique for determination of serum thyroid-stimulating hormone (TSH) concentrations. Blood samples were collected just before rats were rendered pregnant (after ca. 4 weeks on diet), one day after delivery (after ca. 7-8 weeks on diet) and at the time when animals were sacrificed (after ca. 14 weeks on diet). The animals were sacrificed at proestrus using Nembutal and O_2/CO_2. The ovaries were excised, fixed for 24h in 4% buffered formalin, embedded in paraffin and stored at room temperature (RT) until further processing for histological analysis.

Radioimmunoassay

Plasma TSH levels were measured by a radioimmunoassay for rat-TSH using materials

(tracer rTSH-I-9 (AFP-1154B) and antiserum TSH-RIA-6) supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, Bethesda, MD, USA) according to the method described by Mattheij et al (16). In short, collected blood was centrifuged at 13000 rpm for 5 min at RT. The supernatant was removed and the serum was stored at –20 C until RIA analysis. The serum was incubated with antiserum and tracer for 96 h at RT in the dark. The second antibody was added to all samples, mixed and incubated for 60 min at RT. At the end of the incubation, polyethylene-glycol (PEG) was added, and after 15 min the samples were centrifuged at 6000 rpm (15 min at 4 C). After removal of the supernatant, the precipitate was counted in a Wallac Wizard 2470 Automatic Gamma Counter (Perkin Elmer, The Netherlands). TSH concentrations were expressed relative to the NIDDK-rTSH-RP-3 reference. The

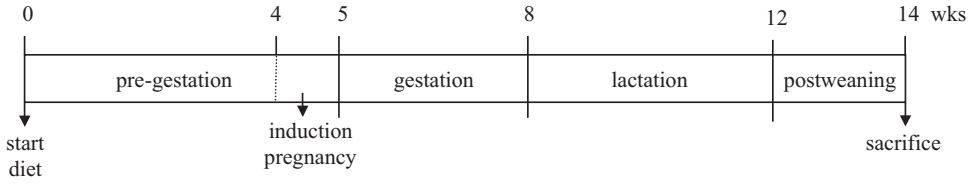

Figure 1. Experimental design. The animals were set on an iodide-free or control diet for 14 weeks and blood samples were taken in the pregestation period (after 4 weeks on the diet), at one day after partus (after 8 weeks on the diet) and at sacrifice (after 14 weeks on the diet).

assay sensitivity was 0.2 ng/ml and the intra-assay variation was 14%.

Determination of the estrous cycle

Vaginal smears were taken daily to determine the stage of the estrous cycle. An estrous cycle was scored as regular (1) when a typical cycle profile of four days was observed, i.e. diestrus II, proestrus, estrous, metestrous /diestrus I. An estrous cycle was scored as irregular (0) when this standard cyclic profile was not found. The regularity of the estrous cycle was determined by dividing the number of regular cycles by the sum of regular (1) and irregular (0) cycles.

Histological evaluation of follicle numbers

The paraffin embedded ovaries were serially sectioned at a thickness of 7 µm. A stratified sample consisting of every fifth section was mounted on glass slides, stained with Periodic Acid Schiff (PAS) and Mayers haematoxylin, and examined by light microscopy in order to estimate the total number of healthy preantral and antral follicles per ovary according to the method of Flaws et al (17). The selected sections from one ovary (approximately 135 sections per ovary) were randomized and the number of healthy follicles was counted in each of these sections. Follicles were identified as healthy when they contained an intact oocyte, organized granulosa and theca cell layers, and when less than 5% of the granulosa cells showed signs of apoptosis. Only follicles with an oocyte containing a visible nucleus with a nucleolus were counted to avoid double counting of the same

follicle. Follicles were scored as preantral if they contained an oocyte with a healthy nucleus, more than one layer of granulosa cells and a developing thecal layer. Follicles were scored as antral if they contained a healthy oocyte and a minimum of two antral spaces of which the diameter had at least the size of the oocyte. To obtain an estimate of the total number of follicles per ovary, the number of preantral and antral follicles present in the marked sections was multiplied by five as every fifth section was used in the analysis. Primordial and primary follicle counts are still under investigation.

Histological evaluation of atresia

The percentage of atretic follicles per ovary was estimated according to the method of Dijkstra et al (10). Based on morphological criteria, follicles were classified as non-atretic or atretic as described previously (18). Non-atretic follicles often showed mitoses of granulosa cells while the percentage of apoptotic granulosa cells was less than 5%. In atretic preantral follicles, the oocyte had in general degenerated and was surrounded by either a disorganized granulosa layer and a granulosa with more than 5% apoptotic cells, and/or a hypertrophied theca layer. In atretic antral follicles the oocyte was often intact, whereas the layer of granulosa cells contained many apoptotic cells; the granulosa cells were lost as atresia proceeded. The theca layer showed signs of hypertrophy. In three sections per ovary (at a quarter, half and three-quarters of the ovary), all preantral and antral healthy and atretic follicles were counted. Because the counted numbers reflected only part of the total follicle population in an ovary, the mean percentage of atretic follicles was calculated in each group of rats by dividing the number of atretic follicles by the sum of the total number of (atretic plus non-atretic) follicles counted, multiplied by 100, and analyzed statistically. Primordial and primary follicles were often arranged in small or large clusters. The number of follicles in these clusters varied considerably per ovarian section. Therefore, primordial and primary follicles were excluded from these calculations.

Statistical analysis

Statistical analysis was performed by a one-way analysis of variance (ANOVA). Differences among group variances were determined with Tukey's multiply comparison test. An unpaired T-test with the Welch's correction was used to assess significance for the data, when less than 3 groups were compared. Values were considered to be statistically significant when $P<0.05$

Results

Establishment of mild hypothyroidism

To induce mild hypothyroidism, female rats were placed on an iodide-free diet

supplemented with perchlorate to deplete endogenous iodide stores. To investigate whether hypothyroidism was accomplished, we measured TSH levels in serum. Serum TSH levels were significantly higher in hypothyroid animals than in controls (fig. 2, P<0.01). In the hypothyroid rats, TSH levels increased following the onset of the iodide-free diet prior to pregnancy, but also during the period after delivery, lactation

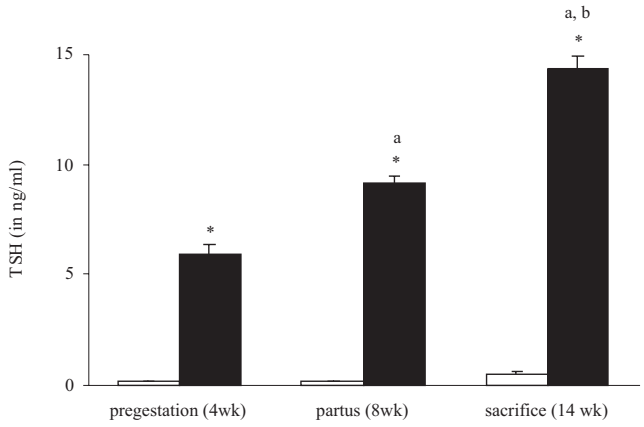

Figure 2. TSH plasma levels in control (open bars; n=6) and hypothyroid rats (filled bars; n=6). The levels of TSH were determined at 4, 8 and 14 weeks after the start of the diet as described in the *Materials and Methods*. Values represent mean ± SEM. * Significantly different from control rats, [a] Significantly different from cyclic (4wk) hypothyroid rats, [b] Significantly different from hypothyroid rats at one day after partus (8wk) P<0.01.

and postweaning. In the control rats, TSH concentrations remained contstant (fig. 2).

Effect of hypothyroidism on the estrous cycle, gestation length, maternal body weights and litter size

To asses whether hypothyroidism affected the estrous cycle profile and gestation, the estrous cycle, gestation length, maternal body weights and litter size were monitored. During the pregestation period, i.e. the first 4 weeks on the diet, the percentage of rats with regular estrous cycles was reduced from 92% in control rats to 61% in hypothyroid females (fig. 3A). The gestation length was not significantly changed in hypothyroid females (22.8 ± 0.5 days vs. 20.3 ± 0.8 days in controls) (fig. 3B). Starting at day 18 of gestation, maternal body weights were slightly reduced in the rats fed with the iodide-free diet compared to control rats. Maternal body weight remained significantly lower in the hypothyroid rats during the lactation/postweaning period (fig. 3C). The average number of pups per litter was reduced from 12 in control to 8 in hypothyroid mothers (fig. 3D). In conclusion, although small effects were observed, mild hypothyroidism allowed a continued reproductive cycle of the majority of rats on the hypothyroidism inducing diet.

Effect of hypothyroidism on the number of heatlhy and atretic follicles

To investigate whether hypothyroidism affected follicular development, we counted the number of healthy follicles at the preantal and antral stage in the rat ovaries as well as the percentage of atretic follicles. The total number of preantral plus antral follicles per ovary was very similar in hypothyroid rats compared to control rats (fig. 4A). However,

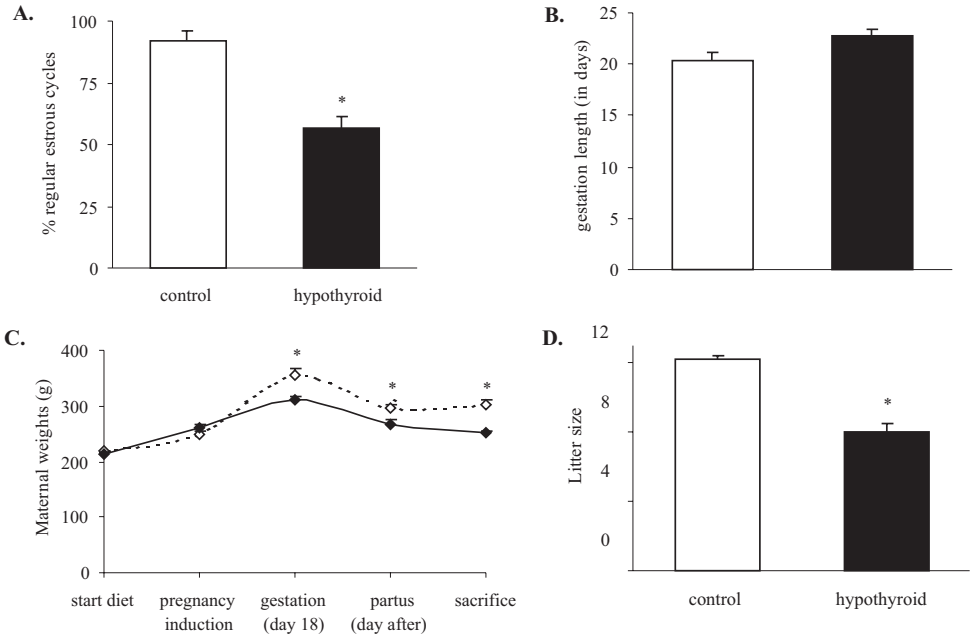

Figure 3. Regularity of the estrous cycle, gestation length, maternal body weight and litter size of control (open bars, n=6) and hypothyroid rats (filled bars, n=6). A. The percentage of regular estrous cycles during the pregestation period was estimated as described in *Materials and Methods*. B. Gestation length. C. Maternal body weights at the start of the diet, pregnancy induction, gestation (day 18), postpartum and sacrifice of control (scattered line) and hypothyroid rats (closed line). D. Total numbers of pups per nest. Values represent mean ± SEM. * Significantly different from control rats, P<0.05.

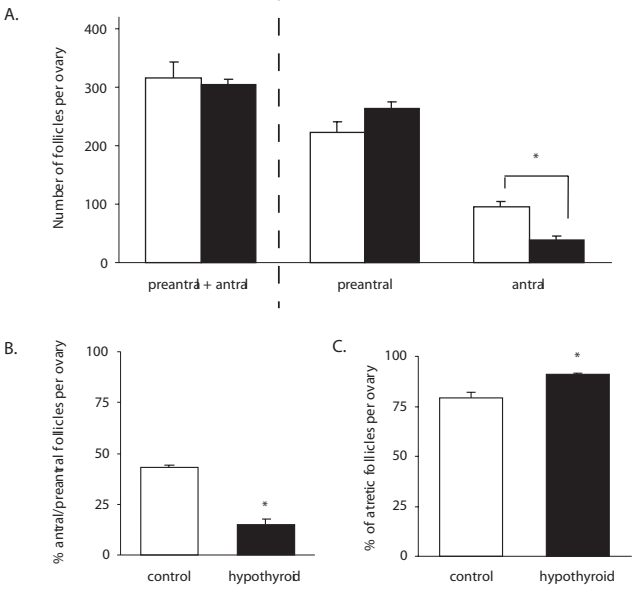

Figure 4. Number of healthy and atretic follicles per ovary in control (open bars; n=6) and hypothyroid rats (filled bars; n=6). A. Total follicle count excluding primordial and primary follicles. B. The percentage of healthy antral follicles versus preantral follicles per ovary. C. The percentage of atretic follicles per ovary. Values represent mean ± SEM. * Significantly different from control rats, P<0.05

in hypothyroid females the ratio of antral follicles versus preatral follicles was decreased more than 2-fold as compared to control rats (fig. 4C, P<0.01). The reduced number of healthy growing antral follicles in hypothyroid rats was accompanied by a significant 14% increase in the percentage of atretic follicles (fig 4D, P<0.01), suggesting reduced follicular survival. The modest increase in atretic follicles in hypothyroid rats may be explained by the observation that atretic follicles already greatly outnumbered healthy antral follicles in control rats.

Discussion

In the present study we found that hypothyroidism can be induced in female rats by an iodide-free diet supplemented with perchlorate to deplete the endogenous iodide stores. Serum TSH levels significantly increased over time following the start of the iodide-free diet. Hypothyroidism only slightly reduced maternal body weights during the lactation/postweaning period. In addition, hypothyroidism moderately affected fertility and caused reduced litter sizes and relatively more irregular estrous cycles. However, the animals remained cyclic even after 14 weeks on the diet. These observations suggest that the diet induced a relative mild form of hypothyroidism in the rats, comparable to the situation in many hypothyroid women. In earlier reported animal models, i.e. thyroidectomy or PTU-treatment in rats, or thyroid hormone receptor deficient mice, a severe form of hypothyroidism was induced resulting in anoestrus. Therefore, our study may provide a more suitable model for the study of the effects of hypothyroidism on ovarian activity in women.

The number of healthy growing follicles beyond the primordial/primary stage was not different in hypothyroid females compared to control rats. However, hypothyroid rats contained higher numbers of preantral follicles (P=0.06), lower numbers of antral follicles, and an increased percentage of atretic follicles compared to control rats. These data suggest that hypothyroidism affects follicular survival from the preantral to the antral stage due to inhibited follicular growth, stimulated atresia or both. Follicle stimulating hormone (FSH) has widely been demonstrated to be the crucial survival factor of follicles during the early antral stage. FSH enhances granulosa cell proliferation (19) and IGF-I production of granulosa cells, which further augments FSH responsiveness (20). In hypothyroid rats treated with PTU, decreased IGF-I serum levels were observed (11). Moreover, thyroid hormone (both T_3 as well as T_4) could stimulate the action of FSH on granulosa cell function *in vitro* (21-23), suggesting a direct effect of thyroid hormone on granulosa cells. Indeed, mammalian granulosa cells express functional thyroid hormone receptors (TR-α1 and 2, TR-β1 and 2) (4-6). Hence, in hypothyroid animals diminished FSH responsiveness of granulosa cells may lead to a reduction of IGF-I production and

a subsequent decline in the number of healthy antral follicles.
Concomitant treatment with T3 and FSH has been shown to stimulate follicular survival of early antral, but not of preovulatory follicles in vitro (24). The number of active caspase-3, an enzyme responsible for the execution of the apoptotic program, positive granulosa cells of early antral follicles was decreased in response to this treatment compared to treatment with FSH alone, whereas T3 alone did not affect active caspase-3 expression in this experimental set-up (24). In addition, T_3 binding activity was highest in granulosa cells of early antral follicles compared to preovulatory follicles in porcine ovaries (25), suggesting that thyroid hormone is important for survival of follicles at the early antral stage. In line with these data, we observed reduced follicular development and increased atresia in ovaries of hypothyroid rats, which was accompanied with increased expression of pro-apoptotic caspase-3 and reduced expression of the anti-apoptotic Bcl-2 protein (preliminary results). Together, these data suggest that thyroid hormone synergizes with FSH to inhibit apoptosis in granulosa cells of small antral follicles. Since decreased FSH responsiveness is associated with follicular atresia, hypothyroidism may diminish the responsiveness of follicles to FSH. Considering that hypothyroid rats display hypersecretion of prolactin in (3,11), which can inhibit the synthesis of pituitary LH and FSH, the secretion of gonadotropins is most likely decreased in severe hypothyroid rats. Indeed, serum FSH and LH levels were decreased in severely hypothyroid adult animals treated with PTU (3,12,26), though significantly only in one study (26). We therefore speculate that hypothyroidism may reduce FSH receptor expression in granulosa cells. Hence, decreased numbers of antral healthy follicles during hypothyroidism may account for reduced responsiveness of the granulosa cells to FSH and as a consequence a decrease in sex steroid hormone production in the ovary. On the other hand, thyroid hormones could also affect the ovaries indirectly via the somatrophic axis. Hypothyroidism have been shown to result in decreased synthesis of hypothalamic somatostatin, growth hormone releasing factor, and their pituitary receptors and subsequently reduced GH secretion (11,27). Therefore, the objective of the next study is to investigate also whether hypothyroidism affects the gene expression of GH in the pituitary as well as IGF-I, IGF-I receptors, GH receptors, LH receptors and FSH receptors at the ovarian level.

The observation that litter sizes were smaller in hypothyroid animals is in line with previous data (2,11) and may be due to the increased percentage of atretic follicles and the lower number of healthy antral follicles that ovulate. On the other hand, it may also be the result of disturbed steroid production of the functional corpora lutea, which may lead to impaired implantation or embryonic death (8,28).

In conclusion, the ovaries of adult hypothyroid female rats contained less healthy antral follicles and more atretic follicles compared to control rats, suggesting that thyroid hormone promotes the survival of follicles at the early antral stage. Whether the follicular survival effect of thyroid hormone is direct or indirect via stimulation of

hormone receptors and/or locally produced growth factors remains to be investigated.

Acknowledgments

We would like to thank Prof. W. den Otter and Prof. W. Stoorvogel (Department of Biochemistry & Cell Biology, Utrecht University) for critically reading this paper.

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Chapter 5

Irregularly shaped inclusion cysts display increased expression of Ki67, Fas, Fas ligand and procaspase-3 but relatively little active caspase-3

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Abstract

Human ovarian cancers are thought to arise from sequestered ovarian surface epithelial (OSE) cells that line the wall of inclusion cysts. Nevertheless, the early events towards neoplasia are not well understood. In this study, immunoreactivity for apoptotic proteins in human OSE of control and tumor ovarian sections were examined. Ki67, a marker for cell proliferation, was generally absent in flat-to-cuboidal OSE cells on the ovarian surface and in regularly shaped inclusion cysts. Fas, Fas ligand and caspase-3, components of the apoptotic pathway, were also largely absent. Ki67, Fas, Fas ligand and procaspase-3 expression, though not active caspase-3 expression, were more frequently observed in epithelial cells lining irregularly shaped inclusion cysts, particularly in the columnar and Müllerian-like OSE cell types that resembled ovarian tumor OSE cells. Immunoreactivity for these factors as well as active caspase-3 was found more frequently in ovarian tumors.We postulate that the appearance of the Fas system and its related proteins in sequestered columnar OSE cells of irregularly shaped inclusion cysts may contribute to balance cell growth with cell death, although little active caspase-3 expression was observed. Further studies are required to identify whether inhibition of apoptosis in inclusion cysts is an early event in ovarian carcinogenesis.

Introduction

Cancer of the ovarian surface epithelium (OSE) is the leading cause of gynecological cancer deaths in the Western world. The aetiology of this disease is poorly understood, although it has generally been accepted that ovarian epithelial cancers originate from OSE cells that line the wall of inclusion cysts (1). Some investigators have suggested that such inclusion cysts develop as a consequence of a dynamic interplay between OSE cells and the underlying stroma cells (1). Other groups have suggested that the formation of inclusion cysts is related to the repair process of the ovarian surface that takes place after ovulation (2-4). Independent of how these inclusion cysts are formed, it has been proposed that entrapment of OSE in inclusion cysts results in enhanced exposure of OSE cells to the ovarian stromal microenvironment (5). Under certain conditions the high amounts of stroma-derived growth factors and steroids may then induce mutations in these cells (6). Normally, such mutated cells will not accumulate, but are presumably eliminated by apoptosis. It has been suggested that OSE cells may persist when the apoptotic process is impaired, leading to propagation of mutations and an increased risk of transformation and tumour formation (7).

Evidence supporting the concept that ovarian tumor progression might involve alterations in cell death control has been provided by the observation that apoptosis and the expression of individual apoptotic proteins in OSE tumors depend on the type and grade of the tumor (8-11). Apoptosis increases during the course of ovarian tumor development, but is outnumbered by proliferatively active OSE cells[8,9], which clearly demonstrates a disturbed balance between tumor OSE cell growth and loss to the detriment of apoptosis. Furthermore, mutations in the p53 tumor suppressor gene are a common aspect in serous OSE tumors (9,12,13). Such mutations are associated with a loss of p53 function and thereby, in response to DNA damage, impaired p53 production may inhibit cell cycle arrest or apoptosis. Taken together, these observations suggest that inhibited apoptosis may be a causal or contributing factor in the aetiology of OSE cancer.

A major pathway that triggers apoptosis in the ovary is the Fas system. Fas receptor (CD95) is a member of the tumor necrosis factor/nerve growth factor family that is activated by binding of Fas ligand, leading to activation of downstream death executioner factors, such as caspase-3, and eventually cell death (14). Expression of Fas and Fas ligand has been demonstrated in many OSE cancers (8,10,15). Nevertheless, their expression in normal OSE cells and the apoptotic and proliferative capacity of OSE cells lining the ovarian surface and (ir)regular inclusion cysts remain to be determined. In order to obtain more insight in the balance between cell growth and death in possible precursor lesions that may develop into ovarian cancer, we have examined immunoreactivity for

Ki67, Fas, Fas ligand, pro- and active caspase-3 protein in epithelial inclusion cysts and compared this with the immunoreactivity pattern in ovarian tumors.

Patients and methods

Patients

Formalin-fixed, paraffin-embedded ovarian tissue sections together with their pathological examination reports were obtained from the Department of Pathology, University Medical Center Utrecht, The Netherlands. The ovarian tumor tissues were obtained from 13 patients undergoing surgery for ovarian cancer, while control ovarian tissues were obtained from 19 patients undergoing oophorectomy for non-ovarian pathology (Table 1). Criteria for the classification of inclusion cysts were as follows: round-to-oval shaped inclusion cysts without invaginations consisting of flat-to-cuboidal OSE cells were classified as regularly shaped inclusion cysts, while cysts with irregular folds that were lined with a combination of normal flat-to-cuboidal and columnar types of OSE cells were assigned as irregularly shaped inclusion cysts. The ethics committee of the University Medical Center Utrecht approved our studies using human tissues.

Table 1. Characteristics of patients (n=32)

Patients	*n (%)*
Ovarian tumor patients	
Median age:	
54 years (range 39 - 74 years)	13
Subtype:	
Serous	8 (62%)
Mucinous	5 (38%)
Type of ovarian lesions:	
Benign cysts	5 (38%)
Borderline tumors	3 (24%)
Carcinomas	5 (38%)
Control patients	
Median age:	
55 years (range 39 - 81 years)	19
Non-ovarian pathology:	
Cervix or endometrium carcinoma	12 (63%)
Genetic predisposition	7 (37%)

Immunohistochemistry

Immunohistochemistry was performed as has been described by Teerds *et al.* (16). Briefly, 5 µm thick paraffin embedded ovarian sections were deparaffinized and treated with 1% H_2O_2 in methanol for 30 min to block endogenous peroxidase activity. The slides were subsequently washed in 0.01 M Tris-buffered saline (TBS pH 7.4), incubated with 0.1 M glycine in TBS for 30 min, and then rinsed with TBS. Sections were blocked for 30 min with 10% normal goat or rabbit serum, and then incubated overnight at 4 ^{0}C with rabbit anti-human polycloncal antibodies against Fas (dilution 1:100, sc-715 (C-20), Santa Cruz Biotechnology, SanverTech Heerhugowaard, the Netherlands), Fas ligand, (dilution 1:100, sc-956 (Q-20), Santa Cruz Biotechnology), active caspase-3 (concentration 10 µg/ml, AF835, R&D systems, ITK Diagnostics Uithoorn, The Netherlands), with a goat anti-human polyclonal antibody against procaspase-3 (dilution 1:100, sc-1226 (N-19), Santa Cruz Biotechnology), or a mouse anti-human monoclonal antibody against Ki67 (dilution 1:50, Dako A/S, Glostrup Denmark). All antibodies were diluted in TBS containing 10% normal goat or rabbit serum, depending on the species the secondary antibodies were raised in. After this incubation, the slides were washed with TBS and incubated for 60 min with the corresponding biotinylated goat anti-rabbit, rabbit anti-goat or goat anti-mouse IgG, respectively (ABC-peroxidase complex staining kit Elite, Vector Laboratories Inc., Burlingame, CA) diluted 1:200 (Fas, Fas ligand, procaspase-3) or 1:100 (active caspase-3 and Ki67) in TBS containing 10% normal goat or rabbit serum. Sections were washed again in TBS and subsequently incubated for at least 60 min with the components avidin (A) and biotin (B) of the ABC staining kit. Both components (A and B) were diluted 1:500 (Ki67), 1:1000 (procaspase-3) or 1:1500 (Fas, Fas ligand and active caspase-3) and prepared at least 15 min before use. Slides were washed in TBS, then rinsed in 0.05 M Tris-HCl (pH 7.5) and finally bound antibody was visualized after addition of a 0.6 mg/ml solution of 3,3'-diaminobenzidine tetrachloride (Sigma Chemical Co., St. Louis, MO) in Tris-HCl to which 0.03% H_2O_2 was added. The slides were subsequently counterstained with Mayer's hematoxylin. Control sections, in which normal rabbit, goat, or mouse serum replaced the primary antibody, were similarly processed. No staining was observed in these controls (Fig. 2F, 3I and 4F). As a positive control for Fas, Fas ligand, pro- and active caspase 3 human and rat ovarian tissues were used. In these tissues Fas, Fas ligand and pro-caspase 3 staining was always observed in theca cells, while active caspase 3 was only observed in granulosa and some theca cells that showed morphological signs of apoptosis (data not shown). The percentage of positively labeled cells for each primary antibody was quantified by counting 500 OSE cells at five different areas per tumor section. In the ovarian sections of patients undergoing oophorectomy for non-ovarian pathology, the percentage of positively stained OSE cells was determined by counting all OSE cells within all (ir)regularly shaped inclusion cysts.

Statistical analysis

We are aware that grouping of different classes (i.e. serous and mucinous) and/or stages (i.e. benign cysts, tumors of low malignant potential and adenocarcinomas) of ovarian cancers is not very appropriate, however, due to the small cohort size, we were forced to group them to power the statistical analysis. Statistics was performed by a one-way analysis of variance (ANOVA) for all studies. Differences between group variances were determined with Tuckey's multiply comparison test. A relationship between the relative content of immunoreactive OSE cells and the stage of ovarian cancer was tested with Spearman's nonparametric correlation test. Benign cysts, borderline tumors and ovarian carcinomas were ranked as 1, 2 and 3, respectively. The variables were considered to be statistically significant different when $P<0.05$.

Results

Morphological characterization of OSE cells

The different OSE cell types that could be observed in the ovarian sections are illustrated in figure 1. The most common found OSE cell type lining the ovarian surface, clefts and regularly shaped inclusion cysts was flat-to cuboidal (Fig. 2A-C, 3A-C and 4A-C). In irregularly shaped cysts, the population of OSE cells was heterogeneous, i.e. a combination of normal flat-to-cuboidal OSE cells, columnar OSE cells and intermediate stages between cuboidal and columnar type of OSE were observed (Fig. 2D). Such irregularly shaped inclusion cysts were often found in ovarian tissues obtained from patients undergoing oophorectomy for genital tract pathology (6 out of 12 patients with endometrial or cervical carcinomas) but also observed in the ovaries of patients with a family history of breast and/or ovarian cancer (2/7 patients).

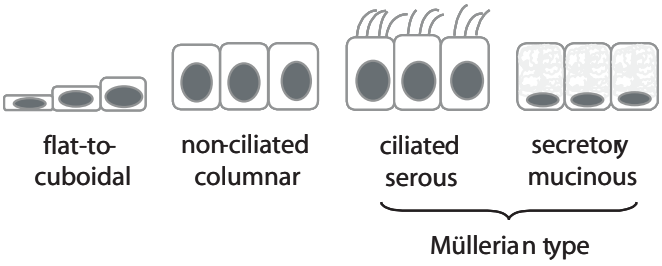

Figure 1. Schematic overview of different OSE cell types in the human ovary. The most common OSE cell type lining the ovarian surface, clefts and regularly shaped inclusion cysts is flat-to cuboidal, while irregularly shaped inclusion cysts and tumors are often lined with columnar or Müllerian type of morphology. The predominant columnar ciliated epithelial cells filled with a clear watery fluid (serous epithelium) resemble that of the Fallopian tube, while the rare tall columnar epithelial cells with basal nuclei and mucin-containing cytoplasm (mucinous epithelium) are thought to resemble endocervical epithelial cells.

The columnar OSE cells within irregularly shaped inclusion cysts could be subdivided into three different cell types: predominantly (1) non-ciliated (Fig. 2D, 3G and 4G), (2) ciliated serous (Fig. 3D) and rarely (3) secretory mucinous (Fig. 4D) OSE cells. The latter secretory mucinous OSE cell type lining irregularly shaped inclusion cysts was only seen in one patient who had undergone oophorectomy due to a uterine carcinoma. The columnar ciliated and secretory OSE cells shared morphological characteristics with epithelial cells lining the Müllerian-duct derivates, such as the fallopian tubes and the endocervix. These types of epithelial cells could also be observed in serous (Fig. 3E and 4E) and mucinous (Fig. 3F and 4H-I) types of benign cysts, borderline tumors and carcinomas.

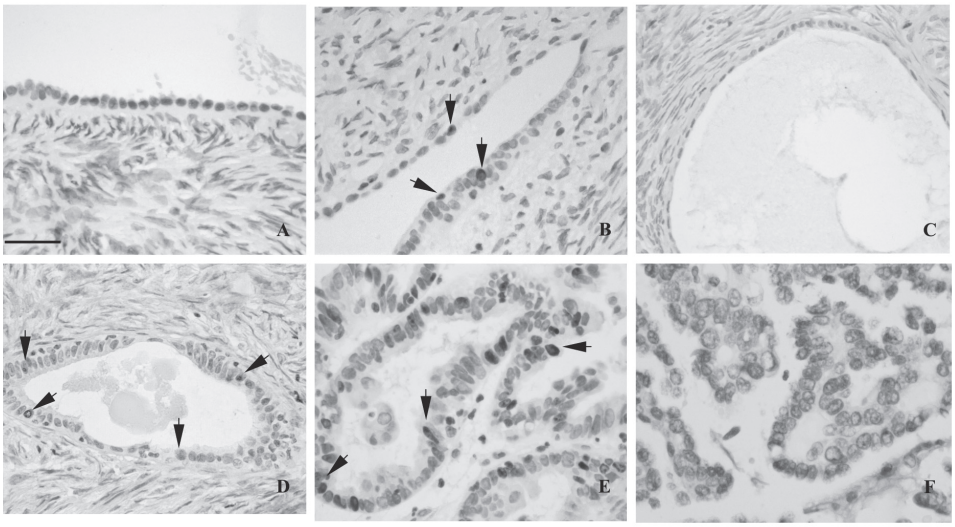

Figure 2. Immunohistochemical localization for Ki67 on the ovarian surface (A), in clefts (B), regularly (C) and irregular shaped inclusion cysts (D) and mucinous adenocarcinomas (E-F). Note positive Ki67 immunostaining (arrows) in the nuclei of OSE cells with columnar or Müllerian morphology (B and D) or tumor OSE cells (E). Control tumor section of borderline malignancy, in which the primary antibody was replaced by normal rabbit serum (F). Bars = 5 μm (C) 10 μm (A-B and D) or 20 μm (E-F).

Immunohistochemical staining of Ki67

The proliferative capacity of OSE cells was assessed by Ki67 labeling. The Ki67 antibody reacts with a human nuclear antigen that is present only in the nucleus of cells that have proliferating capacity (during G1, S, G2 and M phase of the cell cycle), but not in resting cells (in G0) (17). Ki67 immunostaining was absent in flat-to-cuboidal OSE cells on the ovarian surface, in clefts and in regularly shaped inclusion cysts (Fig. 2A and 2C). Immunoreactivtiy for Ki67 was mainly restricted to the nuclei of OSE cells with columnar and/or Müllerian morphology as detected in approximately 4% of OSE cells lining irregularly shaped inclusion cysts (in 5 out of 6 patients with irregular inclusion cysts) (Fig. 2D). The percentage of Ki67 labeled OSE cells lining irregularly shaped inclusion cysts was significantly lower than in benign cysts (11%), borderline tumors

(14%) and carcinomas (21%), respectively (Fig.5). Within the tumor group, there appeared to be a positive correlation between the number of Ki67 labeled cells and the stage of ovarian cancer ($r=0.85$; $p<0.05$).

Immunohistochemical staining of Fas and Fas ligand

Fas and Fas ligand expression was generally absent in flat-to-cuboidal OSE cells on the ovarian surface and in regular inclusion cysts (Fig. 3A-C). Immunoreactivity for Fas and Fas ligand was frequently found in the columnar OSE cells with a Müllerian morphology that lined the irregular inclusion cysts (Fig. 3D and 3G) and benign cysts (Fig. 3E), borderline tumors (Fig. 3F) and carcinomas (Fig. 3H). Since Fas is a membrane receptor, Fas staining was primarily found in the plasma membrane of serous OSE cells, which covers the apical site of the cells around the cilia, and to a lesser extent in the cytoplasm of these cells (Fig. 3E). Membrane receptors like Fas are produced in the cytoplasm and then transported to the plasma membrane.

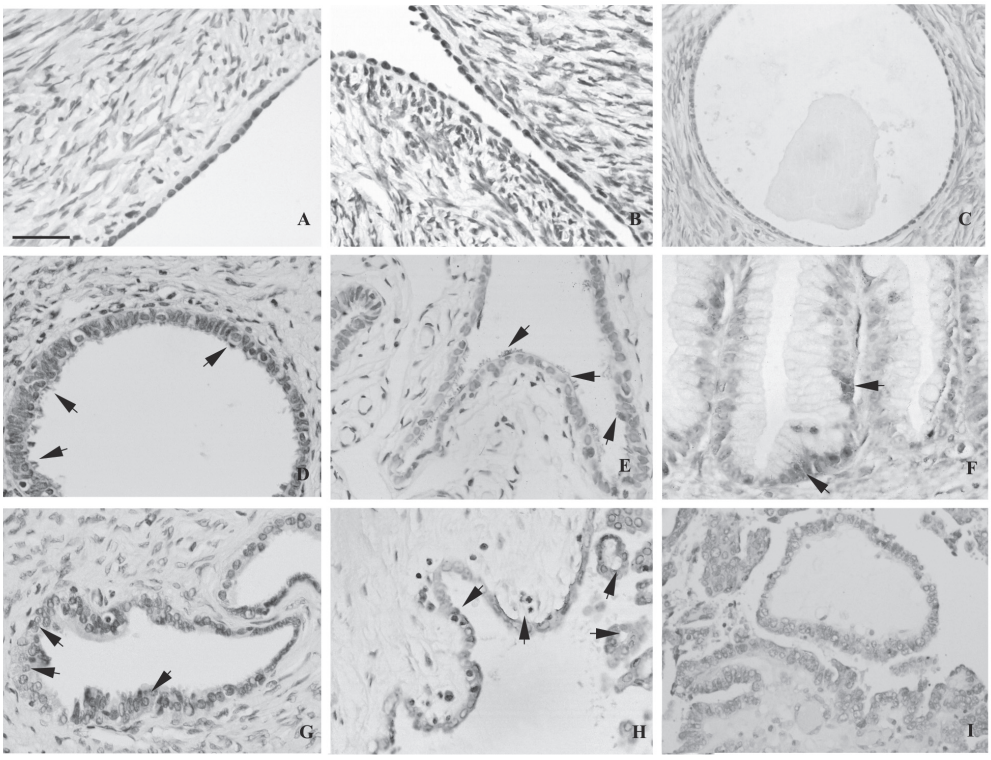

Figure 3. Immunohistochemical localization for Fas (A and D-F) and Fas ligand (B-C and G-H) on the ovarian surface (A), in clefts (B), regularly (C) and irregular shaped inclusion cysts (D and G), serous benign cystadenoma (E), mucinous epithelial borderline tumor (F) and poorly differentiated cystadenocarcinoma (H). Note the presence of immunoreactivity for Fas and Fas ligand immunostaining in columnar or Müllerian-like OSE cells (arrows). Control section of a poorly differentiated cystadenocarcinoma, in which the primary antibody was replaced by normal rabbit serum (I). Bars = 10 μm (A and D-E and G-I) or 20 μm (B-C and F).

This explains why Fas staining can also be observed in the cytoplasm of cells. Fas ligand staining in these cells was confined to the cytoplasm. In the mucinous OSE cell type, Fas and Fas ligand were located in the cytoplasm, primarily in the basal part of the cells (Fig. 3F). Immunoreactivity for both Fas and Fas ligand was significantly higher ($p < 0.01$) in OSE cells lining irregularly shaped inclusion cysts as compared to those in regularly shaped inclusion cysts but significantly lower than in benign cysts, borderline tumors and carcinomas (Fig. 5). There was no correlation between Fas or Fas ligand expression and type or grade of ovarian cancer (not shown).

Immunohistochemical staining of pro- and active caspase-3

To determine whether increased immunostaining for Fas and Fas ligand in columnar and Müllerian OSE cells was accompanied by downstream activation of procaspase-3, expression of both the inactive procaspase-3 and active caspase-3 was examined.

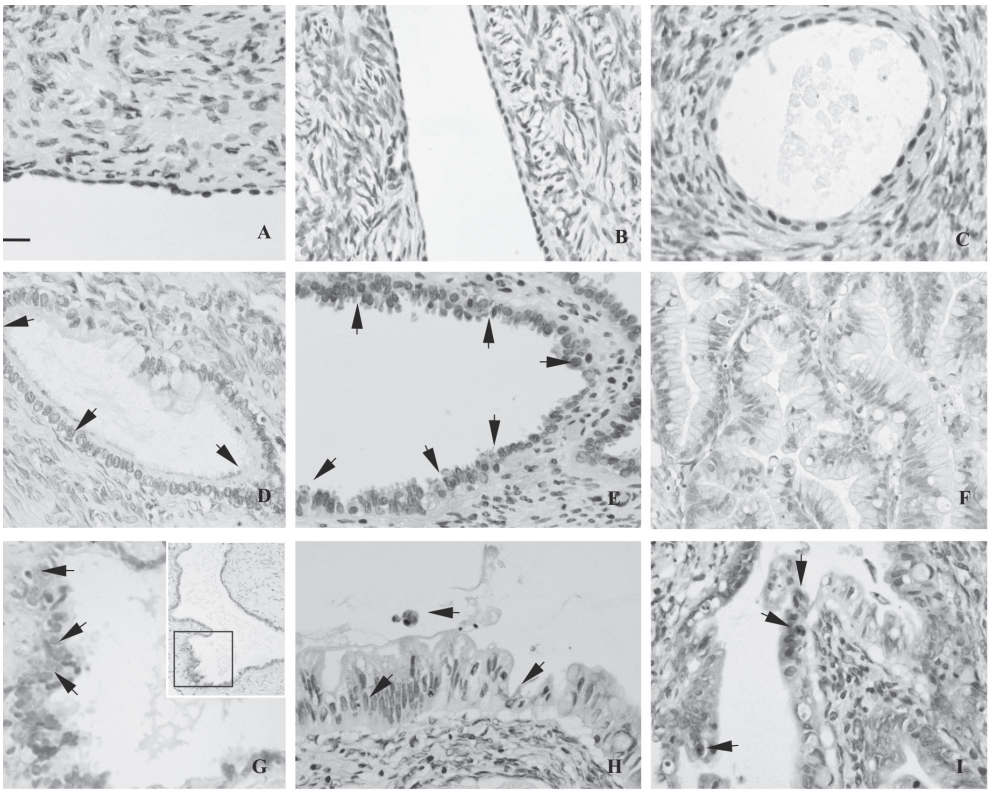

Figure 4. Immunohistochemical localization for procaspase-3 (B-E) and active caspase-3 (A and G-I) on the ovarian surface (A), in clefts (B), regularly (C) and irregular shaped inclusion cysts (D and G), serous benign cystadenoma (E) and mucinous adenocarcinomas (H-I). Note that cells positive for active caspase-3 often had condensed and fragmented nuclei (arrows), which are characteristic for apoptosis (H-I). Control section of a serous papillar cystadenoma, in which the primary antibody was replaced by normal goat serum (F). Bars = 10 µm (A-E, G) or 20 µm (F, H-I).

Immunostaining for procaspase-3 was weak to undetectable in normal flat-to-cuboidal OSE cells on the surface, clefts and regularly shaped inclusion cysts (Fig. 4A-C). Staining was significant but heterogeneous in the cytoplasm of OSE cells with a columnar and Müllerian-like morphology within irregularly shaped inclusions cysts (Fig. 4D) or within benign cysts, borderline tumors and carcinomas (Fig 4E). The percentages of procaspase-3 labeling in OSE cells lining regularly shaped inclusion cysts (1%), irregularly shaped inclusion cysts (33%) and tumors (51%) were very similar to that of Fas and Fas ligand as described above (see for more details also Fig 5).

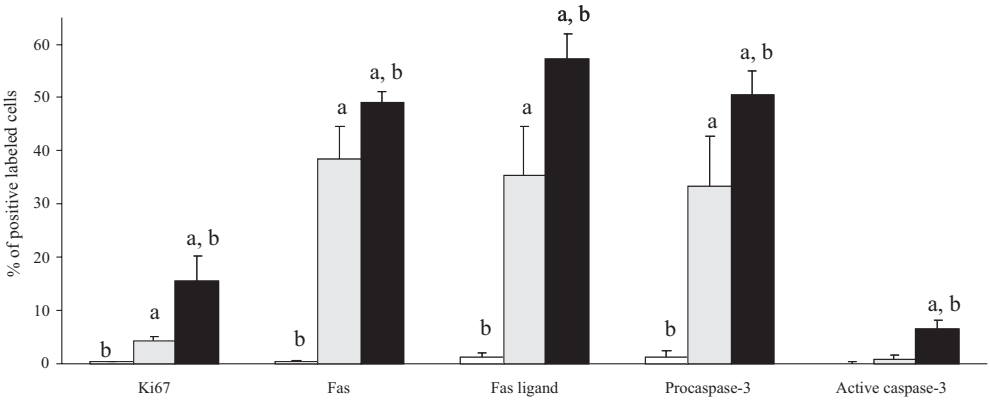

Figure 5. Semi-quantitative analysis of immunoreactivity for Ki67, Fas, Fas ligand, procaspase-3 and active caspase-3 in OSE cells lining regularly (white bars; n=11) and irregularly shaped inclusion cysts (grey bars; n=9) and tumors (black bars; n=13). The percentage of positively labeled OSE cells was determined as described in *Material and Methods*. No active caspase-3 expression was observed in regularly shaped inclusion cysts. Values represent mean ± SEM. [a] Significantly different from regularly shaped inclusion cysts. [b] Significantly different from irregularly shaped inclusion cysts ($P<0.05$).

As caspase-3 activation is essential for the execution of Fas-mediated apoptosis, we have investigated its expression with an antibody directed against the active caspase-3 subunits. Interestingly, in spite of the substantial procaspase-3 expression in OSE cells lining irregularly shaped inclusion cysts and tumors, little active caspase-3 expression was observed. Expression of active caspase-3, which was found exclusively in the cytoplasm of OSE cells, was limited to 0.8% of OSE cells lining irregularly shaped inclusion cysts (Fig. 4G) and 6.7% of tumor OSE cells (Fig. 4H and 4I). Such cells often had the morphological characteristics of apoptosis, i.e. with condensed and/or fragmented nuclei (Fig. 4H and 4I). The percentage of active caspase-3 labeling in OSE cells was significantly higher in tumors compared to in irregularly shaped inclusion cysts (Fig. 5). Moreover, the number of OSE cells staining positively for active caspase-3 was lower than that for Ki67 in irregularly shaped inclusion cysts, benign cysts, borderline tumors and carcinomas (Fig. 5). Calculation of the ratio Ki67/active caspase 3 confirms the data in figure 5. This ratio in irregular inclusion cysts is 10:2, while in tumors this ratio is

5:2, suggesting that the turn over rate of the tumor cells is higher than of the OSE cells lining the irregularly shaped inclusion cysts. This limited number of apoptotic cells in the presence of significant numbers of proliferating OSE cells may lead to a disturbed balance between cell growth and death in the irregularly shaped inclusion cysts.

Discussion

The results of this study demonstrate that the expression of some components of the Fas system is related to the morphological appearance of human OSE cells. Immunohistochemical staining for Fas, Fas ligand, procaspase-3, active caspase-3 and Ki67 was absent in normal flat-to-cuboidal OSE cells on the ovarian surface and in normal round-to-oval shaped inclusion cysts. The appearance of components of the Fas pathway in irregularly shaped inclusion cysts lined by OSE cells with a Müllerian-like morphology, however, was not accompanied by a significant increase in the number of apoptotic OSE cells. In tumors, the percentage of active caspase-3 labeling in OSE cells was significantly higher compared to irregularly shaped inclusion cysts. Moreover, the number of Ki67 positive cells exceeded the number of active caspase-3 OSE labeled cells in these cysts and in OSE tumors. Our observations suggest that the Fas apoptotic pathway is functional in OSE cells lining irregularly shaped inclusion cysts and tumors but that possibly the balance between cell proliferation and death is disturbed.

The Fas system plays a major role in triggering apoptosis in the ovary during fertile life. A role for Fas-induced apoptosis has also been implicated in normal OSE cells to facilitate ovulation. In postmenopausal ovaries, Fas signalling proteins appear to be negligible in OSE cells on the ovarian surface, in clefts and in regularly shaped inclusion cysts, as well as spontaneous apoptosis or proliferation of these cells. Presumably the absence of ovulatory bursts after menopause makes the continuous renewal of OSE cells on the ovarian surface redundant. This does, however, not exclude that OSE cells lining the regularly shaped inclusion cysts are eventually eliminated via Fas activation or another apoptotic pathway. Interestingly, OSE cells lining irregularly shaped inclusion cysts expressed Fas, Fas ligand, procaspase-3 and active caspase-3. The observation that only a very limited number of cells lining irregularly shaped inclusion cysts stained positively for active caspase-3 may be explained by the fact that the manifestations of apoptosis have been suggested to be of short duration and therefore difficult to detect histologically (18).

The physiological trigger for Fas and Fas ligand expression in human OSE cells lining the irregularly shaped inclusion cysts remains to be determined. Possible candidates might be interleukin-1ß, TNFα and IFNγ, as these cytokines have shown to increase Fas

antigen expression in several cell types including mouse OSE cells (19). Furthermore, numerous reports suggest that the Fas/FasL system operates downstream of other cellular control mechanisms, such as p53 and Ras, to protect against neoplastic transformation by inducing apoptosis (20,21). The appearance of the Fas system and its related proteins in sequestered OSE of irregularly shaped inclusion cysts, however, was accompanied by significant Ki67 expression and relatively little active caspase-3 expression, an early marker of apoptosis. Low apoptotic indices have been demonstrated before in OSE cells within tumors (8,9), however, in these studies only the final stage of apoptosis was assessed, i.e. cells with fragmented nuclei (TUNEL labeling). Though we cannot exclude that the duration of active caspase-3 expression is shorter than that of Ki67, our observations suggest that apoptosis might be overruled by proliferation in irregularly shaped inclusion cysts. An alternative way to investigate this may be by using comparative genomic hybridization and microdissection of these lesions. The limited number of apoptotic cells in the presence of significant numbers of proliferating OSE cells in these cysts may lead to a disturbed balance between cell proliferation and death and concomitantly persistence and growth of these cysts (7). Interestingly, the immunostaining patterns for the various components of the Fas system and proliferation in these cysts, were very similar to those observed in tumor tissue.

Although it has been widely accepted that the majority of OSE cancers arise from OSE cells on the surface and/or cells sequestered in inclusion cysts, this hypothesis is based only on a few histological observations (1,22-24). The lack of evidence together with the fact that OSE tumors are morphologically similar to tumors arising from the Müllerian duct derivatives has led to an alternative hypothesis for the origin of these tumors; namely that OSE tumors originate from the Müllerian duct system (25). Most irregularly shaped inclusion cysts lined with Müllerian-like epithelium in the present study were found in ovarian tissues that were obtained from patients undergoing oophorectomy for genital tract pathology (i.e. endometrial or cervical carcinomas) supporting the hypothesis by Dubeau (25). It does not, however, seem likely that these cysts are metastases of these Müllerian duct carcinomas, as the irregularly shaped inclusion cysts were often found in the deep layers of the cortex of the ovary and not on the surface. Moreover, in support of the first hypothesis that OSE cells lining the ovarian inclusion cysts are the preferred site for the development of ovarian carcinomas, we observed: 1) transitions from normal flat-to-cuboidal OSE cells to a more columnar type in inclusion cysts, and 2) different columnar types of OSE cells, such as non-ciliated, ciliated serous or secretory mucinous cells, inside the same inclusion cysts of several patients. The histology of these cells was comparable to those seen in serous and mucinous OSE borderline tumors and carcinomas. These data, together with the substantial Ki67, Fas, Fas ligand, procaspase-3 expression and relatively little active caspase-3 expression as discussed above, suggest that some of these irregularly shaped inclusions cysts may represent potential (neoplastic) precursor lesions of OSE cancers.

The expression of Fas and Fas ligand in OSE tumor cells, its localization and heterogeneity are in accordance with previously published data (8,10,15,26,27). Nevertheless, the literature on the appearance of Fas during the course of ovarian cancer development is contradictory. Baldwin *et al.* (26) reported increased levels of Fas proteins in transformed epithelial cells compared to normal human OSE cells, while Das *et al.* (15) showed decreased levels of Fas gene products. In the latter study RNA was obtained from whole human ovaries including the cancer tissues and not from isolated tumor cells. Therefore, one could question whether this truly represents expression of Fas in tumor OSE cells or in other ovarian cell types as well (28-30). In various other types of tumors, either increased (e.g. in leiomyomas and liposarcomas), unchanged (e.g. in colorectal adenomas and neurofribomas) or decreased Fas expression (e.g. in lung and colorectal carcinomas) has been shown, compared to the normal tissues from which these tumors developed (31).

It has been suggested that OSE tumor cells expressing membrane bound Fas ligand or cleaved soluble Fas ligand (sFasL) have a survival advantage by their ability to kill Fas expressing lymphocytes (32,33). Consistent with this, we observed apoptotic lymphocytes in the vicinity of Fas ligand positive OSE tumor cells. Additionally, deregulation of the Fas pathway in such tumor cells, e.g. when activation of caspases is affected, may further protect the OSE cells from programmed cell death resulting in tumor promotion as has been demonstrated for instance in human colon cancer (34). In this light, the presence of low active caspase-3 expression in benign cysts, borderline tumors and carcinomas may explain the low incidence of apoptosis at these tumor sites in our study.

In summary, this is the first comparative histological report describing OSE morphology in relation to the presence of components of the Fas system, proliferation and apoptosis in human OSE cells during ovarian cancer development. Activation of the key effector molecules for apoptosis may be a crucial step deciding whether such sequestered OSE cells will undergo apoptosis or survive and possibly proliferate with the chance of the development of a neoplasm. Further research is required in a larger cohort of patients to identify whether inhibited apoptosis in irregularly shaped inclusion cysts is an early event in ovarian carcinogenesis.

Acknowledgments

This study was supported in part by a grant from the Jan Dekkerstichting and dr. Ludgardine Bouwmanstichting, The Netherlands. We would like to thank Prof. W. Stoorvogel, Prof. W. den Otter and Dr. C. van de Lest (Department of Biochemistry & Cell Biology, Utrecht University) for their valuable comments and support in the statistical evaluation.

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Chapter 6

Luteinizing hormone inhibits Fas-induced apoptosis in an ovarian surface epithelial cell line

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Abstract

Gonadotropins including luteinizing hormone (LH) have been suggested to play an important role in the etiology of epithelial ovarian cancers. The goal of the present study was to obtain more insight in the mechanism of gonadotropin action on ovarian surface epithelial (OSE) cells. As the Fas system is known to be a major player of apoptosis in the ovary, we investigated whether LH interfered with the process of Fas-induced apoptosis in the human OSE cancer cell line HEY. Activation of Fas receptor by an agonistic anti-Fas receptor antibody induced apoptosis as was evaluated by caspase-3 activation, poly (ADP-ribose) polymerase (PARP) fragmentation, phosphatidylserine externalization and morphological changes characteristic for apoptosis. Co-treatment with LH reduced the number of apoptotic cells in a transient manner, while LH by itself did not affect apoptosis or cell proliferation. The anti-apoptotic effect of LH could be mimicked by the membrane-permeable cAMP analogue 8-CPT-cAMP, and blocked by H89, a specific inhibitor of protein kinase A (PKA). These findings suggest that LH protects HEY cells against Fas-induced apoptosis through a signaling cascade involving PKA. It is plausible that in vivo LH might enhance OSE tumor growth through inhibition of apoptosis.

Introduction

Although epithelial ovarian cancer is the most lethal of all gynecological cancers in women in the western world, the etiology of this disease remains poorly understood (1). An inherited mutation in the BRCA1 gene and to a lesser extent in the BRCA2 gene has been implicated in 5-10% of all cases (2). Another major risk factor for epithelial ovarian cancer development is related to the number of lifetime ovulations (3). The subsequent repeated rupture and repair of ovarian surface epithelium (OSE) at the ovulation site requires a high proliferative activity of the epithelium, resulting in an increased risk of mutations in these cells (4). Moreover, OSE sequestered in crypts and inclusion cysts is exposed to high levels of stroma-derived growth factors and steroids, which might also contribute to ovarian cancer development (5).

Evidence in support of hormonal involvement in ovarian carcinogenesis is given by the observation that lower basal and peak gonadotropin levels, as occurring in women during oral contraceptives use, pregnancy and breastfeeding, were found to protect against ovarian cancer (6,7). Conversely, the incidence of ovarian surface epithelium cancer is increased in women during the years after menopause when serum gonadotropin levels are high (8). Moreover, cases have been reported of OSE cancers arising in infertile women during or after prolonged treatment with gonadotropins (9,10). Considering the number of women receiving assisted reproductive treatment nowadays and the expected growing demand for ovulation induction during the coming years, it is of major importance to elucidate the role of gonadotropins in the development and progression of epithelial ovarian cancer.

Until now, it is unclear whether gonadotropins, like luteinizing hormone (LH), act in an indirect endocrine manner (via stimulating steroid production in ovarian follicular cells), or directly target OSE cells. Expression of LH receptors has been demonstrated in normal OSE (11), in epithelial cells lining ovarian inclusion cyst (10) and in approximately 50% of the ovarian carcinomas (12). However, controversy exists whether there is a direct effect of LH on OSE cell survival, as increased (3,11,13-16), unchanged (17-19) as well as decreased (20,21) proliferation rates in response to LH have been reported. Many OSE tumors have been shown to contain low apoptotic indices and therefore it is possible that LH might influence OSE tumor growth and survival through inhibition of apoptosis. Indeed, it has been demonstrated that LH suppressed apoptosis of ovarian granulosa cells (22). However, not much is known about the effects of LH on apoptosis in OSE cells.

A major pathway triggering apoptosis in OSE cells involves the Fas system (23). Fas receptor (CD95) is a member of the tumor necrosis factor/nerve growth factor family, which is expressed in many OSE cancers (24,25). Fas is activated by binding of Fas ligand, leading to activation of downstream death executioner factors, such as caspase-3, and eventually resulting in cell death (26). Fas-induced apoptosis is also believed to be one of the mechanisms involved in cisplatin cytotoxity in OSE cancer cells (27,28), a therapy often used for OSE cancer treatment. We have examined the effect of LH on the occurrence of Fas-induced apoptosis in the human ovarian epithelial cancer cell line HEY and determined whether signaling occurs via PKA and/or PKC activation.

Materials & Methods

Reagents

All cell culture reagents were purchased from Gibco BRL (Grand Island, NY, USA), except for the culture medium, MEM without phenol red (M3149), which was obtained from Sigma (St. Louis, MO, USA). H89 an inhibitor of PKA, 8-(4-chlorophenylthio)cyclic AMP (8-CPT-cAMP), H7 an inhibitor of PKC, 4-β-phorbol-12-myristate-13-acetate (PMA) an activator of PKC, 4-α-phorbol-12-myristate-13-acetate an inactive phorbolester and IBMX a phosphodiesterase inhibitor were also purchased from Sigma. The agonistic mouse-anti-human Fas IgM (clone CH11) was obtained from Upstate Biotechnology (Campro Scientific, Veenendaal, The Netherlands), the caspase inhibitor Z-vad-fmk from Promega Corporation (Madison, WI, USA) and ovine LH (NIH-LH-S20) was a gift from NIDH (Bethesda, MD, USA) and. The antibody against the LH receptor (P1B4) was a gift from Dr. J. Wimalasena (University of Tennessee, Knoxville, TN, USA). This antibody was raised against purified rat LH receptors, as described by Indrapichate et al. (29) and has been shown to bind specifically to LH receptors in different tissues (30). Antibodies against Fas receptor (sc-715), Fas ligand (sc-956) and procaspase-3 (sc-1226) were purchased from Santa Cruz Biotechnology (SanverTech Heerhugowaard, the Netherlands). The antibodies against PARP p85 (G7341), active caspase-3 (AF835) and caspase-3 (A3537) were obtained from Promega Corporation (Madison, WI, USA), R&D systems (ITK Diagnostics Uithoorn, The Netherlands) and DakoCytomation (Heverlee, The Netherlands), respectively. Secondary biotinylated goat anti-rabbit, rabbit-anti-goat or goat-anti-mouse IgG were purchased from Vector Laboratories (Vectastain kit Elite, Burlingame, CA, USA) and horseradish peroxidase-conjugated goat-anti-rabbit IgG from Nordic Immunological Laboratories (Tilburg, The Netherlands). The bromodeoxyuridine (BrdU) incorporation assay detection kit and the Annexin-V-Fluos staining kit were purchased from Roche Diagnostics GmbH (Mannheim, Germany), the cAMP Biotrak enzyme-immunoassay system from Amersham Biosciences (Freiburg, Germany) and the supersignal chemiluminescent substrate kit (ECL) was from Pierce (Tattenhall, Cheshire, UK).

Cell culture

For this study, the human ovarian surface epithelial cell line HEY was used, which was originally derived form a papillary cystadenocarcinoma (31). The HEY cell line was kindly provided by Dr D. Lobb (McMasters University, Hamilton, Canada) and was routinely kept in culture at 37 C in a 5% CO_2 incubator in Eagle's minimal essential medium without phenol red. The culture medium was supplemented with 0.1 mM non-essential amino acids, 2 mM glutamine, 50 U/ml penicillin/streptomycin, 1.5 mM Hepes and 10% heat-inactivated FCS. The cells were sub-cultured twice a week and only those cells in the exponential growth phase were used in the experiments described.

Immunohistochemistry

To detect the LH receptor (LH-R), Fas receptor (Fas), Fas ligand, procaspase-3, active caspase-3 and fragmented PARP in ovarian surface epithelial cells, HEY cells were grown on glass coverslips placed in 24-multiwell culture dishes and seeded at a density of 2.5 x 10^4 cells/well. After incubation overnight the culture medium was replaced by serum-free medium and cells were cultured for another 24 h. Then, the cells were fixed for 15 min in 4% buffered formalin pH 7.4 and immunohistochemistry was performed as has been described previously (32). Briefly, cells were washed in 0.01 M Tris-buffered saline (TBS; pH 7.4), and incubated with 0.1 M glycine in TBS for 30 min. After rinsing, cells were permeabilized with 0.1% Triton X-100 in TBS for 5 min. Cells were, depending on the species in which the secondary antibody was raised, blocked for 30 min with either 10% normal rabbit, goat or mouse serum in TBS, and then incubated overnight at 4 C with either rabbit anti-human polycloncal antibodies against Fas, Fas ligand (dilution 1:100), active caspase-3 (concentration of 0.5 µg/ml) and PARP p85 (dilution 1:100), with a goat anti-human polyclonal antibody against procaspase-3 (dilution 1:100) or with a mouse anti-rat monoclonal antibody against LH-R (dilution 1:3000). All antibodies were diluted in TBS containing 0.05% acetylated BSA (Aurion, Wageningen, The Netherlands). After washing the cells in TBS, the cells were incubated with the corresponding biotinylated goat-anti-rabbit (Fas, Fas ligand, active caspase-3 and PARP p85), rabbit-anti-goat (procaspase-3) or goat-anti-mouse (LH-R) antibodies diluted 1:200 in TBS containing 0.05% acetylated BSA for 60 min. Cells were again washed in TBS and subsequently incubated for at least 60 min with the components avidin (A) and biotin (B) of the ABC staining Elite kit. Both components (A and B) were diluted 1:1000 and prepared at least 15 min before use. Then, cells were washed in TBS, and bound antibody was visualized after the addition of a 0.6 mg/ml solution of 3,3'-diaminobenzidine tetrachloride (Sigma) in TBS to which 0.03% H_2O_2 was added. Finally, the cells were counterstained with Mayer's hematoxylin. Controls, in which the primary antibody was replaced by normal rabbit, goat or mouse serum respectively, were processed similarly. No staining was observed in these controls.

Western blot analysis

HEY cells seeded at an initial density of 2.5 x 10^5 cells/dish in 20 cm^2 culture dishes were incubated overnight in culture medium. Next, the culture medium was replaced by serum-free culture medium supplemented with or without agonistic anti-Fas antibody (50 ng/ml CH11) and a caspase inhibitor Z-vad-fmk (10^{-5} M), and incubated for 4, 8 or 24 h. Culture medium was collected and centrifuged for 5 min at 6000 g to spin down the detached cells. Adherent cells were scraped from the surface following the addition of SDS-sample buffer. Both cellular samples were pooled together in SDS-sample buffer separated by 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (1 x 10^4 cells/lane) and then transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in TTBS (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween-20) for 1 h, and incubated overnight at 4 C with a rabbit anti-human polyclonal antibody against Fas, Fas ligand, and caspase-3 (that reacts with both the 32 kDa proenzyme and the spliced 20 kDa active form of caspase-3) respectively, with a goat anti-human polyclonal antibody against procaspase-3, diluted 1:500 in blocking buffer, or with only blocking buffer (negative control). After washing three times with TTBS, the membranes were incubated for 1 hour with horseradish peroxidase-conjugated goat-anti-rabbit IgG (Fas, FasL, caspase-3) diluted 1:10000 or rabbit-anti goat (procaspase-3) diluted 1:5000 in blocking buffer. The presence of antibody-protein complexes was detected by enhanced chemoluminescence. A human fibroblast lysate in SDS-PAGE buffer (Becton Dickinson, Franklin Lakes, NY, USA) was used as a positive control and bands were detected at the appropriate sizes. In the absence of the primary antibodies no specific bands were detected on the blot.

Cell death analysis

Apoptotic cells were identified by analysis of phosphatidylserine at the outer leaflet of apoptotic cell membranes using Annexin-V and propidium iodiode (33). OSE cells were seeded at a density of 2.5 x 10^4 cells/well in 24-multiwell culture dishes and cultured overnight, followed by treatment in serum-free culture medium supplemented with or without an agonistic mouse-anti-human Fas IgM (50 ng/ml clone CH11) at various times in the absence or presence of various compounds; Z-vad-fmk (10^{-5} M), LH (0.1, 1 or 10 ng/ml), 8-CPT-cAMP (10^{-6} M), H89 (10^{-6} M), PMA (10^{-6} M), inactive phorbolester (10^{-6} M) or H7 (10^{-6} M). Detached cells were retained by centrifugation of the culture medium for 5 min at 6000 g and adhesive cells were harvested after 5 min of trypsinization. Adhesive and floating cells were pooled together and were incubated with Annexin-V-fluorescein (dilution 1:200) containing propidium iodide (dilution 1:500) in Hepes buffer for 10 min in the dark. The percentage of Annexin-V labeled cells was measured per 7500 cells and quantified by flow cytometry, which was performed on a FACS-Scan flow cytometer (Becton Dickinson).

Cell growth analysis

The dose-response effect of LH and 8-CPT-cAMP on the proliferative activity of the ovarian cancer cell lines was assessed by a bromodeoxyuridine (BrdU) incorporation assay using a 5-bromo-2'deoxy-uridine labeling and detection kit. BrdU is an analog of thymidine that is incorporated into the DNA of cells in the S-phase of the cell cycle. Cells, seeded at a density of 2.5 x 10^4 cells/well, were grown on glass coverslips placed in 24-multiwell culture dishes in culture medium. After culture overnight the medium was discarded and replaced by serum-free culture medium with or without supplementation of LH (0.1, 1 or 10 ng/ml) or 10^{-6} M 8-CPT-cAMP, followed by another culture period of 8 or 24 h. Three h prior to the end of each culture period, BrdU was added to the culture medium in a final concentration of 3 µg/ml. Cells were fixed in methacarn (60% methanol, 30% chloroform and 10% acetic acid) for 10 min at room temperature. After fixation the cells were rinsed with 70% ethanol, PBS, and finally distilled water. The cells were pretreated with 1% periodic acid at 55 C for 20 min, followed by immersion in respectively tap water and PBS. Next, the cells were blocked for 10 min with 5% BSA diluted in PBS and then incubated for 1 h with a mouse-anti-BrdU antibody diluted 1:100 in PBS containing 0.05% acetylated BSA. The coverslips were again washed in PBS and incubated for 1 h with a goat anti-mouse IgG horse radish peroxidase labeled diluted antibody 1:100 in PBS containing 0.05% acetylated BSA. After this incubation the cells were washed in PBS and bound antibody was visualized after the addition of a 0.6 mg/ml solution of 3,3'-diaminobenzidine tetrachloride in PBS to which 1% nickelamminosulphate and 0.05% H_2O_2 was added. The cells were subsequently counterstained with Mayer's hematoxylin. Black staining of the nuclei identified BrdU incorporation in the nuclei. To examine the specificity of the immunostaining, control coverslips were incubated with normal mouse serum instead of primary antibody and similarly processed. No staining was observed in these controls. HEY cells cultured in the presence of serum were used as a positive control and processed as described above. The percentage of BrdU labeled cells was determined by counting 500 (labeled and unlabeled) cells per coverslip under a light microscope. Values are presented as means ± SEM from one representative experiment using quadruplicates. Experiments were repeated three times with similar results.

cAMP enzyme-immunoassay

To measure total cellular (intracellular en secreted) cAMP levels, HEY cells (2.5 x 10^4 cells/well) were plated in 24-multiwell dishes and grown overnight in culture medium. The medium was discarded and cells were pre-incubated in serum-free culture medium for 1 h and then treated with LH (0.1 and 10 ng/ml) for 60 min in the presence of 1mM IBMX (3-isobutyl-1-methylxanthine). Incubations without IBMX were below the detection level of the assay used. Therefore, all cAMP measurements were performed in the presence of IBMX, which resulted in a significant increase in basal cAMP levels

making a stimulus detectable. Treatment with IBMX (1mM) alone was used as a control. Total cellular cAMP levels were measured using a cAMP Biotrak™ competitive enzyme-immunoassay (EIA) system, according to the manufacturer's protocol.

Statistical analysis

Statistics was performed by a one-way analysis of variance (ANOVA), unless otherwise stated. Differences between group variances were determined with Tuckey's multiply comparison test. Values were considered to be statistically significant when $P<0.05$.

Results

Expression of Fas, Fas ligand and procaspase-3

To investigate whether LH affects Fas-induced apoptosis in OSE cells, we first demonstrated the presence of the Fas pathway in HEY cells. The Fas receptor and signaling molecules were detected by both Western blot analysis and immunohistochemistry. HEY cells expressed the Fas receptor (45 kDa), Fas ligand (40 kDa) and procaspase-3 (32 kDa) proteins under basal conditions (fig. 1). Fas immunostaining resulted in staining of an additional protein band of approximately 40 kDa, which has also been reported by Kamitani et al (34).

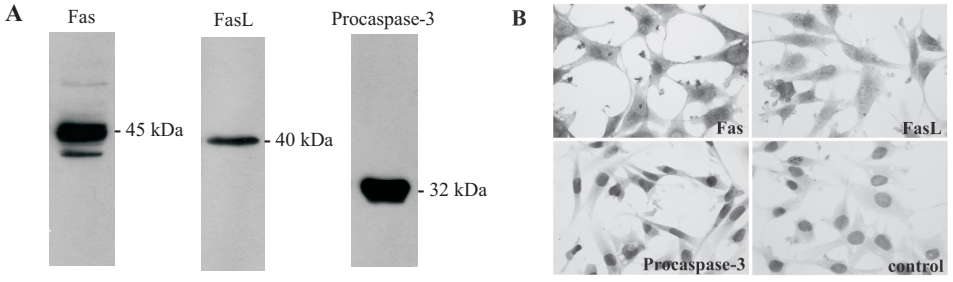

Figure 1. Expression of Fas, Fas ligand and procaspase-3 in HEY cells. HEY cells (5×10^{-4} cells/ml) were maintained overnight and subsequently serum and growth factor starved for 24 h. Fas, Fas ligand and procaspase-3 protein expression was determined by Western blot analys (A) and immunohistochemistry (B) as described in the *Materials and Methods*. The control represents a DAB staining in which the primary antibody was replaced by normal rabbit serum. A representative experiment is shown, which was repeated three times with similar results.

Activation of Fas induces apoptosis

To determine whether Fas, Fas ligand and procaspase-3 expression in HEY cells was accompanied by a functional Fas pathway, cells were treated with an agonistic anti-Fas receptor antibody (CH11). Significant apoptosis occurred in HEY cells upon CH11 treatment. Dramatic changes in cell morphology, characteristic for apoptosis, were

observed in response to treatment with 50 ng/ml CH11 (Fig. 2A). Cells shrank, rounded up, showed blebbing in quick succession, and detached from the culture dish in a time and concentration-dependent manner (data not shown). In addition, Fas receptor activation also resulted in splicing of procaspase-3 and the immunocytochemical detection of active caspase-3 and PARP cleavage fragments (fig. 2A), indicating that CH11 induced apoptosis in these cells. Caspase-3 activation was further confirmed by Western blot analysis (fig. 2B). This stimulatory effect on caspase-3 activity was completely reversed by co-treatment with Z-vad-fmk, a well-known inhibitor of caspase-3 processing (fig. 2B). Fas-induced apoptosis resulted in a significant translocation of phospatidylserine (PS) to the outer leaflet of the plasma membrane as quantified by FACS analysis using Annexin-V/propidium iodide double staining (fig. 2C).

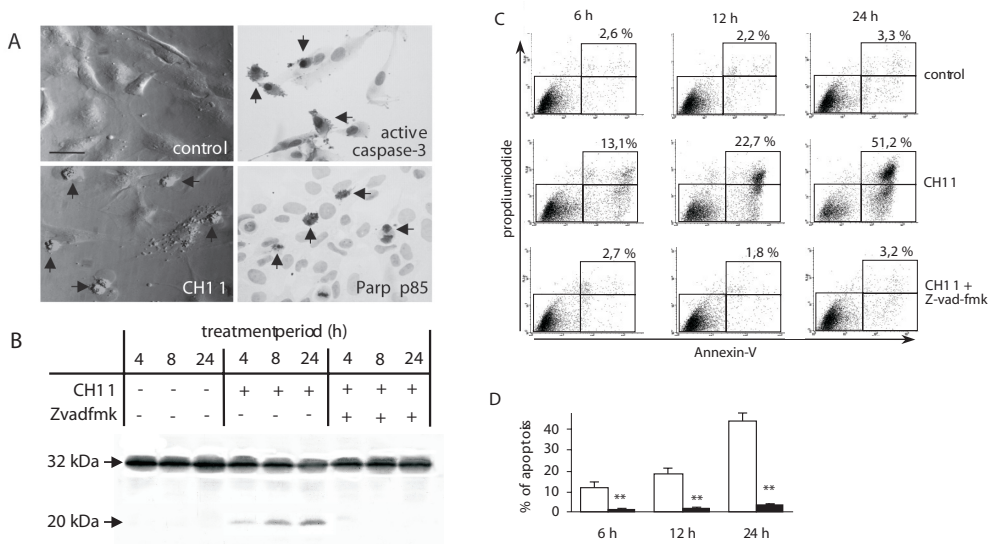

Figure 2. Effect of Fas stimulation on various parameters indicative for apoptosis in HEY cells. A. HEY cells (5 x 10^{-4} cells/ml) were maintained overnight and then treated for 8 h without or with an agonistic anti-Fas antibody (50 ng/ml CH11) in serum-free medium. The left of panel A shows phase contrast images of non-stimulated (control) and CH11-stimulated HEY cells. The right panel shows immunohistochemical staining for active caspase-3 and PARP p85 in CH11-treated HEY cells. Arrows indicate apoptotic cells. B, HEY cells were treated without or with 50 ng/ml CH11 in the presence or absence of 10^{-5}M Z-vad-fmk for 4, 8 and 24 h. Expression of procaspase-3 (32kDa) and active caspase-3 (20 kDa) was determined in whole cell lysates as described in the *Materials and Methods.* C, PS exposure was determined by FACS analysis at various times after treating cells without additions (control), 50 ng/ml CH11 or 50 ng/ml CH11 plus 10^{-5}M Z-vad-fmk as described in the *Materials and Methods.* Values on top of the right quadrant are the sum of the relative number of cells positive for Annexin-V. Data shown are from one representative experiment, which was repeated three times with similar results. D, Quantification of Fas-induced apoptosis in HEY cells following treatment with 50 ng/ml CH11 in the absence (open bars) or presence of 10^{-5}M Z-vad-fmk (closed bars) as described in panel C. Values shown are means ± SEM from four independent experiments. Significantly different from CH11-treated cells; **, P<0.01.

The number of cells displaying PS at the outer leaflet of the plasma membranes increased over time with 12.1 ± 2.4%, 18.3 ± 2.7% and 44.1 ± 4.2% of HEY cells staining positive for Annexin following 6, 12 and 24 hours of CH11 treatment, respectively (fig 2D). Viable cells that lack Annexin staining remained in culture even after 72 hours, indicating that not all HEY cells became apoptotic during CH11 treatment (data not shown). Z-vad-fmk administration significantly inhibited Fas-induced PS externalization within the time frame studied to similar levels ($P<0.01$) as observed in controls (fig. 2C-D).

Expression of LH receptor and LH responsiveness

HEY cells expressed the LH receptor under basal conditions. LH receptor immunostaining was detected intracellularly and on the plasma membrane of HEY cells (fig. 3A). To test whether the LH receptor was responsive to LH in these cells, HEY cells were treated without or with LH (0.1 and 10 ng/ml) in the presence of IBMX (1 mM), a phosphodiesterase inhibitor and total cAMP levels were measured. Treatment with 0.1 ng/ml and 10 ng/ml LH resulted respectively in a 1.3 and 1.4-fold increase in total cAMP levels compared to IBMX control after 60 min (fig. 3B).

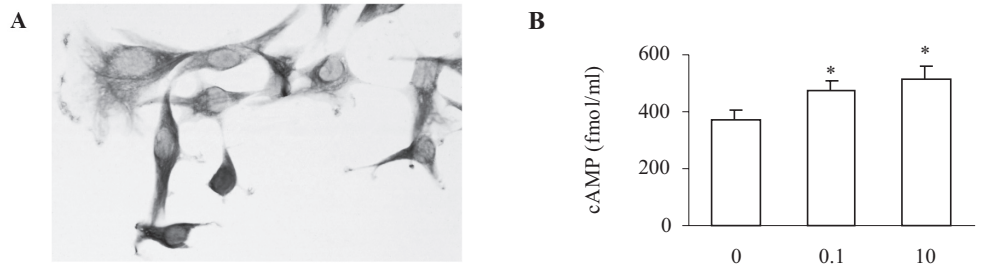

Figure 3. Expression of functional LH receptors in HEY cells. A, HEY cells (5 x 10^{-4} cells/ml) were maintained overnight, then serum-starved for 24 h and LH-R protein expression was determined by immunohistochemistry as described in *Materials and Methods*. B, HEY cells were incubated in serum-free medium for 1 h and then further cultured in the absence (0 ng/ml LH) or presence of (0.1 or 10 ng/ml) LH for 60 min. Total cAMP levels were measured as described in the *Materials and Methods*. Values are expressed as the means ± SEM of quadruplicate incubations from one representative experiment. The experiment was repeated two times. A student's T-test was used to compare the mean of cAMP levels among the groups. Significantly different from control (0 ng/ml LH + IBMX); *, $P<0.05$

LH promotes cell survival by suppressing Fas-induced apoptosis

To asses whether LH modulates the survival of OSE cancer cells, we tested the effect of LH on Fas-induced apoptosis in OSE cells. Treatment of HEY cell cultures with LH resulted in a significant inhibition of CH11-induced apoptosis in a concentration-dependent manner as measured by FACS analysis, using Annexin-V/propidium iodide double staining. A representative experiment is shown in fig 4A. When treated with low doses of LH (0.1 or 1 ng/ml) for 12 hours, the percentage of apoptotic cells was significantly reduced by respectively 22.7 ± 5.3% (n=7) and 29.2 ± 3.1 % (n=11)

compared to CH11-treated cells (fig. 4B). Z-vad-fmk inhibited Fas-induced apoptosis (for a representative experiment see fig 2D). Treatment with a higher dose (10 ng/ml) had no modulating effect on apoptosis in HEY cells. The anti-apoptotic effect of LH observed at 12 hours of culture was transient, as after 24 hours of incubation the amount of Fas-induced apoptosis was similar to cultures treated without LH (fig. 4A). A similar trend was observed when apoptosis was determined by flow cytometry for DNA content in permeabilized cells (data not shown) as described by Guthrie et al (35). The level of apoptosis was somewhat lower with this assay compared to the Annexin-V assay. This may be expected as DNA fragmentation is a late apoptotic event and the detection of Annexin-V is a relatively early apoptotic marker.

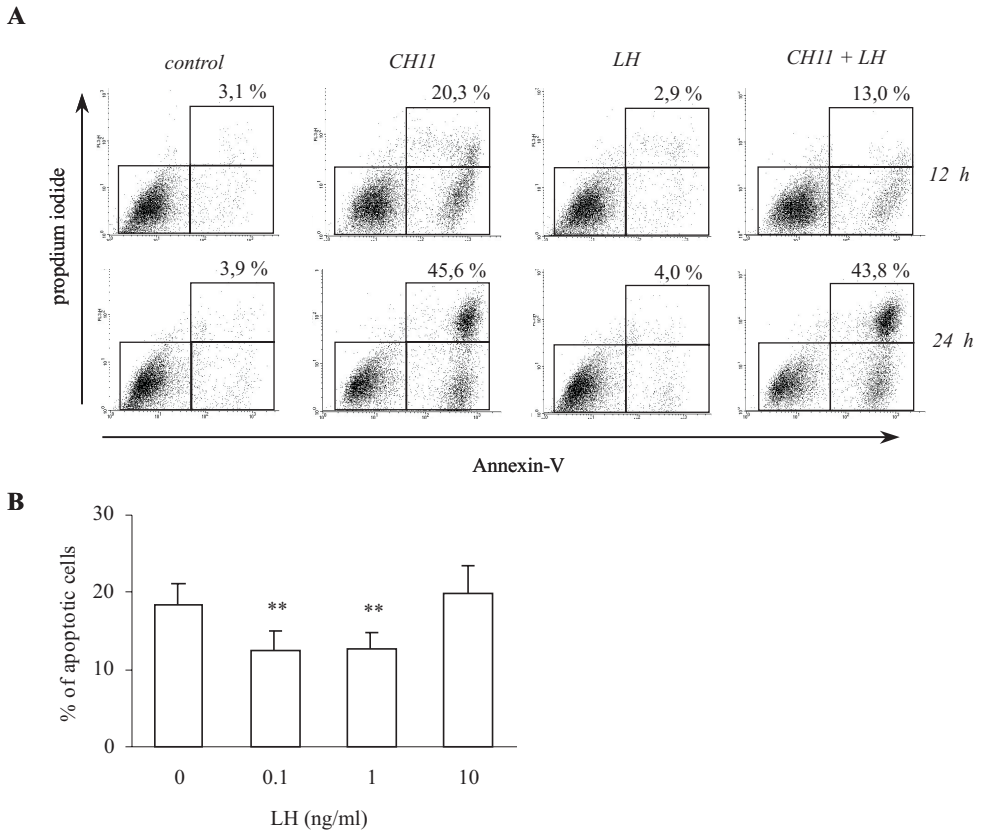

Figure 4. Effect of LH on Fas-induced apoptosis in HEY cells. A, FACS analysis of apoptosis in HEY cells after incubation in serum-free medium in the absence (control) or presence of 1 ng/ml LH and/or 50 ng/ml CH11 for 12 and 24 h. Values on top of the right quadrant are the sum of the relative number of cells positive for Annexin-V. Data shown are from a representative experiment. B, Quantification of Fas-induced apoptosis in HEY cells 12 h after treatment with various concentrations of LH. Apoptotic indices were determined as described in panel A. Values shown are means ± SEM from eight independent experiments. Significantly different from CH11-treated cells; **, P<0.01.

LH does not promote OSE cell survival by cell proliferation

To verify whether the anti-apoptotic effect of LH is caused by increased cell growth, we measured incorporation of BrdU by HEY cells in the absence of serum. HEY cells were proliferating under these serum-free culture conditions. The number of cells that incorporated BrdU decreased over time with 28.7 ± 0.8 % and 7.8 ± 1.2% of HEY cells staining positive for BrdU following 8 and 24 hours of culture, respectively (n=3). LH did not change the levels of BrdU incorporation in HEY cells. After 8 hours of incubation, the percentage of BrdU labeled cells in 0.1, 1 and 10 ng/ml LH-treated HEY cells was 29.2 ± 0.9%, 29.3 ± 1.3% and 29.3 ± 1.3%, respectively, versus 8.1 ± 0.8%, 8.2 ± 1.1% and 8.3 ± 1.1% following 24 hours of culture.

The anti-apoptotic effect of LH on Fas-induced apoptosis was mimicked by PKA activation and reversed by blocking of PKA activity

In steroid producing ovarian follicular cells, the binding of LH to its cognate receptor stimulates adenylyl cyclase, resulting in subsequent production of cyclic AMP and activation of protein kinase A (PKA) (36,37). To investigate whether the LH anti-apoptotic pathway in OSE cancer cells includes cAMP/PKA, we tested whether addition of a cell membrane permeable cAMP analogue (8-CPT-cAMP) mimicked the anti-apoptotic effect of LH in HEY cells. Addition of 10^{-6} M 8-CPT-cAMP has been demonstrated in other cell culture systems to mimic the effects of LH on steroidgenesis (36). Figure 5 shows the effects of respectively an activator and inhibitor of PKA and PKC activity on Fas-induced apoptosis in HEY cells. The percentage of Fas-induced apoptotic cells was reduced significantly by 37.0 ± 2.0% ($p<0,01$) following 8 hours treatment with 8-CPT-cAMP (10^{-6} M). This reduction was similar to what was observed after treatment with 1 ng/ml LH (42.1 ± 3.7%) (fig. 5). No significant additive effects on the inhibition of Fas-induced apoptosis were observed after combined treatment of 10^{-6} M 8-CPT-cAMP and 1 ng/ml LH, compared to single LH or 8-CPT-cAMP treatment. The anti-apoptotic effect of 8-CPT-cAMP was not due to increased cell growth, as no significant differences were observed in the percentage of BrdU labeled HEY cells in the absence or presence of 10^{-6} M 8-CPT-cAMP (respectively 28.7 ± 0.8% versus 30.7 ± 0.9%). The effect of the cAMP analogue was, however, biphasic. In contrast to the inhibitory dose of 10^{-6} M 8-CPT-cAMP, no effect was observed in HEY cells treated with 10^{-5} M 8-CPT-cAMP while a high dose of 8-CPT-cAMP (10^{-4} M) significantly enhanced Fas-induced apoptosis by approximately 40%. High doses of cAMP analogues have been shown before to induce or accelerate apoptosis in various human cancer cell lines (38). Moreover, BrdU incorporation was also significantly increased in 10^{-4} M 8-CPT-cAMP treated HEY cells (34.7 ± 1.2 % versus 28.7 ± 0.8% in non-treated cells ($P<0.05$)) suggesting an enhanced cell turnover in the presence of high concentrations of cAMP analogues. Furthermore, we have investigated whether an inhibitor of protein

kinase A activity (H89) could antagonize the anti-apoptotic effect of LH in HEY cells (fig. 5). H89 by itself did not affect the level of apoptosis (data not shown), but the anti-apoptotic effect of LH (fig. 5) and of 10^{-6} M 8-CPT-cAMP (data not shown) on Fas-induced apoptosis was almost completely reversed when 10^{-6} M H89 was added to the culture medium.

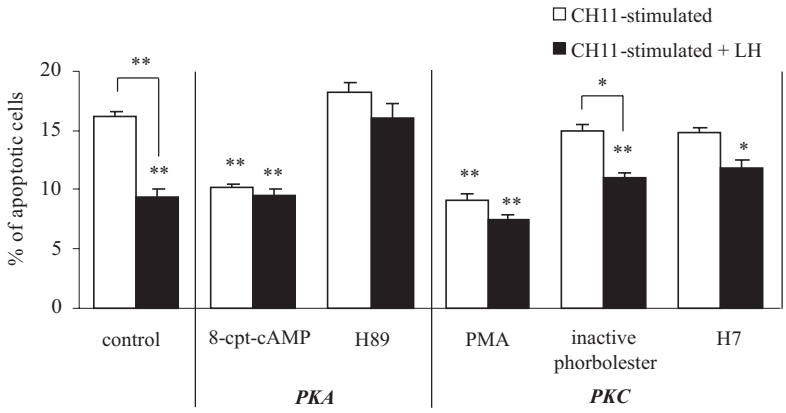

Figure 5. Effect of PKA/PKC signaling on Fas-induced apoptosis. HEY cells were treated for 8 h with 50 ng/ml CH11 (open bars) or 50 ng/ml CH11 plus 1 ng/ml LH (closed bars) in the absence or presence of various compounds: 10^{-6}M 8-CPT-cAMP (n=8), 10^{-6}M H89 (n=8), 10^{-6}M PMA (n=8), 10^{-6}M inactive phorbolester (n=6) or 10^{-6}M H7 (n=4). Apoptotic indices were determined by FACS analysis and quantified as described in the legend of *Fig. 4*. Values represent the mean ± SEM from four to eight independent experiments. Significantly different from cells treated only with CH11; **, P<0.01 or *, P<0.05

To investigate the possibility that LH also signals via protein kinase C, HEY cells were treated with respectively an activator and inhibitor of PKC activity (fig. 5). In the presence of the phorbolester PMA (10^{-6} M), a stimulator of protein kinase C activity, the number of Fas-induced apoptotic cells was significantly reduced compared to untreated Fas-stimulated cells, while no significant additive effect on the inhibition of Fas-induced apoptosis was observed after combined treatment with LH and PMA. Treatment with an inactive phorbolester (10^{-6} M) in either the absence or presence of LH did not affect the number of Fas-induced apoptotic cells. Additionally, treatment with H7 (10^{-6} M), an inhibitor of protein kinase C activity by itself did not affect the level of apoptosis (data not shown). H7 only partially prevented the anti-apoptotic effect of LH in HEY cells (fig. 5). Moreover, no significant differences were observed between CH11-stimulated HEY cells treated with LH in the absence or presence of H7. Thus, the anti-apoptotic effect of LH on CH11-induced apoptosis was mimicked by PKC activation, but not completely reversed by blockade of PKC activity. Though the involvement of PKC cannot be completely excluded, our observations suggest that LH exerts its anti-apoptotic effect predominantly through activation of the cAMP/PKA system.

Discussion

The results of the present study suggest a direct effect of LH on the survival of the OSE cancer cell line HEY following Fas-receptor activation. HEY cells expressed both functional LH and Fas receptors; addition of LH resulted in cAMP production, suggesting that the LH receptor is functional, while stimulation of the Fas receptor induced apoptosis in these cells. LH did not affect cell proliferation in the absence of serum and growth factors, but affected OSE cell survival through the inhibition of Fas-induced apoptosis. This anti-apoptotic effect of LH could be mimicked by a membrane-permeable cAMP analogue (8-CPT-cAMP), while treatment with a protein kinase A (PKA) inhibitor (H89) reversed the anti-apoptotic effect of LH. These observations suggest that LH exerts its anti-apoptotic effect predominantly through activation of PKA.

The Fas system has been shown to play a major role in regulating ovarian homeostasis by triggering apoptosis in various ovarian cell types, such as granulosa and theca cells (22,23,39). A role for Fas-induced apoptosis has also been implicated in the normal ovarian surface epithelium. Ovarian surface epithelial cells undergo apoptosis to facilitate ovulation as has been demonstrated in the goat (40) and rat (see chapter 2). This apoptotic process presumably takes place via activation of the Fas pathway, as OSE cells at the ovulatory site expressed Fas, Fas ligand and active caspase-3 (see chapter 2). The Fas apoptotic machinery is also present in OSE cells at the tumor site and in OSE cells lining irregular inclusions cysts which are thought to have under certain conditions the capacity to develop into epithelial cancers (41). Despite the presence of the Fas pathway, apoptotic cells were relatively scarce in OSE tumors (see chapter 5).

Fas and Fas ligand expression in HEY cells is in accordance with previously published data in OSE tumor cells (23-25,42,43). The presence of the Fas pathway seems to reflect the ability of OSE cancer cell lines to undergo Fas-induced apoptosis. The degree of Fas-induced cell death may vary, as we found low numbers of apoptosis and immunostaining for Fas, Fas ligand and procaspase-3 in another OSE cell line, Caov-3 cells (data not shown), while abundant expression of these antigens and much higher sensitivity to Fas activation were found in the HEY cell line. Dysregulation of the Fas system may contribute to ovarian tumor development and progression as has been suggested by Ghahremari et al (44). Nevertheless, the mechanisms by which apoptosis is inhibited and the survival factors involved remain to be determined.

Gonadotropins have been reported to play a major role in the regulation of ovarian apoptosis. It has been shown that FSH or human chorionic gonadotropin (hCG) suppressed the spontaneous onset of follicular DNA fragmentation in serum free cultures

of bovine preovulatory follicles (45). Furthermore, treatment of hypophysectomized immature rats with FSH decreased granulosa cell apoptosis in vivo (46). Despite these observations, not much is known about the effects of gonadotropins on OSE cell growth or survival. In the present study, we demonstrated that activation of Fas-induced apoptosis in the OSE cancer cell line HEY could be blocked, in part, by treatment for 12 hours with the gonadotropin LH, in a relative low concentration of 0.1-1.0 ng/ml. The observation that 10 ng/ml LH did not inhibit Fas-induced apoptosis may be due to desensitization of the LH-receptor (37). Doses of LH, in the range of 10-100 ng/ml, have been described to induce a rapid desensitization of the LH receptor in different cell systems (47,48). In granulosa cells, on the other hand, it has been found that such high doses of gonadotropic hormones induced a short-lasting cAMP increase, with no effect on apoptosis (49). A similar transient inhibitory effect of LH on Fas-induced apoptosis was observed in response to a membrane permeable cAMP analogue, which is in line with some previous studies (50,51). Cyclic AMP might act upstream of caspase-3 activation to delay the activation of the apoptotic pathway as has been postulated in human neutrophils (50,51). Eventually, such an anti-apoptotic block may be overruled by other death signaling pathways. A delay in cell death as induced by LH may under certain circumstances increase the chance of survival of mutated OSE cancer cells *in vivo*. Moreover, it might change the ability of cancer cells to respond to chemotherapeutic agents. For example, cisplatin, which is the preferred chemotherapeutic agent for treatment of OSE cancer, has been shown to sensitize cancer cells to Fas-mediated apoptosis, by modulating several components of this apoptotic pathway. In ovarian cancer cell lines, cisplatin can upregulate Fas and Fas ligand (27,28) resulting in caspase-3 activation and eventually in apoptosis. Moreover, it was demonstrated that hCG, an LH homologue, lowered the chemosensitivity to the anticancer drug cisplatin in the OSE cancer cell line OVCAR-3 (10). It was hypothesized that the hCG-induced inhibition of ciplatin-induced apoptosis was dependent on the presence of LH-Rs, as in cells lacking LH-Rs, i.e. the SKOV-3 cell line, hCG failed to block cisplatin-induced cell death (10).

To obtain more insight in the role of LH in the inhibition of Fas-induced apoptosis in OSE cells, we have investigated whether the survival effect was indeed the result of inhibition of apoptosis or was caused by stimulation of HEY cell growth. Several studies have demonstrated that certain OSE cancer cells undergo enhanced cellular proliferation following LH addition (3,13-16,52). However, also decreased (20,21) or unchanged (17-19) OSE cell proliferation in response to LH have been reported. In the HEY cell line, proliferation rates as measured by the use of a BrdU incorporation assay were not affected following supplementation with LH under serum and growth factor free conditions. This suggests that the observed increased OSE survival was due to inhibition of apoptosis and not to a modulation of cell proliferation.

We have also analyzed the signal transduction pathway of LH in these cells. In ovarian follicular cells, it is well established that LH interacts with its cognate receptor and stimulates a G_s-protein that leads to the production of cyclic AMP, followed by activation of PKA and subsequently steroid production. Interestingly, the second messenger molecule cAMP also modulates the apoptotic program in these cells; when follicles were treated in vitro with cAMP analogues, apoptosis was prevented (46,53). In non-follicular cells, cAMP and cAMP-dependent PKA have been shown to modulate apoptosis in a wide variety of ways. In human neutrophils, cAMP analogues activate PKA resulting in a delay or suppression of apoptosis induced by either TNF-α or Fas. Inhibitors of PKA activity could antagonize the suppressive effect of cAMP on TNF-α-induced apoptosis (50,51,54). In contrast, in various human cancer cells, cAMP analogues have been demonstrated to induce or accelerate apoptosis (38).

Up to now, there was no evidence for activation of PKA in OSE cancer cells, nor on the regulation of PKA activity by LH in these cells. In the present study, we have demonstrated that HEY cells respond to LH with enhanced cAMP production. Moreover, we showed that a cAMP analogue could mimic the anti-apoptotic effect of LH, and that an inhibitor of PKA could reverse this effect. Previously, it has been demonstrated that the extent and the duration of the intracellular rise in cAMP might play an important role in controlling the rate and extent of apoptosis in cultured granulosa cells (49). In addition, very low doses of LH have been shown to elicit physiological actions in a variety of cells concomitant with barely detectable cAMP responses (55,56). A small but significant 15% increase in cAMP levels in a lung cancer cell line was able to inhibit UV-induced apoptosis (57). Possibly, the anti-apoptotic effect of LH is exerted by small increases of cAMP concentrations acting at discrete cellular locations and by activation of multiple signaling pathways in addition to PKA, such as the PKC pathway (58). Activation of PKC by treatment with the phorbol ester PMA has been demonstrated to block Fas-induced apoptosis in human T-cells and Jurkat cells (59). The present study demonstrates that Fas-induced apoptosis is also blocked by PKC activation in the OSE cell line HEY. However, the PKC inhibitor, H7 could not completely antagonize the effect of LH, implicating that the anti-apoptotic effect of LH most likely does not signal predominantly through PKC.

Gonadotropins have been suggested to play a role in the etiology of ovarian surface epithelium cancer, as several cases have been reported of OSE cancers arising in infertile women during or after prolonged treatment with gonadotropins. Moreover, the incidence of OSE cancer is increased in women during the years after menopause when serum gonadotropin levels are high, i.e. within the range of 7 to 47 IU/L (60), which corresponds to approximately 1 - 7 ng/ml LH. The transient anti-apoptotic effect of LH in OSE cancer cells, as demonstrated in the present study may be relevant for future

treatment of OSE cancer. Further research is required to extrapolate our *in vitro* results to the *in vivo* situation.

Acknowledgments

This study was supported in part by a grant from the Jan Dekkerstichting and dr. Ludgardine Bouwmanstichting, The Netherlands. We would like to thank Dr. C. van de Lest and Prof. W. Stoorvogel (Department of Biochemistry & Cell Biology, Utrecht University) for their support in the statistical evaluation and for reviewing this paper, respectively.

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Chapter 7

Summarizing Discussion

SUMMARIZING DISCUSSION

Cell biologists have spent many decades searching for regulatory mechanisms that sustain the life span of cells. These efforts are based predominantly on the clinical implications of modulating inappropriate cell survival in diseases associated with excessive accumulation or degeneration of cells. Research has revealed that constant trophic receipt of growth factors and hormones is essential for cells to stay alive. If trophic support is absent or withdrawn, cells often undergo programmed cell death (apoptosis). Consequently, knowledge of the process of apoptosis is needed in order to understand the regulation of cell survival. This thesis focuses on the hormonal regulation of apoptosis in the ovary. Complex interactions with various hormones and growth factors are required to maintain ovarian homeostasis, which makes the ovary a suitable model to study the hormonal control of apoptosis. Moreover, any imbalance between proliferation and apoptosis may contribute to the pathogenesis of a variety of ovarian diseases. For example, excessive apoptosis may result in early exhaustion of follicle stores with the consequence of reduced fertility. Conversely, moderate or deficient apoptosis may promote unwanted tissue growth, contributing to ovarian cancer development. Hence, a better understanding of the hormonal control of programmed cell death in the ovary may help to obtain more insight in the basic principles of apoptosis and, moreover, to provide new treatment strategies for ovarian disorders, such as premature ovarian failure and ovarian cancer.

Mammalian ovary as a model to study the hormonal regulation of apoptosis

The ovary might initially seem an unlikely place to search for death, as it harbors the germ cells that potentially give rise to future life. Nevertheless, cell death plays an important role in this tissue. In the human ovary, approximately 6 million germ cells are formed during fetal life, but due to massive loss during prenatal and postnatal life, only 400,000 are left at the onset of puberty. Approximately, only 400 of these follicles will ovulate during fertile life. At the time of menopause just a few follicles are left in the ovary, suggesting that more than 99.9% of human follicles undergo degenerative changes during female reproductive life (1). Such massive degeneration of follicles occurs throughout all vertebrate classes. It may seem an incredibly wasteful and expensive way in terms of biological energy costs, to generate such an oocyte surplus in great excess, which is eliminated thereafter at different stages of development. One possible evolutionary advantage of atresia may be to remove all damaged or unwanted follicles, for instance follicles that contain chromosomal abnormalities, and to select only the fittest and healthiest oocytes to forward their DNA towards the next generation (2). Although the underlying mechanisms for the massive death of both somatic and germ cells are not yet clearly defined, it is generally accepted that follicle degeneration is the result of the process of follicular atresia, which involves apoptosis of oocytes, granulosa

cells and eventually theca cells. Atresia is considered to be the default pathway of follicles that occurs when cells fail to receive sufficient trophic support required to suppress the apoptotic command.

Apoptosis in the ovary is tightly linked to the estrous cycle

The major regulatory players in the process of atresia are assumed to be the Fas system and the Bcl-2 family members (3-7). However, little is known regarding the hormonal factors that trigger expression of components of the Fas signaling pathway. So far, the general idea of the hormonal regulation of apoptosis has been extrapolated from *in vitro* and *in vivo* models, in which apoptosis is often induced artificially. Surprisingly, not much is known about follicle survival and atresia under normal physiological conditions even though apoptosis in the ovary is linked to estrous cycle related variations in hormonal secretions. In a straightforward investigation, as described in chapter 2, we have examined whether the components of the Fas signaling pathway were differentially expressed throughout the estrous cycle in the rat ovary. We have demonstrated that the expression levels of components of the Fas apoptotic pathway change in the ovary throughout the estrous cycle and are related to fluctuations in hormone levels. The observed reduction in expression of the proapoptotic proteins Fas, Bax and caspase-3, and the stable expression of Fas ligand and anti-apoptotic Bcl-2 in the rat ovary around the time of ovulation may reflect physiological survival mechanisms to ensure ovulation of the dominant follicles, corpus luteum formation and ovarian epithelial (OSE) cell repair (chapter 2). We have concluded that apoptosis of the various ovarian cell types under physiological conditions seems to depend on hormonal support as well as on the presence of the Fas system and Bcl-2 family members.

Regular menstrual/estrous cycles and follicular survival are dependent on the cyclic release of gonadotropins. The fluctuating pattern of gonadotropin release in fertile women is dependent on a complex interplay of positive and negative feedback regulation by hypothalamic gonadotropin-releasing hormone (GnRH) as well as by ovarian steroids (fig. 1). The key controller of the hypothalamic-pituitary-ovarian axis in all vertebrate species is GnRH, which is synthesized by the hypothalamus and released into the hypophyseal-portal circulation. GnRH drives the synthesis and secretion of the gonadotropins, i.e. follicle stimulating hormone (FSH) and luteinizing hormone (LH), into the systemic circulation. These gonadotropins, but also ovarian sex steroids (estrogens, androgens and progesterone) that are released under the influence of gonadotropins contribute to follicular survival. Changes in the hypothalamic-pituitary-ovarian axis resulting in dysregulation of GnRH, gonadotropin, or ovarian steroid hormone production, have been linked to abnormalities in the menstrual/estrous cycle, reduced ovarian function and fertility. Several conditions, such as reproductive aging, exposure to chemical

endocrine disruptors, physical and emotional stress, biological clock rhythm disturbers, and extreme energy expenditure or reduced energy intake have been shown to modify the cyclic release of gonadotropins and thus fertility.

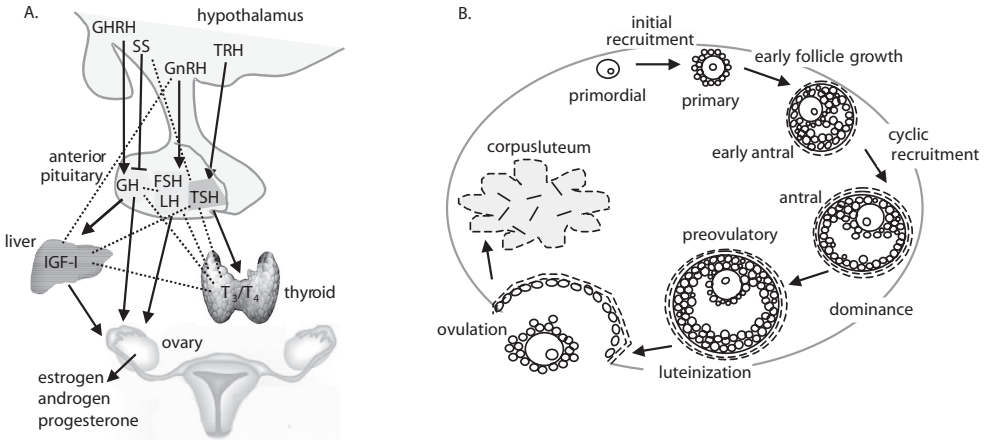

Figure 1. Hormonal regulation of follicular survival. A. Simplified chart illustrating the interactions of the gonadotropic, somatropic and thyrotropic axes (excluding feedback mechanisms). Numerous interactions link these 3 axes at the hypothalamic, pituitary and ovarian level that in turn may affect follicular survival B. Diagram of the ovary showing the various stages of follicles through development, ovulation, corpus luteum formation and regression.

Hormonal regulation of follicular fate by thyroid and growth hormone

Hormones involved in the regulation of the energy metabolism, such as growth hormone (GH), insulin-like growth factor (IGF-I), insulin, leptin or thyroid hormone (T_3/T_4) control many aspects of growth and nutrient metabolism. They also seem to play a role in ovarian function by interacting with the hypothalamic-pituitary-ovarian axis, thereby modulating the secretion of GnRH, gonadotropins, prolactin, androgens and estrogens (8). Women with GH and thyroid hormone disorders often require assisted reproductive treatment to improve fertility, which demonstrates the important role of these hormones in normal female reproduction (8,9). The importance of thyroid hormone on ovarian function was tested in adult rats in which a mild form of hypothyroidism was induced (chapter 4). Our data suggest that hypothyroidism resulted in irregular estrous cycles and reduced litter sizes. At the ovarian level, hypothyroidism reduced follicular survival from the preantral to the antral stage due to inhibited follicular growth and increased levels of follicular atresia. Since granulosa cells express thyroid hormone receptors, it is very well possible that thyroid hormone may directly mediate follicular function (10,11). Thyroid hormones may also affect the ovary indirectly via the gonadotropic or somatropic axes. Since hypothyroid rats display hypersecretion of prolactin, which can block the production and action of gonadotropins (12), the reduced follicular survival during hypothyroidism may be due to diminished FSH responsiveness. Moreover, hypothyroid

rats showed an increased activity of somatostatin neurons in the hypothalamus and reduced levels of GH and IGF-I in the circulation (13). Consequently, it was hypothesized that a decline in GH and IGF-I production may lead to a decline in the number of healthy growing follicles (9). Indeed, GH receptor null mice (GHR/GHBP-KO mice; chapter 3) displayed irregular estrous cycles and reduced litter sizes (14-16), comparable to hypothyroid rats (chapter 4). Our study indicated that adult GHR/GHBP-KO mice contained higher numbers of primordial follicles, lower numbers of healthy growing primary, preantral and antral follicles and had increased numbers of atretic follicles (chapter 3). Moreover, we demonstrated that GH might affect the primordial follicle pool through IGF-I, since postnatal IGF-I treatment of GHR/GHBP-KO mice decreased the number of primordial follicles to the level observed in wild-type mice. Since the absence of GH signaling increases GH levels but decreases IGF-I, insulin and estradiol levels in serum, it is likely that GH also influences follicular survival due to indirect alterations in systemic IGF-I, insulin and/or estradiol levels. As a consequence, this may lead to decreased GnRH and gonadotropin secretion at the hypothalamic and pituitary level respectively, or diminished gonadotropin responsiveness at the ovarian level (17).

Due to the close functional interaction between the gonadotropic, somatropic and thyrotropic axes in the regulation of follicular development and degeneration in the ovary (fig. 1), the individual contributions of the various hormones are difficult to establish by an *in vivo* approach. Additionally, it is difficult to determine whether the observed effects of GH or thyroid hormone on follicular survival are mediated via changes in gonadotropin release or caused by direct effects on the ovary. Potential roles for GH and thyroid hormone on, for instance, growth factor production, might be explored by using genomic and proteomic databases. Such knowledge provides new possibilities for studying the apoptotic pathway in follicular atresia and to allow designing treatments to improve fertility. It should, however, be taken into account that age-related increased chromosomal defects in the oocyte may occur upon prolonging the fertile lifespan. A recent study suggested that the fertile life span was prolonged in Bax deficient mice through reduced rates of follicular atresia (18). Such interferences with apoptotic machineries can, however, not be translated to clinical applications due to the negative side effects that would be imposed on health. At present, the most logical and possible approach to expand the reproductive life span and fertility in women, is to improve survival of the reduced number of follicles left in the ovary by hormone replacement therapy (HRT). The obvious drawback of HRT is the increased risk of cancer in steroid-sensitive reproductive tissues, such as breast tissue, the uterus and the ovary. Recent studies have indeed demonstrated that the risk of developing ovarian cancer increases with HRT (19) and the duration of postmenopausal estrogen replacement therapy (20,21).

Apoptosis and the etiology of ovarian cancer

In cancer, the balance between proliferation and programmed cell death is disturbed and diminished apoptosis allows cells with genetic abnormalities to survive. Previous research has suggested that inhibited apoptosis may contribute to ovarian cancer progression (22-24) (reviewed in chapter 1). The majority of the human ovarian cancers are thought to arise from sequestered OSE cells that line the wall of inclusion cysts (fig. 2A). In order to identify whether inhibition of apoptosis in inclusion cysts is an early event in ovarian carcinogenesis, we have examined the expression pattern of various markers for apoptosis and cell proliferation in regularly and irregularly shaped inclusion cysts (chapter 5). According to our findings, sequestered columnar OSE cells of irregularly shaped inclusion cysts display increased expression of Ki67, Fas, Fas ligand and procaspase-3, suggesting that the Fas system and its related proteins may contribute to balance cell growth with cell death. Apoptosis and active caspase-3, however, are largely absent, despite increased proliferating activity, indicating that apoptosis through activation of Fas might be impaired. Such defects in the apoptotic pathway may allow sequestered OSE cells with genetic abnormalities to survive. This may lead to the persistence of these cysts and propagation of mutations, therewith increasing the chance of tumor formation and progression.

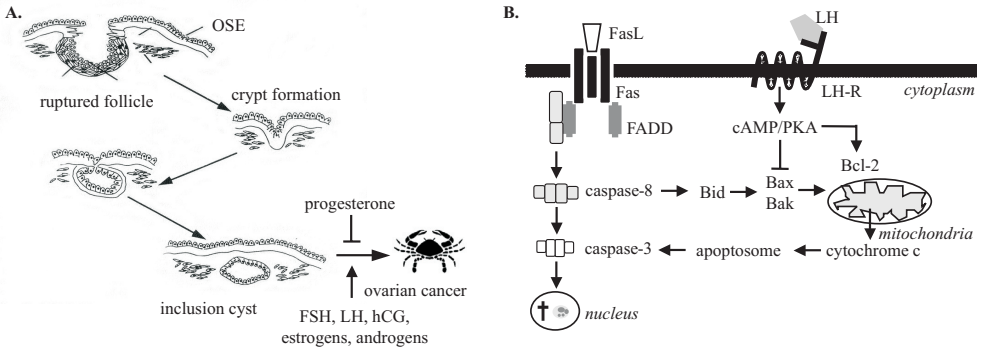

Figure 2. Hormonal regulation of ovarian cancer cell survival. A. Hypothetical illustration showing a role for pituitary and sex hormones in the etiology of ovarian cancer. B. Hypothetical mechanism of gonadotropin action on ovarian surface epithelial cells demonstrating that LH might affect OSE cell survival through inhibition of Fas-induced apoptosis.

Hormonal regulation of ovarian cancer cell fate

A growing body of evidence indicates that gonadotropins play an important role in the etiology of ovarian cancer; reduced gonadotropin levels due to the use of oral contraceptives, pregnancy or breastfeeding reduce the risk of ovarian cancer (25,26) (reviewed in chapter 1). Case studies have reported development of ovarian cancers in women undergoing prolonged fertility treatment with high doses of gonadotropins (27,28). It is possible that the development of ovarian cancer in these young women is

due to the direct action of gonadotropins on OSE cells. A role for sex steroids in the etiology of ovarian cancer can, however, not be excluded, though this seems less likely. The incidence of ovarian cancer peaks in the years after menopause when gonadotropin levels are elevated (29), and ovarian production of progesterone and estrogen has ceased. Moreover, the majority of ovarian cancers express gonadotropin receptors and some, but not all, primary ovarian cancer cells and cell lines have been shown to respond to gonadotropins (FSH and/or LH/hCG) with stimulated cell proliferation (30-35). We have found that LH did not affect cell proliferation but enhanced OSE cancer cell survival through affecting protein kinase A (PKA) activity (chapter 6). Moreover, it was demonstrated that activation of Fas-induced apoptosis in the OSE cancer cell line HEY could be blocked, in part, by treatment with LH. Although the observed inhibitory effect of LH on Fas-induced apoptosis was transient, a delay in cell death induced by LH might under certain circumstances increase the chance of survival of mutated OSE cancer cells *in vivo*. Further research is required to extrapolate our *in vitro* results to the *in vivo* situation. Considering the number of women receiving fertility treatment nowadays and the expected growing demand for ovulation induction during the coming years, it is of major importance to elucidate the role of gonadotropins in the development and progression of epithelial ovarian cancer. This putative risk should be discussed with all patients before assisted reproductive treatment is commenced.

Additional information on the regulatory mechanism of gonadotropins in suppressing apoptosis of OSE cancer cells may also have therapeutical value in treatment of ovarian cancer. Since anti-cancer drugs kill cancer cells by inducing apoptosis, the efficiency of cancer treatments depends on the ability of the cell to respond to apoptotic stimuli (36). For example, cisplatin, which is the preferred chemotherapeutic agent for treatment of OSE cancer, sensitizes cancer cells to Fas-mediated apoptosis by modulating several components of this apoptotic pathway (37,38). *In vitro*, hCG has been shown to lower the chemosensitivity to cisplatin by inhibiting apoptosis (39). In a preliminary study, we showed that the inhibitory effect of LH on Fas-induced apoptosis in OSE cancer cells was accompanied by an increased ratio of anti-apoptotic versus proapoptotic Bcl-2 family proteins (40). It is possible that PKA activation enhances OSE cancer survival through inhibition of apoptosis by modulating the expression of Bcl-2 protein family members (fig. 2). Further studies are required to find out whether gonadotropins or downstream signaling components might indeed change the ability of ovarian cancer cells to respond to a chemotherapeutic agent. Similarly, the potency of using GnRH analogs, that lower the gonadotropin levels, in combination with chemotherapeutic regimes, have to be tested *in vivo* to evaluate such a combination therapy can be used as an effective anti-ovarian cancer therapy.

In conclusion

This thesis provides substantial data about a functional relationship between hormonal support, the presence of components of the Fas signaling pathway and apoptosis *in vitro* and *in vivo* in a variety of ovarian cell types. Continuous hormonal support may suppress the Fas apoptotic pathway, whereas diminished hormonal support may trigger expression of the Fas signaling components and eventually apoptosis in ovarian cells. Future research should focus on the identification of the hormonal and molecular mechanism of follicular atresia and ovarian cancer cell death, as this may facilitate new perspectives for the treatment of infertility and ovarian cancer.

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Appendices

NEDERLANDSE SAMENVATTING

Eén van de verschijnselen die celbiologen de afgelopen jaren uitvoerig hebben bestudeerd is het mechanisme dat de levensduur van een cel bepaalt. Dit wordt met name ingegeven door de mogelijke klinische implicatie die dit kan hebben voor ziekten geassocieerd met een overmatige degeneratie of ophoping van cellen, zoals bijvoorbeeld het geval is bij het ontstaan van kanker. Onderzoek heeft aangetoond dat de aanwezigheid van bepaalde 'overlevingsfactoren', zoals hormonen en groeifactoren, essentieel is voor cellen om in leven te blijven. Daalt de concentratie van deze factoren in de directe omgeving van cellen, dan ondergaan ze dikwijls 'apoptose', een vorm van geprogammeerde celdood. Het woord apoptose is afgeleid van het Griekse woord dat vrij vertaald 'het vallen van bladeren van een boom in de herfst' betekent. Celbiologen gebruiken deze term ter aanduiding van het feit dat cellen in weefsels volgens een vastgesteld programma zelfmoord kunnen plegen in het geval ze onherstelbaar beschadigd of overbodig zijn geworden. Apoptose speelt dus een cruciale rol in het correct functioneren van een weefsel.

Het doel van het in dit proefschrift beschreven onderzoek is het verkrijgen van meer inzicht in de hormonale regulatie van apoptose in de ovaria. In de ovaria bestaan complexe interacties tussen verschillende hormonen en groeifactoren die de balans tussen celdeling en celdood nauwkeurig reguleren. Deze balans kan echter onder bepaalde omstandigheden ontregeld raken waardoor een verstoring kan optreden in de het proces van follikelontwikkeling. Een overmaat aan apoptose kan resulteren in een verstoorde follikelontwikkeling hetgeen kan leiden tot een vroegtijdige uitputting van de follikelvoorraad met als mogelijk gevolg onvruchtbaarheid (prematuur ovarium falen). Anderzijds kan verminderde apoptose ongewenste weefselgroei bevorderen, hetgeen tot een verhoogde kans op tumorvorming kan leiden. Onderzoek naar de manier waarop hormonen celdoodprogramma's in de ovaria kunnen activeren dan wel onderdrukken, kan bijdragen aan een beter inzicht in de basis principes van apoptose, en bovendien nieuwe aangrijpingspunten bieden voor de behandeling van ovariële aandoeningen, zoals prematuur ovarium falen en ovariumkanker.

De ovaria lijken mogelijk in eerste instantie een onwaarschijnlijke plek voor de bestudering van celdood, omdat hier de eicellen liggen opgeslagen, de potentiële kiemcellen van nieuw leven. Desalniettemin speelt celdood een belangrijke rol in dit weefsel. Hoewel een grote hoeveelheid eicellen (ongeveer 6 miljoen primaire eicellen) wordt aangelegd tijdens de foetale ontwikkeling, gaan veel eicellen en follikels reeds verloren gedurende het prenatale en vroege postnatale leven, nog voordat de vruchtbare periode (menarche) is bereikt. In tegenstelling tot de testes van een man, worden er in de eierstokken van een vrouw, nauwelijks nieuwe geslachtscellen gevormd na de geboorte. Het gevolg is dat er ten tijde van de start van de puberteit nog slechts ongeveer 400,000 eicellen in de vorm van follikels over zijn. Ongeveer 400 van deze follikels komen tot volledige maturatie en ovulatie gedurende de

vruchtbare periode van het leven van de vrouw. De resterende follikels gaan tussentijds ten gronde. Indien nog aanwezig in het ovarium rond de menopauze zijn ze (± 1000 follikels) veelal niet of nauwelijks nog in staat te reageren op stimulatie door gonadotrope hormonen, waardoor zowel de vruchtbaarheid als een adequate productie van vrouwelijke hormonen niet langer gewaarborgd kan blijven. Dit betekent dat in het humane ovarium tijdens de vruchtbare periode meer dan 99,9% van de follikels, die aanwezig zijn ten tijde van de start van de puberteit vroegtijdig degenereren. Een dergelijke massale teloorgang van follikels vindt overigens ook plaats in andere zoogdieren. In termen van biologische energetische kosten lijkt het een onwaarschijnlijke verspilling en kostbare manier om eicellen in overschot te genereren, waarna het merendeel tijdens verschillende fasen van de ontwikkeling verloren gaat ten gevolge van 'follikulaire atresie'. Een mogelijk evolutionair voordeel van atresie kan zijn dat op deze wijze voorkomen wordt dat eicellen met ernstige DNA schade hun genetisch materiaal kunnen overdragen aan een volgende generatie en dus slechts de beste eicellen in staat zijn om hun erfelijk materiaal door te geven.

Er zijn aanwijzingen dat verschillende signaaleiwitten, in het bijzonder het Fas systeem en leden van de Bcl-2 familie, een belangrijke rol spelen bij de regulatie van apoptose in het ovarium. Er is echter weinig bekend over de wijze waarop de activiteit van het Fas systeem gereguleerd wordt in het ovarium onder normale fysiologische omstandigheden. In hoofdstuk 2 is daarom het voorkomen van apoptose in relatie tot het Fas systeem onderzocht gedurende de oestrische cyclus in de rat. De rat is een veel gebruikt modeldier voor onderzoek naar de regulatie van de voortplanting. In dit hoofdstuk werd aangetoond dat de expressie niveaus van verschillende componenten van het Fas systeem in de ovaria afhankelijk zijn van het stadium van de oestrische cyclus. Deze verandering bleken gerelateerd te zijn aan schommelingen in hormoonspiegels. De waargenomen afname in de expressie van apoptose inducerende eiwitten zoals Fas, Bax en caspase-3, en de stabiele expressie van Fas ligand en het apoptose remmende eiwit Bcl-2 ten tijde van de ovulatie lijkt mogelijk een fysiologisch overlevingsmechanisme. Dit mechanisme maakt achtereenvolgens ovulatie van dominante follikels, vorming van corpus lutea en het sluiten van het oppervlakte epitheel rondom de ovulatiewond mogelijk (hoofdstuk 2).

Een regelmatige menstruele of oestrische cyclus en de overleving van follikels zijn afhankelijk van de cyclische afgifte van gonadotrope hormonen. De fluctuatie in de afgifte van gonadotrope hormonen staat op zijn beurt onder invloed van een complex hormonaal samenspel tussen hypothalamus, hypofyse en ovarium. De hypothalamus is het gebied in de hersenen, dat ondere andere het hormoon 'gonadotropin releasing hormone' (GnRH) produceert, dat de hypofyse, een klier gelegen aan de basis van de hersenen, aanzet tot de uitscheiding van een tweetal gonadotrope hormonen; het follikel stimulerend hormoon (FSH) en het luteïniserend hormoon (LH). De gonadotrope hormonen op hun beurt beïnvloeden de productie en afgifte van de de geslachtshormonen (oestrogenen, progestagenen

en androgenen) door de ovaria. Zowel de gonadotrope hormonen als ook de geslachtshormonen reguleren de overleving van follikels door beïnvloeding van het Fas systeem (hoofdstuk 2). Veranderingen in het functioneren van deze zogenaamde hypothalamus-hypofyse-ovarium as kan door verschillende omstandigheden worden beïnvloed, zoals emotionele factoren, stress, blootstelling aan toxische hormoonverstorende stoffen, verminderde voedsel opname en extreme energie uitgave. Deze veranderingen kunnnen uiteindelijk resulteren in abnormaliteiten in de menstruele of oestrische cyclus en verminderde ovariumfunctie en fertiliteit.

Hormonen die een rol spelen bij de groei en energiestofwisseling, zoals groeihormoon (GH), 'insulin-like growth factor' (IGF-I), insuline, leptine of schildklierhormoon (T3/T4), beïnvloeden eveneens het functioneren van de hypothalamus-hypofyse-ovarium as. Daarmee moduleren ze de secretie van GnRH, FSH, LH, prolactine, androgenen and estrogenen, hormonen betrokken bij de follikelontwikkeling. Vrouwen met te lage groeihormoon of schildklierhormoonspiegels hebben vaak problemen om zwanger te worden en doen daarom dikwijls een beroep op de gyneaecoloog voor de toepassing van moderne voortplantingstechnieken. De invloed van schildklierhormoon op de ovariumfunctie werd gestest in volwassen vrouwelijke ratten, nadat in deze dieren een milde vorm van hypothyreoïdie was geinduceerd (hoofdstuk 4). De bevindingen van dit onderzoek tonen aan dat hypothyreoïdie resulteerde in een onregelmatige oestrische cyclus. In de ovaria werd een geringer aantal follikels aangetroffen in het antrale stadium en een hogere mate van atresie. Naast verhoogde atresie treedt er onder invloed van de hypothyreotische status mogelijk ook een remming op in de groei van follikels, waardoor minder preantrale follikels kunnen ontwikkelen tot het antrale stadium en uiteindelijk kunnen ovuleren. Daar granulosa cellen receptoren voor schildklierhormoon tot expressie brengen, is het mogelijk dat schildklierhormoon een direct effect uitoefent op de functie en ontwikkeling van de follikels. Anderzijds kan schildklierhormoon ook indirect de ovaria beïnvloeden via de hypothalamus-hypofyse-ovarium as, of via GH/IGF-I. Bovendien is het mogelijk dat chronische verlaagde schilklierhormoon niveaus indirect de afgifte van LH en FSH door de hypofyse kunnen beïnvloeden. Een verlaging in FSH niveaus in de circulatie kan op zijn beurt weer de follikelontwikkeling in het ovarium aantasten. FSH is vooral van belang voor de groei van follikels van het preantrale naar het antrale stadium. Verder onderzoek heeft aangetoond dat in het brein van hypothyreotische ratten een verhoogde activiteit van somatostatine neuronen, een remmer van de GH afgifte, in de hypothalamus wordt waargenomen. De GH spiegels en de daarmee samenhangende IGF-I spiegels in de circulatie van deze dieren zijn dan ook verlaagd. Op grond van deze gegevens zou men derhalve kunnen veronderstellen dat vermindering van het aantal gezonde groeiende follikels mogelijk deels een gevolg is van een verlaging in GH en IGF-I productie. Uit eerder onderzoek in GH receptor 'knock-out' muizen (GHR/GHBP-KO muizen; hoofdstuk 3), waar GH door afwezigheid van functionele GH receptoren zijn werking niet kan uitoefenen, werd vastgesteld dat in GHR/GHBP-KO muizen de oestrische cycli onregelmatig zijn en de nest grootte gereduceerd,

een situatie vergelijkbaar met hypothyreotische ratten (hoofdstuk 4). De bevindingen beschreven in hoofdstuk 3 tonen aan dat de ovaria van volwassen GHR/GHBP-KO muizen een groter aantal primordiale follikels bevatten, een lager aantal gezond groeiende primaire, preantrale en antrale follikels en een verhoogd aantal atretische follikels. Daarnaast wordt in dit hoofdstuk aangetoond dat GH de primordiale follikel pool beïnvloedt mogelijk via de werking van IGF-I. Postnatale behandeling van GHR/GHBP-KO muizen met IGF-I resulteerde in een reductie van het aantal primordiale follikels tot een vergelijkbaar niveau zoals waargenomen in wild-type muizen. Het kan echter niet worden uitgesloten dat GH de overleving van follikels ook indirect beïnvloedt, aangezien de GH spiegels in de circulatie van GHR/GHBP-KO muizen verhoogd zijn, terwijl IGF-I, insuline en oestradiol spiegels verlaagd zijn. Als gevolg van de aanwezigheid van verschillende terugkoppelingsmechanismen is het mogelijk dat de afgifte van GnRH en dus LH en FSH beïnvloed worden, hetgeen weer een direct effect kan hebben op de follikelontwikkeling in de ovaria.
Doordat er een nauwe functionele interactie bestaat tussen de gonadotrope, somatrope and thyreotrope assen met betrekking tot de regulatie van follikulaire ontwikkeling en degeneratie in de ovaria, is het moeilijk te achterhalen wat de individuele contributies van de verschillende hormonen zijn in een in vivo model. De potentiële rol voor GH en schilklierhormoon op, bijvoorbeeld groeifactor productie, zou onderzocht kunnen worden met specifieke genoom- en proteoom databestanden. Dergelijke kennis kan nieuwe inzichten verschaffen voor het bestuderen van de apoptotische signaalroutes die geactiveerd worden tijdens follikulaire atresia en indirect bijdragen aan de behandeling van vrouwen met fertiliteitsklachten.

Uit een recente studie is gebleken dat de vruchtbare periode verlengd kon worden in Bax deficiënte muizen, doordat in deze knock-out muizen de follikulaire atresie geremd is en dus meer follikels overblijven om te ovuleren. Op dit moment is er voor een dergelijk manipulatie van de apoptotische machinerie nog geen directe klinische toepassing, omdat onder deze omstandigheden apoptose niet alleen in het ovarium geremd wordt, maar ook in andere organen met alle gevolgen van dien voor de gezondheid. Een meer logische en veilige benadering om de voortplantingsduur en vruchtbaarheid te verlengen in vrouwen, is om de overleving van de overgebleven follikels in de ovaria te stimuleren met behulp van hormoonbehandelingen. De effectiviteit van dergelijke hormoonbehandeling is mede afhankelijk van de omvang van de functionele eicelvoorraad in de ovaria. De keerzijde van hormonale ondersteuning van de fertiliteit is echter het verhoogde risico op kankerontwikkeling in steroïdgevoelige weefsels, zoals borstweefsel, baarmoeder en ovaria. Recente epidemiologische studies hebben bevestigd dat het risico om ovariumkanker te ontwikkelen verhoogd is na fertiliteitsbehandelingen met hormonen. Daarnaast zijn er aanwijzingen dat ook hormoonsubstitutie therapieën tijdens de menopauze een verhoogd risico geven op de ontwikkeling van gynaecologische vormen van kanker.

Na kanker in de borst, darm, long en baarmoeder is ovariumkanker de meest voorkomende vorm van kanker bij vrouwen. Jaarlijks wordt er in Nederland bij ongeveer 1500 vrouwen dit type van kanker vastgesteld. De prognose voor ovariumkanker patïenten loopt sterk uiteen, afhankelijk van de mate van agressiviteit en het stadium waarin de ziekte ontdekt wordt. Bij het merendeel van de patïenten wordt de ziekte pas in een ver gevorderd stadium ontdekt, wanneer de tumor zich al heeft uitgebreid tot in de buikholte. Chirurgische verwijdering van de tumor is daardoor vaak niet meer mogelijk. Als gevolg hiervan kent ovariumkanker de hoogste mortaliteit van alle gynaecologische kankers: de gemiddelde 5-jaar overlevingskans is minder dan 20%.

Er bestaan verschillende soorten ovariumkanker, waarvan de meest voorkomende vorm (80-90 %) onstaat uit de laag cellen die de ovaria omkleed (het oppervlakte epitheel), ook wel epitheliale ovariumkanker genoemd. Alhoewel er helaas weinig bekend is over de oorzaak van deze vorm van kanker, denkt men dat deze tumoren onstaan uit epitheelcellen die ingesloten raken in de ovaria waardoor cysten (holte met vloeibare inhoud) gevormd worden (fig. 2A, hoofdstuk 7). Meestal zijn deze cysten goedaardig, en gaan ze in de loop van tijd ten gronde door middel van apoptose. Recent onderzoek heeft aangetoond dat verminderde apoptose kan resulteren in een toenemende progressie van ovariumkanker. In hoofdstuk 5 van dit proefschrift is onderzocht of de remming van het apoptotische mechanisme van epitheelcellen in inclusiecysten een vroege stap is in het ontstaan van ovariumkanker. Daartoe hebben we het expressiepatroon van verschillende markers voor apoptose en celdeling bestudeerd in rond-tot ovale en onregelmatig gevormde inclusiecysten. Er werd aangetoond dat de expressie van Ki67 (een marker voor celdeling), Fas, Fas ligand en procaspase-3 verhoogd was in de epitheelcellen die de wand van onregelmatig gevormde inclusie cysten bekleden. Dit suggereeert dat het Fas systeem en zijn gerelateerde signaaleiwitten een rol spelen bij de balans tussen celdeling en celdood in deze cysten. De balans lijkt echter door te doen slaan in de richting van celdeling, aangezien een verhoogde celdelingsactiviteit werd waargenomen terwijl apoptose en caspase-3 activiteit zo goed als afwezig waren. Deze studie duidt op het onvermogen van de ingesloten epitheelcellen in inclusiecysten met een onregelmatige vorm om in apoptose te gaan door middel van Fas activatie. Wellicht dragen deze defecten in de apoptotisch machinerie bij aan het voortbestaan van dit type inclusiecysten. Als gevolg van de verhoogde celdelingsactiviteit van de ingesloten epitheelcellen bestaat er een toegenomen kans op de inductie van DNA mutaties. Als deze cellen dan vervolgens zich blijven vermenigvuldigen en niet apoptotisch worden kan dit op den duur leiden tot de vorming van een gezwel of tumor.

Uit de meest recente cijfers van de Nederlandse Kankerregistratie die begin december 2004 zijn gepubliceerd, blijkt dat zowel de incidentie als de sterftekansen van ovariumkkanker in de afgelopen 10 jaar licht gedaald zijn in Nederland. Er zijn aanwijzingen dat het wijdverbreide gebruik van de anticoneceptie pil heeft bijgedragen aan de afname van het aantal

vrouwen met ovariumkanker. Uit eerdere wetenschappelijke studies is gebleken dat anticonceptie gebruik, maar ook zwangerschap en borstvoeding het risico op ovariumkanker vermindert, vermoedelijk als gevolg van verlaagde gonadotrope hormoonspiegels. Onlangs is gesuggereerd dat gonadotrope hormonen mogelijk een belangrijke rol kunnen spelen in de etiologie van ovariumkanker. Dit idee wordt ondersteund doordat bij een aantal vrouwen die fertiliteitsbehandeling ondergingen met hoge doses gonadotrope hormonen, op relatief jonge leeftijd reeds ovariumkanker is geconstateerd. Het is mogelijk dat het ontstaan van ovariumkanker in deze jonge vrouwen een direct gevolg is van de blootstelling van ovariële oppervlakte epitheelcellen aan gonadotrope hormonen (zie literatuuroverzicht hoofdstuk 1). Het toedienen van hormonen tijdens de fertiliteitsbehandelingen resulteert in een verhoogde productie van de geslachtshormonen oestradiol en progesteron. Een rol voor geslachtshormonen in de etiologie van eierstokkanker kan daarom niet worden uitgesloten, doch lijkt minder aannemelijk aangezien het voorkomen van ovariumkanker het hoogst is in de jaren na de menopauze, wanneer gonadotrope hormoonspiegels verhoogd zijn en de productie van de geslachtshormonen nagenoeg nihil is. Onderzoek heeft aangetoond dat in veel gevallen van ovariumkanker, de tumorcellen receptoren voor gonadotrope hormonen tot expressie brengen. In hoofdstuk 6 wordt aangetoond dat behandeling met LH resulteerde in een toename van de overleving van ovariële oppervlakte epitheel kankercellen, een proces dat werd gemediëerd door verhoogde protein kinase A (PKA) activiteit. Daarnaast werd aangetoond dat LH behandeling de delingsactiviteit van kankercellen niet beïnvloedde. In vitro onderzoek met behulp van een ovariële oppervlakte epitheel cellijn toonde aan dat de door LH geïnduceerde overleving tot stand kwam doordat LH de Fasgeïnduceerde apoptose in deze cellen remde. Alhoewel het waargenomen remmend effect van LH op Fas- geïnduceerde apoptose tijdelijk was, is het mogelijk dat een vertraging in de inductie van celdood onder omstandigheden kan leiden tot een verlengde levensduur van gemuteerde ovariële epitheel kanker cellen in vivo. Verder onderzoek is noodzakelijk om deze in vitro resulaten te herleiden naar de in vivo situatie. Gezien het aantal vrouwen dat tegenwoordig fertiliteitsbehandeling ondergaat en de verwachte toename in de vraag naar ovulatie inductie in de komende jaren, is het van groot belang om de rol van gonadotrope hormonen in het onstaan en de voortwoekering van ovariumkanker op te helderen. Vanwege de nog bestaande twijfels over de veiligheid van IVF is het noodzakelijk dat alle patiënten goed worden geïnformeerd over de aard van de mogelijke risico's voordat met de fertiliteitsbehandeling wordt aangevangen.

Nieuwe informatie over de regulatoire anti-apoptotische mechanismen van gonadotrope hormonen kan verder wellicht andere mogelijkheden bieden in de behandeling van ovariumkanker. Gezien anti-kanker drugs (cytostatica) cellen doden door middel van de inductie van apoptose, is de de effectiviteit van de therapie, oftewel de gevoeligheid voor cytostatica, afhankelijk van het vermogen van de cel om in apoptose te gaan. Het blootstellen van tumorcellen aan het cytostaticum cisplatinum, het meest gebruikte cytostaticum voor

de behandeling van ovariumkanker, maakt kankercellen ontvankelijk voor Fas-gemedieerde apoptose. In vitro onderzoek heeft aangetoond dat hCG, een LH analoog, de gevoeligheid voor cisplatinum verlaagd, en daarmee het vermogen tot in apoptose gaan verminderd is. In een pilot studie hebben we gevonden dat het remmende effect van LH op Fas-geïnduceerde apoptose in ovariële oppervlakte epitheel kankercellen gepaard gaat met een toename in de ratio van anti-apoptotische versus pro-apoptotische Bcl-2 eiwitten. Het is mogelijk dat PKA activatie leidt tot een verhoogde overleving van deze kankercellen door remming van apoptose via modulatie van Bcl-2 eiwit familiegenoten (fig. 2, hoofdstuk 7). Vervolgstudies zijn essentiëel om te onderzoeken of gonadotrope hormonen dan wel geactiveerde 'downstream' signaalmolekulen inderdaad in staat zijn om de reactie van de cel op cytostatica te moduleren. Ook zal de therapeutische potentie van GnRH analogen (die gonadotrope hormoonspiegels verlagen) in combinatie met cytostatica behandelingen getest moeten worden op hun effectiviteit in de behandeling van bepaalde vormen van ovariumkanker.

Dit proefschrift heeft geleid tot de identificatie van een aantal hormonen die de activatie van het Fas systeem en apoptose kunnen beïnvloeden in verscheidene ovariële celtypen. Continue hormonale blootstelling kan de Fas apoptotische signalering onderdrukken, terwijl verminderde hormonale invloeden kan resulteren in verhoogde expressie van verschillende componenten van het Fas systeem en zo apoptose beïnvloeden. Verdere karakterisering van de hormonale en moleculaire regulatie van follikulaire atresie in het ovarium en epitheliale celdood in ovariumkanker, zou waardevolle nieuwe informatie kunnen op leveren voor zowel de behandeling van infertiliteit als ovariumkanker.

DANKWOORD

Hier ligt dan het boekje, een 'slot-product' waaraan velen een steen(tje) hebben bijgedragen. Er zijn zoveel collega's, mensen buiten de afdeling en vrienden die ik moet bedanken, dat ik in een lastig parket zit. Om hen allemaal uitvoerig te bedanken zou ik nog een een heel boek kunnen schrijven. Alleen een lijst met namen noemen zou van individuen een menigte maken en dan zou ik bovendien bang zijn dat ik iemand vergat. De mensen die hieraan hebben bijgedragen weten wie ze zijn. Kortom bedankt allemaal, dit boekje is ook een beetje voor jullie. Toch ontkom ik er niet aan om enkele namen apart te noemen. Allereerst natuulijk Katja; mijn blikveld in de eierstokwereld zou een stuk beperkter zijn, als ik niet zo af en toe op je schouders had mogen staan. Gedurende het gehele wordingsproces heb je fantastisch werk gedaan en ondanks je verhuizing naar Wageningen is je betrokkenheid bij het onderzoek er zeker niet minder op geworden. Bedankt voor alle steun, vertrouwen, sturing en ruimte die je me al die jaren gegeven hebt. Verder wil ik Wim, promotor van het eerste uur, bedanken voor het vertrouwen dat je in me hebt gesteld, en dat je me neemt zoals ik ben. Ik heb onze gesprekken altijd erg gewaardeerd. Willem, promotor op de valreep, bedankt voor al je inspanningen en kritische commentaar gedurende de laatste fase van het schrijfproces.

Een deel van het werk is tot stand gekomen dankzij de inbreng van Marsha en de studenten (Kirsten en Wendy). Marsha, bedankt voor de inspanningen en enthousiasme, samen met jou is onderzoek doen geweldig. Mieke, bedankt voor je hulp en toewijding bij de diverse immuunhistochemische bepalingen en de thecacel isolaties. Pachi, bedankt voor de lol tijdens onze samenwerking. Alle overige mede-auteurs, bedankt voor de waardevolle suggesties en de fijne samenwerking. Verder wil ik Martin H bedanken voor de ondersteunende nuchterheid en voor het oogje in het zeil houden. Chris, bedankt voor de hulp bij computersores en statistiek. Ger en Bart, bedankt voor de hulp bij de FACS-bepalingen. Daarnaast wil ik de AIO's van het Promovendi Overleg Diergeneeskunde (PODium) bedanken. Gedurende het laatste jaar heeft een deel van het onderzoek zich verplaatst naar de Leerstoelgroep FMD. Hen wil ik bedanken voor de aanvoer van ovaria en het gebruik van de microscoop, maar ook voor de genoten gastvrijheid. FMD-kamergenoot Harmke, ik vond het jammer dat ik niet bij je verdediging aanwezig kon zijn. Ook wil ik graag de leescommissie bedanken voor de snelle beoordeling van dit proefschrift. En Lisanne, bedankt dat je ervoor gezorgd hebt dat het boekje er zo mooi uit ziet.

Bovenal wil ik graag IEDEREEN van de afdeling Biochemie & Celbiologie bedanken, die op een of andere wijze heeft bijgedragen aan de geweldige sfeer op het lab, in de wandelgangen en aan de koffietafel, tijdens de vele vrijdagmiddag sessies in de Poort, weekendjes weg, hardlooptrainingen, estafettes en andere evenementen. Tevens bedankt voor alle hulp, belangstelling, vriendschap en goede herinneringen. In het bijzonder wil ik nog mijn kamergenoten (Evert, TJ en Danny) bedanken voor de afleiding en het geouwehoer op de werkkamer. Agnieszka, na drie jaar intensiviteit binnen en buiten de muren van de celbiologie vond ik het een hele eer om samen met Canan als paranimf-duo te fungeren. Not last and certainly also not the least, lieve jongens van de shoarmaclub (Ed, Frits en Rene met streepje), bedankt voor alle inspirerende emailtjes, lunches en borrels.

Tenslotte wil ik graag mijn familie en vrienden bedanken voor al het aangename wat onschatbare waarde heeft voor een natuurlijke balans tussen werk en privé. Met name wil ik Marion, Sandra en Linette bedanken voor de vertering van de buitensporige portie onderzoeksbezinning. Ernée en Evert, ik vind het erg leuk dat jullie mijn paranimfen willen zijn. Verder wil ik graag de familie Nieuwenhuizen bedanken voor hun oprechte belangstelling. Lieve zussen en broer, gezien het nog steeds groeiende aantal neefjes en nichtjes, kan ik slechts concluderen dat mijn interesse in de biologie van de voortplanting genetisch bepaald is. Lieve ouders, bedankt voor alle liefde, steun en vertrouwen die jullie me altijd gegeven hebben. Lieve Arie, mocht je eraan twijfelen, alle vreugde en gekkigheid hebben zeker bijgedragen aan het tot stand komen van dit boekje. Ik hoop dat wij samen nog een hele geweldige tijd tegemoet gaan, zowel binnen als buiten de driehoek Utrecht-Berlijn-Maastricht.

CURRICULUM VITAE

Karin Annemarie Slot werd geboren op 21 juni 1973 te Borne. Het VWO diploma behaalde zij in juni 1992 aan het Pius X college te Almelo. In datzelfde jaar begon zij met de studie Biologie aan de Rijksuniversiteit Groningen. Tijdens deze studie deed ze drie onderzoekstages: bij de afdeling Voortplantingsbiologie, Disciplinegroep Obstetrie en Gynaecologie, Rijksuniversiteit Groningen; the Zoology Department, University of Lund (Zweden); en the MRC Reproductive Biology Unit, Centre for Reproductive Biology, Edinburgh (UK). In augustus 1997 behaalde zij haar doctoraal examen. Na van mei 1998 tot maart 2000 werkzaam te zijn geweest als Junior Onderzoeker bij Numico Research B.V. (Afdeling Biomedical Research) te Wageningen, trad zij in april 2000 in dienst als Assistent in Opleiding (AIO) bij de Hoofafdeling Biochemie en Celbiologie, Faculteit Diergeneeskunde, Universiteit Utrecht. Onder leiding van Dr. Katja Teerds werd het in dit proefschrift beschreven onderzoek verricht. Een deel van dit onderzoek werd uitgevoerd aan de Leerstoelgroep Fysiologie van Mens en Dier, Departement Dierwetenschappen, Wageningen Universiteit. Na afloop van haar AIO aanstelling heeft ze gedurende 3 maanden als onderzoeksmedewerker gewerkt bij de laatstgenoemde Leerstoelgroep om haar onderzoek af te ronden. Vanaf februari 2004 gaat ze werken als Postdoc bij "The Institute for Zoo and Wildlife Research" (Berlin) onder leiding van Dr. Katarina Jewgenow aan cryopreservatie en xenotransplantatie van ovariumweefsel, een samenwerkingsproject met de Hoofdafdeling Landbouwhuisdieren, Faculteit Diergeneeskunde, Universiteit Utrecht.

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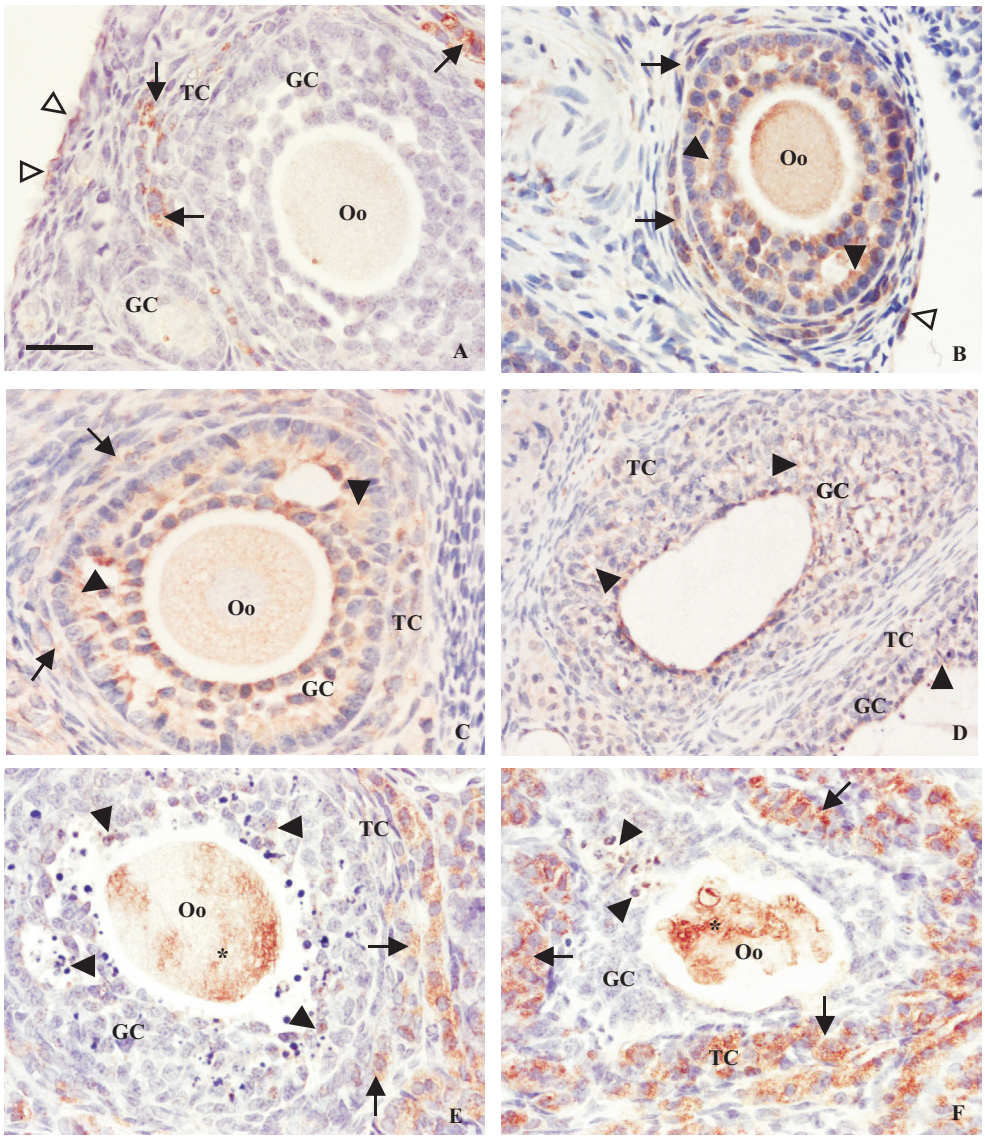
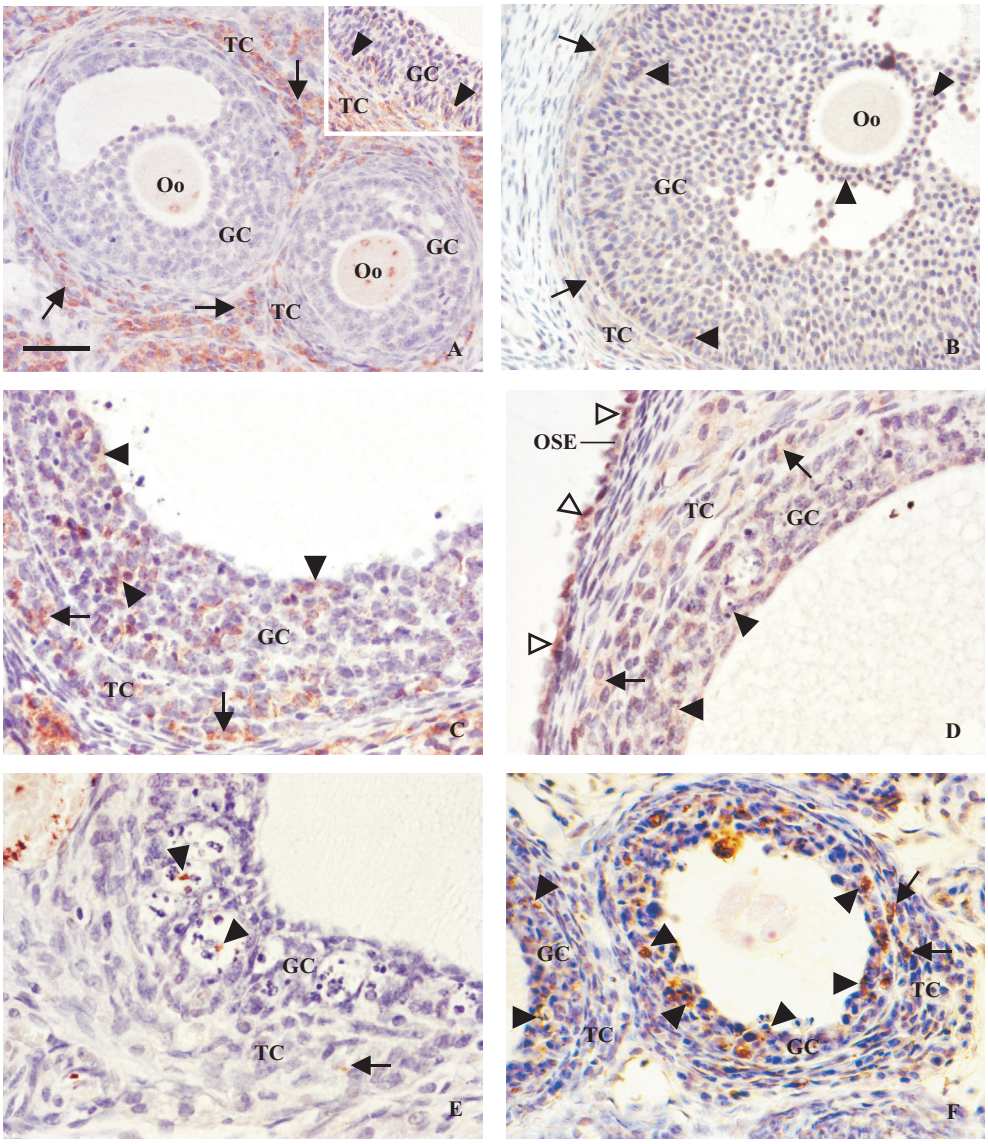
Slot KA, Kastelijn J, Bachelot A, Kelly PA, Binart N, Teerds KJ. Reduced recruitment and survival of primordial and growing follicles in growth hormone receptor deficient mice. Submitted

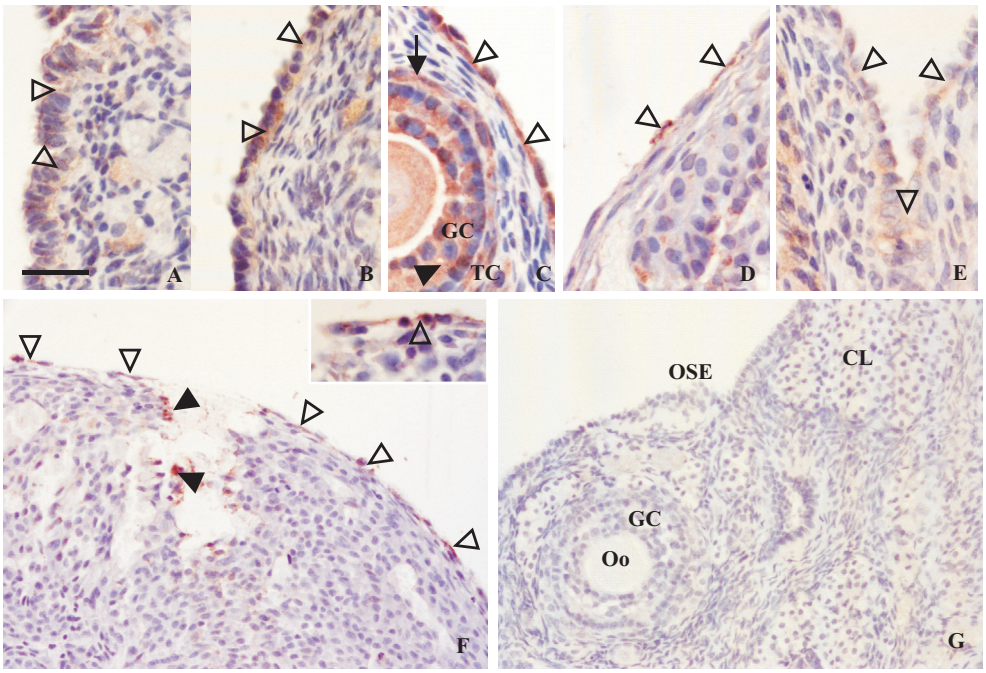
Slot KA and Teerds KJ. Hormonal regulation of apoptosis in the ovary, a review. Submitted



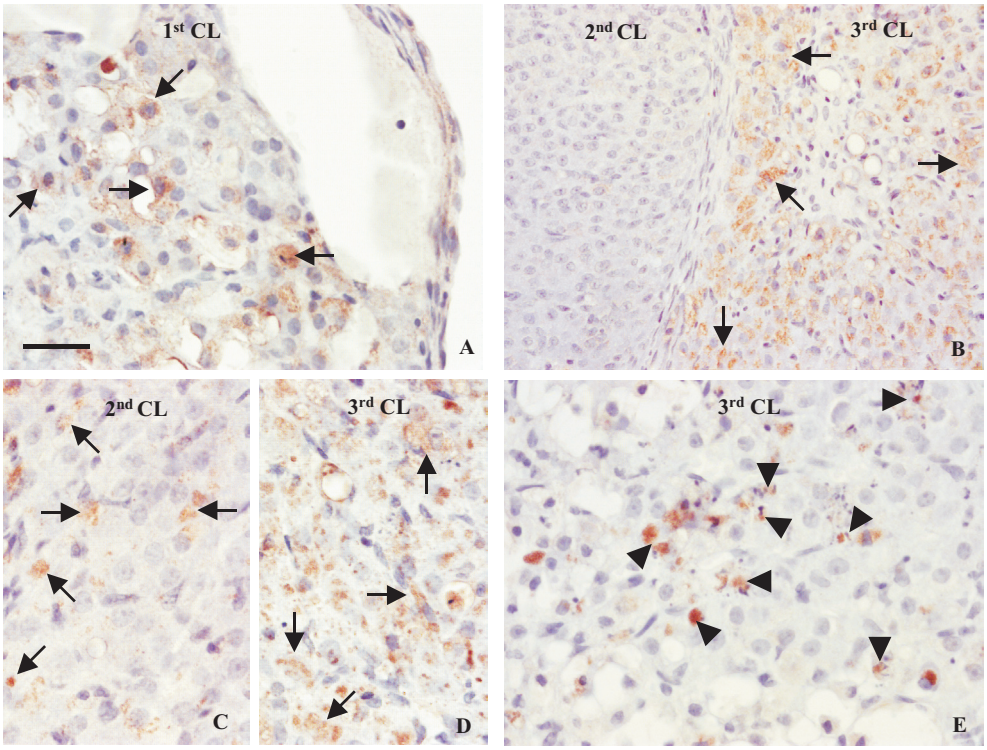
Chapter 2 - Figure 3. Immunohistochemical localization of various apoptotic proteins in preantral follicles of cycling rats. Positive staining is shown in brown and indicated by arrow(head)s. A-B) Healthy preantral follicles; Fas (A) immunostaining was found in theca cells but not in granulosa cells, whereas procaspase-3 (B) and Bcl-2 (C) were observed in both granulosa and theca cells. D-F) In atretic preantral follicles; less Bcl-2 immunopositive cells were observed (D), while Bax (E) and Fas ligand (F) immunostaining was now also present in some granulosa cells of both moderately (E) and severely (F) atretic follicles. The immunohistochemical labeling experiments were repeated at least three times with similar results. Theca cells (TC; arrows), granulosa cells (GC; arrowheads), oocytes (Oo and degenerating oocytes; asterisk), ovarian surface epithelium (open arrowheads). Bar = 20 µm (A-C,E,F) or 40 µm (D).



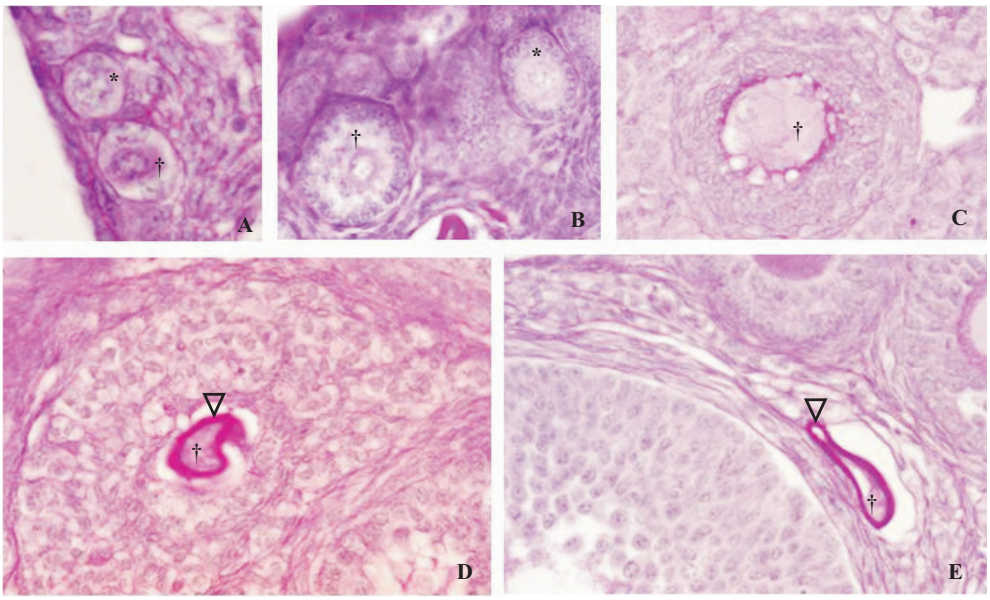
Chapter 2 - Figure 4. Immunohistochemical localization of various apoptotic proteins in antral follicles of cycling rats. Positive staining is shown in brown and indicated by arrow(head)s. A-B) Healthy antral follicles; Fas ligand (A) immunostaining was observed in theca cells only, though at proestrus some Fas ligand was observed in the mural granulosa cells of preovulatory follicles close to the basal membrane (insert), whereas Bcl-2 (B) was present in both granulosa and theca cells. C-F) Atretic antral follicles; Fas (C) and procaspase-3 (D) immunostaining are present in both granulosa and theca cells; staining for active caspase-3 (E) and TUNEL labeling (F) is often observed in granulosa with condensed nuclei but also in some apoptotic theca cells. The immunohistochemical labeling experiments were repeated at least three times with similar results. Theca cells (TC; arrows), granulosa cells (GC; arrowheads), oocytes (Oo), ovarian surface epithelium (OSE; open arrowheads). Bar = 20 µm (C-E) or 40 µm (A,B,F).



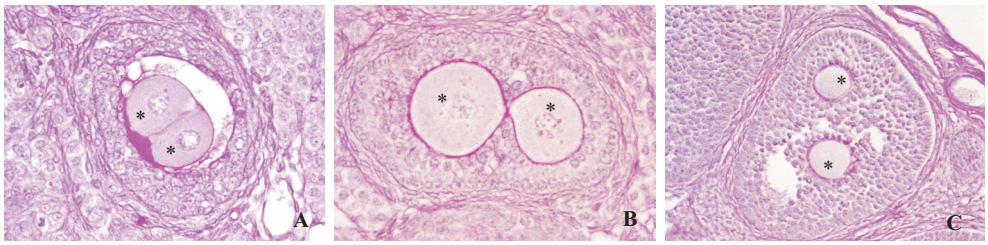
Chapter 2 - Figure 5. Immunohistochemical localization of various apoptotic proteins in ovarian surface epithelial cells (OSE) of cycling rats. Positive staining is shown in brown and indicated by arrow(head)s. A-E) On the ovarian surface; flat-to-columnar OSE cells show a clear positive immunostaining for Fas (A), Fas ligand (B), Bcl-2 (C) and procaspase-3 (E), while immunoreactivity for Bax (D) was mainly restricted to OSE cells at the ovulatory site. F) At the postovulatory site; some OSE cells have now become immunopositive for active caspase-3 (see also insert) as well. G) Control ovarian section incubated with normal rabbit serum instead of primary antibody. The immunohistochemical labeling experiments were repeated at least three times with similar results. Theca cells (TC; arrows), granulosa cells (GC; arrowheads), oocytes (Oo), ovarian surface epithelium (OSE; open arrowheads), corpora lutea (CL). Bar = 10 µm (A-E and insert of F), 40 µm (F) or 100 µm (G).



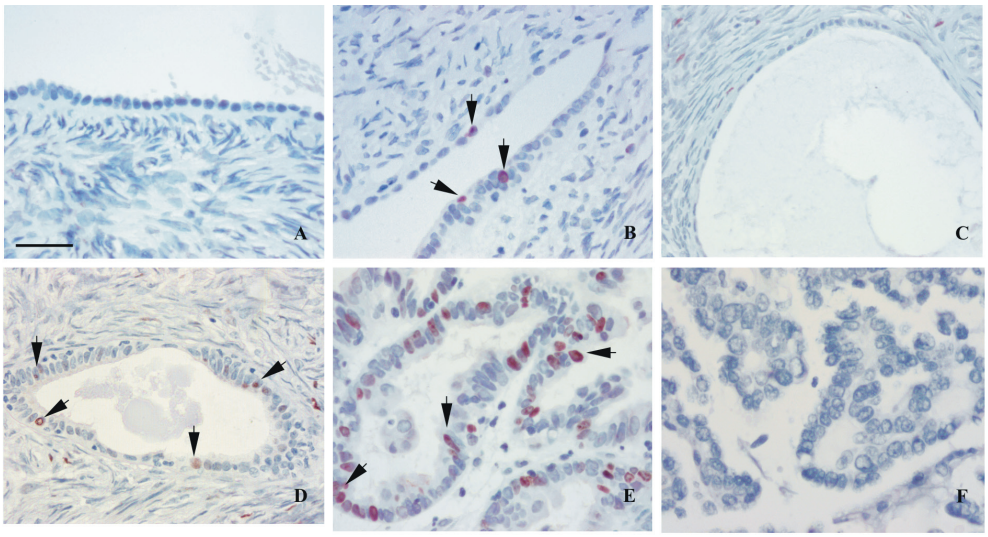
Chapter 2 - Figure 6. Immunohistochemical localization of various apoptotic proteins in corpora lutea (CL) of cycling rats. Positive staining is shown in brown and indicated by arrow(head)s. A) Early corpus luteum of the first generation (1st CL) at metestrus; clear immunostaining for Fas ligand is present. B-D) CL of different age; immunostaining for Bax (B) and Fas (C-D) was faint in the CL of the first and second generation (2nd CL) while high in CL of the third generation (3rd CL). E) CL of the third generation (3rd CL); active caspase-3 immunopositive cells often had condensed and fragmented nuclei (arrowheads) that are indicative for apoptosis. The immunohistochemical labeling experiments were repeated al least three times with similar results. Bar = 20 µm (A,C-E) or 40 µm (B).



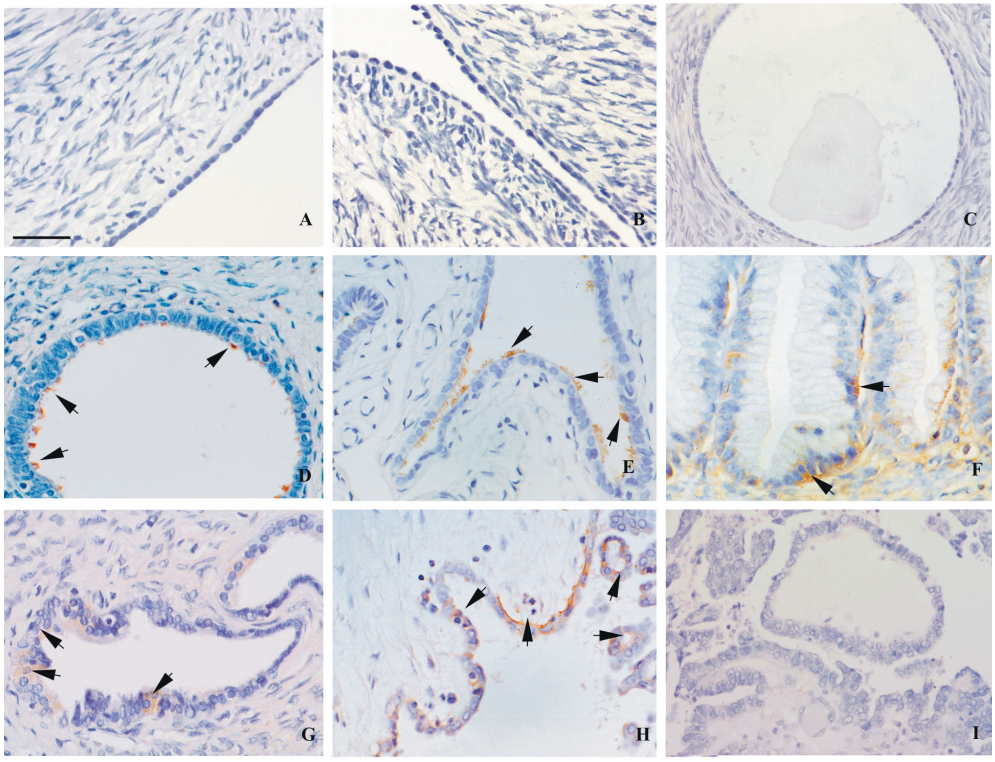
Chapter 3 - Figure 2. Representative photomicrographs of atretic follicles in ovaries of wild-type and/or GHR/BP-KO mice. Atretic follicles were defined as desribed in *Materials and Methods*. A. Atretic (crucifix) and healthy (asterisk) primordial follicle. B. Atretic (crucifix) and healthy (asterisk) primary follicle. C. Early atretic preantral follicle displaying a degenerating oocyte (crucifix). D. Moderate atretic (pre)antral follicle (crucifix) in which granulosa cells have disappeared, while the zona pellucida (arrowhead) is still present. E. Late atretic (pre)antral follicle (crucifix), in which most theca cells have degenerated, while the zona pellucida (arrowhead) is still present. Magnification 40x.



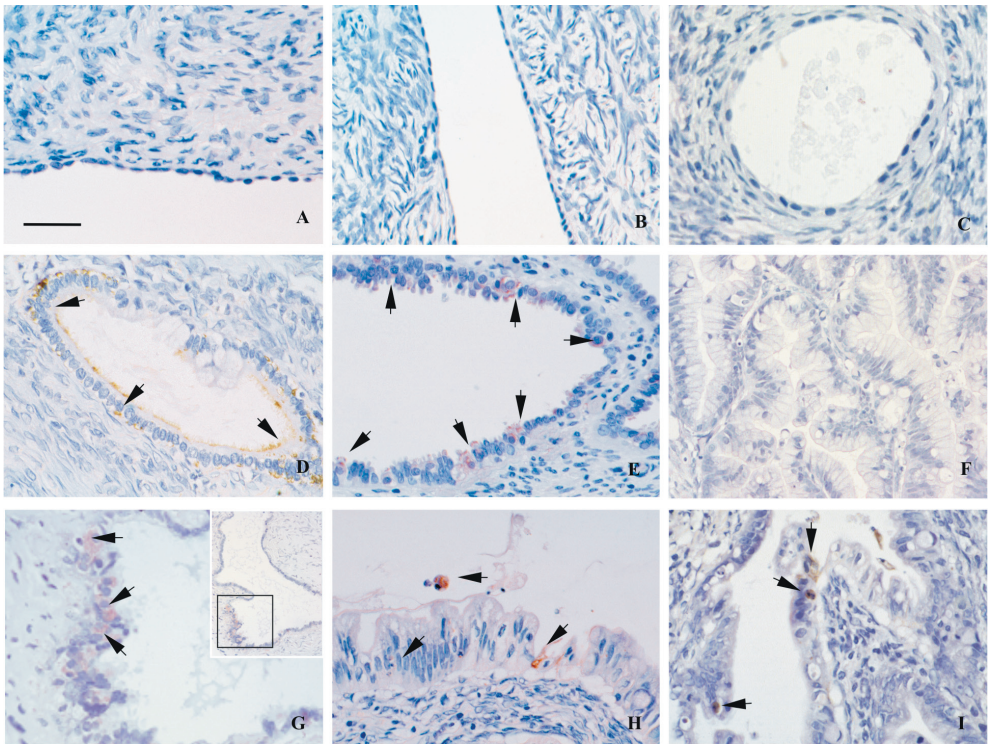
Chapter 3 - Figure 4. Abnormal follicles in GHR/GHBP-KO mice. A-B. Preantral follicles with two oocytes (asterisks). Magnification 40x. C. Early antral follicles follicle with 2 oocytes. Magnification 20x..



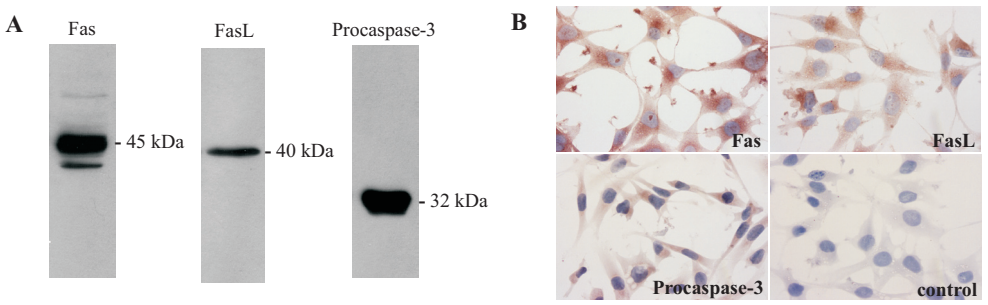
Chapter 5 - Figure 2. Immunohistochemical localization for Ki67 on the ovarian surface (A), in clefts (B), regularly (C) and irregular shaped inclusion cysts (D) and mucinous adenocarcinomas (E-F). Note positive Ki67 immunostaining (arrows) in the nuclei of OSE cells with columnar or Müllerian morphology (B and D) or tumor OSE cells (E). Control tumor section of borderline malignancy, in which the primary antibody was replaced by normal rabbit serum (F). Bars = 5 µm (C) 10 µm (A-B and D) or 20 µm (E-F).



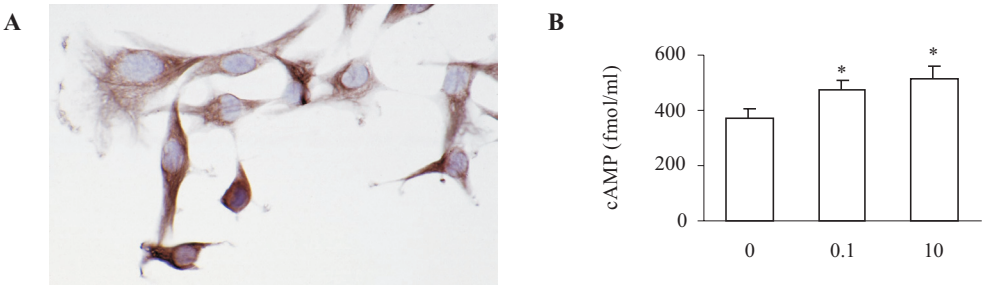
Chapter 5 - Figure 3. Immunohistochemical localization for Fas (A and D-F) and Fas ligand (B-C and G-H) on the ovarian surface (A), in clefts (B), regularly (C) and irregular shaped inclusion cysts (D and G), serous benign cystadenoma (E), mucinous epithelial borderline tumor (F) and poorly differentiated cystadenocarcinoma (H). Note the presence of immunoreactivity for Fas and Fas ligand immunostaining in columnar or Müllerian-like OSE cells (arrows). Control section of a poorly differentiated cystadenocarcinoma, in which the primary antibody was replaced by normal rabbit serum (I). Bars = 10 µm (A and D-E and G-I) or 20 µm (B-C and F).



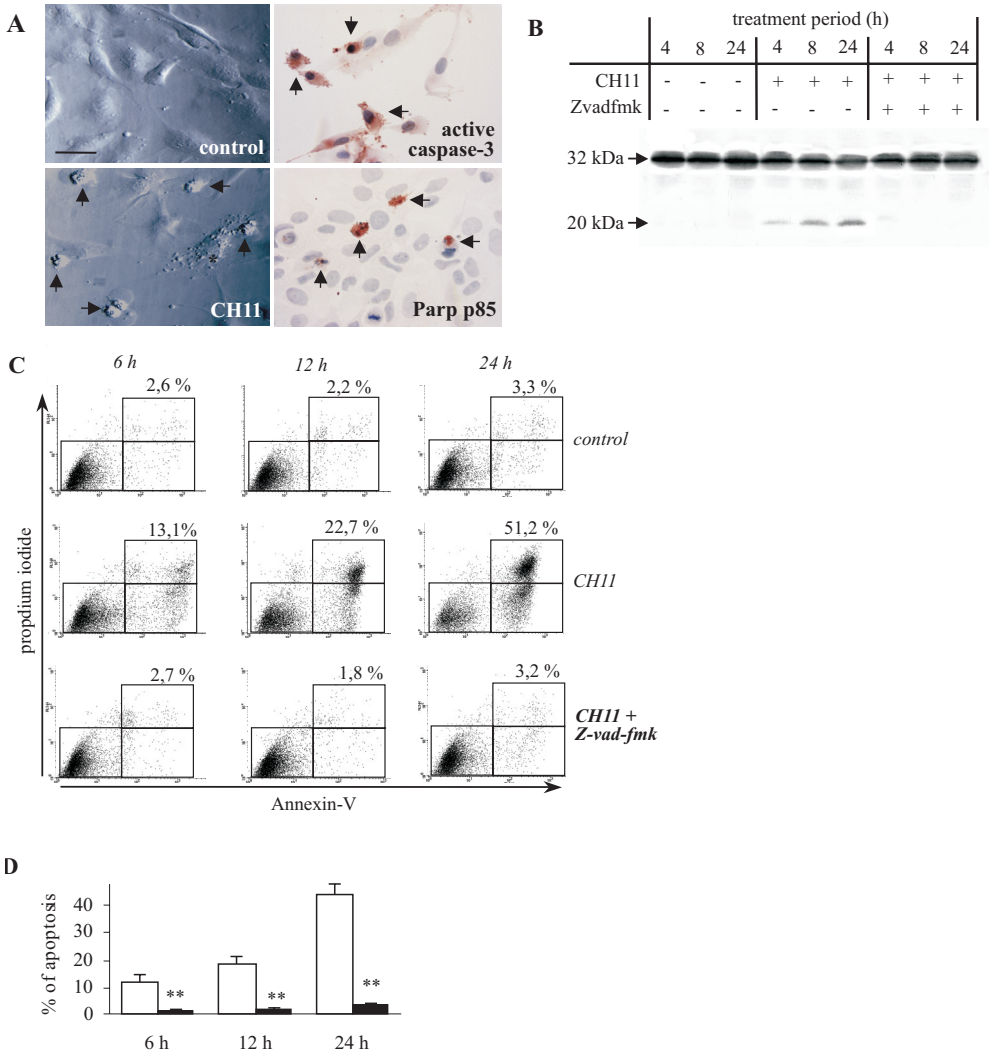
Chapter 5 - Figure 4. Immunohistochemical localization for procaspase-3 (B-E) and active caspase-3 (A and G-I) on the ovarian surface (A), in clefts (B), regularly (C) and irregular shaped inclusion cysts (D and G), serous benign cystadenoma (E) and mucinous adenocarcinomas (H-I). Note that cells positive for active caspase-3 often had condensed and fragmented nuclei (arrows), which are characteristic for apoptosis (H-I). Control section of a serous papillar cystadenoma, in which the primary antibody was replaced by normal goat serum (F). Bars = 10 µm (A-E, G) or 20 µm (F, H-I).



Chapter 6 - Figure 1. Expression of Fas, Fas ligand and procaspase-3 in HEY cells. HEY cells (5 x 10^{-4} cells/ml) were maintained overnight and subsequently serum and growth factor starved for 24 h. Fas, Fas ligand and procaspase-3 protein expression was determined by Western blot analysis (A) and immunohistochemistry (B) as described in the *Materials and Methods*. The control represents a DAB staining in which the primary antibody was replaced by normal rabbit serum. A representative experiment is shown, which was repeated three times with similar results.



Chapter 6 - Figure 3. Expression of functional LH receptors in HEY cells. A, HEY cells (5 x 10^{-4} cells/ml) were maintained overnight, then serum-starved for 24 h and LH-R protein expression was determined by immunohistochemistry as described in *Materials and Methods*. B, HEY cells were incubated in serum-free medium for 1 h and then further cultured in the absence (0 ng/ml LH) or presence of (0.1 or 10 ng/ml) LH for 60 min. Total cAMP levels were measured as described in the *Materials and Methods.* Values are expressed as the means ± SEM of quadruplicate incubations from one representative experiment. The experiment was repeated two times. A student's T-test was used to compare the mean of cAMP levels among the groups. Significantly different from control (0 ng/ml LH + IBMX); *, $P<0.05$



Chapter 6 - Figure 2. Effect of Fas stimulation on various parameters indicative for apoptosis in HEY cells. A. HEY cells (5 x 10^{-4} cells/ml) were maintained overnight and then treated for 8 h without or with an agonistic anti-Fas antibody (50 ng/ml CH11) in serum-free medium. The left of panel A shows phase contrast images of non-stimulated (control) and CH11-stimulated HEY cells. The right panel shows immunohistochemical staining for active caspase-3 and PARP p85 in CH11-treated HEY cells. Arrows indicate apoptotic cells. B, HEY cells were treated without or with 50 ng/ml CH11 in the presence or absence of 10^{-5}M Z-vad-fmk for 4, 8 and 24 h. Expression of procaspase-3 (32kDa) and active caspase-3 (20 kDa) was determined in whole cell lysates as described in the *Materials and Methods.* C, PS exposure was determined by FACS analysis at various times after treating cells without additions (control), 50 ng/ml CH11 or 50 ng/ml CH11 plus 10^{-5}M Z-vad-fmk as described in the *Materials and Methods.* Values on top of the right quadrant are the sum of the relative number of cells positive for Annexin-V. Data shown are from one representative experiment, which was repeated three times with similar results. D, Quantification of Fas-induced apoptosis in HEY cells following treatment with 50 ng/ml CH11 in the absence (open bars) or presence of 10^{-5}M Z-vad-fmk (closed bars) as described in panel C. Values shown are means ± SEM from four independent experiments. Significantly different from CH11-treated cells; **, P<0.01.