Apoptosis, proliferation, and sex steroid receptors in endometrium and endometrial carcinoma

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Front cover picture: Apoptosis is seen in benign endometrium on the first day of menstruation. The apoptotic bodies and cells are stained brownish with TUNEL method. Magnification  x430. Microphotograph by Stefan Cajander.
To Johan
ABSTRACT

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The cyclic changes in the female genital tract require remodeling of the endometrial tissue related to hormonal variations during the menstrual cycle. Apoptosis and proliferation function together in many hormone-dependent organs and during embryogenesis, when rapid growth and regression are needed for tissue modulation. This thesis focuses on the involvement of apoptosis and proliferation in the mechanisms of menstruation and hormonal replacement therapy, HRT, as well as in the mechanisms of progesterone therapy in endometrial carcinoma.

Under the assumption that apoptosis is involved in menstruation, the aim of the first study was to investigate endometrium for 4 days before and for 2 days during menstruation. Endometrium was examined separately in endometrial glands and stroma during declines in levels of serum 17ß-estradiol and progesterone. Different reactions were observed in epithelial and stromal tissues. In the epithelium, decreasing expression of estrogen receptor a (ER) and progesterone receptor (PR), minimal proliferation, and rapid increase in the apoptotic index were observed prior to menstruation. In the stroma, an increase in the expression of ER and PR and proliferation was seen before the final decrease during menstruation. Stromal apoptosis was clearly observed, but later than in the epithelium. Thus, apoptosis is involved in the remodeling of the endometrium during menstruation.

Apoptosis and proliferation, as well as high ER and PR expression, were also observed in postmenopausal endometrium. During substitution therapy, which consisted of 2 different regimens of HRT, the epithelial glands showed unaffected homeostasis with apoptotic index and Ki-67 index as proliferation markers. ER expression was decreased both in the epithelium and stroma, while PR showed different sensitivity, with some increase in receptor expression. The unchanged homeostasis during combined continuous HRT contributes to endometrial safety, while an increase in proliferation was seen in stroma along with a maintained level of apoptosis. This increase in proliferation has not been reported before and its importance should be further evaluated. It could have some effect on breakthrough bleedings, as the stromal support may be important to the vascular stability in endometrium.

Unchanged apoptosis and increasing proliferation were observed with increasing tumor grade in 29 patients with endometrioid endometrial carcinoma, which may contribute to greater aggression as tumor grade increases. The effects of medroxy-progesterone at 20 mg per day were monitored after 14 days of therapy, and decreased proliferation was observed particularly in the foci of maximal proliferation in G1 and G2 tumors, while G3 tumors were unaffected by the progesterone therapy. The expression of ER was unchanged, while PR was decreased in the foci of maximal expression for PR in G1 and G2 tumors. Since high proliferation and PR expression also coexisted in the same foci, evaluated in G1 and G2 tumors, the effect of progesterone could be facilitated in these tumor groups. High expression of sex steroid receptors was also a predicting factor for good response to progesterone (= decrease in proliferation), while the amount of stroma could not predict that effect.
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ABBREVIATIONS

17β-OH-HSD  17beta hydroxy steroid dehydrogenase
ABC     avidin-biotin complex
Ai      apoptotic index
APAAP   alkaline phosphatase-antialkaline phosphatase complex
CDK     cyclin dependent kinase
E1      estriol
E2      17β-estradiol
EGF     epidermal growth factor
ER      estrogen receptor
ET      endotelin
FIGO    Federation Internationale de Gynecologie et Obstetrique
HNPCC   hereditary non polypoid colon carcinoma
hpf     high power field
HRT     hormone replacement therapy
IGF     insulin-like growth factor
IGFBP   insulin-like growth factor binding protein
MMP     matrix metalloproteinase
PCO     polycystic ovary (syndrome)
PG      prostaglandin
SERM    selektive estrogen receptor modulator
SHBG    sex hormone binding globulin
TGF     transforming growth factor
TNF     tumor necrosis factor
TUNEL   terminal uridine deoxynucleotidyl nick end labeling
VEGF    vascular endothelial growth factor
WHO     World Health Organization
PAPERS

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

I

II
Dahmoun M, Ödmark I-S, Risberg B, T. Pavlenko, and Bäckström T. Apoptosis, proliferation and sex steroid receptors in postmenopausal endometrium before and during HRT. Manuscript.

III

IV
Dahmoun M, Boman K, Cajander S, and Bäckström T. Intratumoral effects of medroxy-progesterone on apoptosis, proliferation and sex steroid receptors in endometrioid endometrial adenocarcinoma. Submitted for publication.

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GENERAL INTRODUCTION

Apoptosis and proliferation regulate homeostasis in benign as well as malignant tissue. Proliferation of human endometrium is stimulated mainly by estrogen via nuclear receptors, a process that can be inhibited by progesterone [1]. In its receptor complex, 17β-estradiol participates in cell cycle stimulation. It also prepares the feedback mechanism by stimulating PR synthesis. Progesterone stops the estrogen-stimulated proliferation via nuclear effect, enzyme activation that catalyzes the bioactive 17β-estradiol (E2) to inactive estrone (E1) [2-5]. Hormonal effects are further modulated locally by growth factors both in normal endometrium [6, 7] and in hormone-responsive endometrial carcinoma cells [8].

Apoptosis, programmed cell death, is an active physiological process used by organisms for disposal of unnecessary or potentially harmful cells [9]. Apoptosis is triggered in many reproductive and hormone-dependent organs after ablation of the hormones [10-14]. In normal endometrium, autodigestion of basophilic granules had been observed before apoptosis was described 1972 [15]. Apoptosis was later observed through the luteal phase, menstruation, and the early follicular phase [16-21], and there are indications of low apoptotic activity in postmenopausal endometrium in some studies [3, 18, 22, 23].

Changes in the balance between apoptosis and proliferation may be physiological, such as the changes during the menstrual cycle, when the phases of proliferation, specialization, shedding, and renewal all show specific balance between apoptosis and proliferation both in the epithelium and in the stroma. Unopposed estrogen therapy may lead to hyperplasia and carcinoma [24-26]. However, another pathway of carcinogenesis also exists, one not related to estrogen [27, 28]. Maintaining the balance between apoptosis and proliferation in postmenopausal endometrium during hormonal replacement therapy (HRT) is important for endometrial safety, and it may also be essential for good bleeding control [3]. The mechanisms of cytostatica and radiation are often induction of apoptosis in the tumor cells [9, 13, 29, 30], but progesterone therapy of endometrial carcinoma has been used without knowing the exact mechanisms of action.
BACKGROUND

1. Apoptosis

Apoptosis is an energy-consuming active process that multicellular organisms have developed to dispose of unnecessary or potentially harmful cells. The apoptotic stimuli lead to a cascade of events that are shared by many cell types: the changes in the mitochondrial membrane stop production of ATP and lead to the irreversible pathway of apoptosis with activation of caspase reactions. Finally, the Ca2+/Mg2+ dependent endonuclease is activated and breaks the DNA into 180 base-pair nucleosomal fragments or to fragments in multiples of that size [9, 31-33].

Affected cells first show coarse aggregation of the chromatin that abuts inside the nuclear membrane. The cytoplasm shrinks, and cells diminish in volume. By that time, the apoptotic cells are seen in halos and the epithelial cells have lost the specific microvilli and desmosomal cell-to-cell junctions. The cell continues to shrink, the nucleus is condensed-later fragmented-and blunt protuberances of cytoplasm may develop and later separate from the rest of the cell body by enclosure of the functionally active cell membrane. Using that mechanism, the whole cell breaks up into smaller particles called apoptotic bodies, which include relatively intact cell organelles and parts of the nucleus. Apoptotic bodies are either engulfed by neighboring cells or extruded into the glandular lumen [9, 30, 31] (Fig 1). The lysosomal enzymes of the engulfing cells are involved in the digestion of the apoptotic bodies, but the apoptotic bodies dispersed into lumen or fluid may escape digestion and undergo a spontaneous degeneration process such as necrosis [34].

Figure 1
Apoptosis and necrosis
The two pathways of cell death differ from each other at most points: Apoptosis is genetically programmed active process that starts with DNA fragmentation and increasing density (decreased volume) fragmentation of the cell with active cell membrane action and followed by phagocytosis by neighboring cells or leukocytes without inflammation process. Necrosis starts with membrane damage with subsequent swelling of the cell and lysis of the cellular organelles followed by inflammation process.
2. Proliferation

Cyclic proliferation is characteristic of the female reproductive organs during fertile years, and FSH and estradiol are the most important hormones stimulating proliferation, especially in the ovary and endometrium [35]. The ability to measure proliferation and to know the factors regulating hormonal effects is of great value, since proliferation is associated with carcinogenesis of the endometrium [25, 28]. High proliferation rate in endometrial carcinoma is also associated with aggressive behavior of the tumor [36-39].

The events taking place in proliferating cells are best illustrated in the cell cycle (Fig. 3):

- **G1-phase** represents the period from mitosis to S-phase. Most of the specialized functions of the cells are carried out during this phase and G0, and the DNA content of benign diploid cells in this phase is equivalent with a double set of chromosomes. The cells are under the influence of several growth factors and other stimuli, also oncogenes, that may push the cells to enter the next proliferative phase or to enter the resting phase G0. Before entering the S-phase there is an important restriction point, under control of a tumor suppressor gene, wild type p53, that is able to stop proliferation of genetically defective cells and lead them to G0 to be repaired, or to undergo apoptosis (see Section 2.2.2).
- **S-phase** is the period during which the DNA is duplicated.
- In **G2-phase** the cells show duplicated DNA contents and prepare for mitosis.
- **Mitosis (M)** features cell division, during which the DNA chromatin is divided and condensed to chromosomes distributed equally to each daughter cell.

**Figure 2**

Cell cycle

The specialized action of the cells takes place in G1-phase and the length of this phase is shortened by estrogens, which push the cell to the next phase and towards mitosis, thus shortening the cell cycle. D-type cinases and Cytocin E are the main regulators of both estrogen and progesterone action, but in a different way as progesterone prolongs the G1-phase, at least in breast tissue, thus promoting the specialized function of the cells.
Measurements of the ploidy level can be used to describe the genetic contents of the cell population. The results can reflect the number of cells with aberrant chromosomal contents and the proportions of cells at different points in the cell cycle [40-42].

2.1. Regulation of cell cycle by estrogen and progesterone

In animal studies, 17ß-estradiol reduces the cell-generation time by selectively shortening the time that cells stay in G1-phase and by promoting the G1/S transition of uterine epithelial cells in vivo [43]. D-type cyclins D1 and D3 are cell-cycle-promoting factors induced by estrogen in G1-phase [44]. Cyclin E is also a G1/S regulatory protein shown in endometrial endometrioid carcinoma in a tumor-grade-dependent manner but not in endometrial hyperplasia or in normal endometrium [45]. Unfortunately, little is known about the role of progesterone directly in the cell cycle of endometrial cells.

In breast carcinoma cells, both estrogen and progesterone act mainly via D1 and c-myc as targets. Estrogen stimulates the formation of these proteins as well as the formation of highly specific activity forms of the cyclin E-Cdk2 enzyme complex lacking the cyclin dependent kinase CDK3 inhibitor p21. The delayed growth inhibition of progestins involves decreases in cyclin D1 and E gene expression and recruitment of CDK inhibitors into cyclin D1-Cdk4 and cyclin E-Cdk2 complexes. Progestins promote the cell differentiation in the prolonged G1-phase [46, 47].

2.2. Monitoring proliferation and apoptosis

2.2.1. Proliferation

Proliferation can be studied by morphological identification and counting of the mitotic cells. Later methods have been developed to evaluate the number of cells in other active cell phases as in S-phase (flow cytometric methods) [48, 49], or in phases G1-G2, i.e., all other active phases except G0-phase (immunohistochemical method for staining of Ki-67) [50]. Ki-67 is a nuclear protein (antigen) present only in proliferating cells. This protein can be demonstrated with anti-Ki-67 antibody (MIB-1) during the cell cycle except in G0-phase and the early part of G1-phase [50, 51]. Ki-67 is present in endometrial glands during the proliferative phase and during the first half of the secretory phase but fades off during the second part of the secretory phase [52, 53].

2.2.2. Apoptosis

Apoptosis is first detected and described according to morphologically characteristic signs [9] that make it possible to detect apoptotic cells in routine staining, such as hematoxylin and eosin(H&E) staining in light microscope. In some tissues and conditions, such as inflammation with leukocyte infiltration, the detection of apoptotic cells is difficult. In situ hybridization techniques have been developed to assess identification of the cells under programmed cell death [54, 55]. Generally, these
methods are based on labeling the free 3′hydroxyl ends for marking the fractions of DNA. When terminal deoxynucleotidyl transferase (TdT) is used to incorporate biotinylated deoxyuridine at sites of DNA breaks and the reaction then amplified with avidin-peroxidase for conventional histochemical identification by light microscope, the method is usually called the TUNEL-method (TdT-mediated dUTP-biotin nick end labeling method) [54, 55]. The TUNEL-method has several variations [56, 57] that enable identification of apoptotic cells even at the beginning of the apoptotic process, when the morphological signs are not observed. Using the combination of both morphological and staining criteria may give false low rates of apoptosis, but cells showing unspecific staining of non-apoptotic cells can be excluded [56]. A flow cytometric TUNEL-method has also been developed and is able to give semiquantitative images of apoptosis frequency in pure cell solutions [58].

Radiolabeled nucleotides can also be incorporated to the free 3′hydroxyl ends of the 180-base-pair DNA fragments or their multimers. In agarose gel electrophoresis, a typical ladder pattern is seen [59, 60]. The thickness of the ladders gives only a coarse image of the number of apoptotic cells among the cells studied. This method is suited for evaluation of pure cell cultures or cells easily separated from the tissues.

Quantitative image analyses to detect apoptosis in situ have been developed only in experimental studies [61]. Time-consuming ocular methods are still needed for comparison of apoptosis in different cell populations in the same organ or tumor as well as for observations of staining heterogeneity [62, 63].

2.3. Other regulators of apoptosis and proliferation

2.3.1. Bcl-2

The proto-oncogene bcl-2 (B-cell lymphoma/leukemia 2) [64] is implicated in controlling the cell cycle together with other members of the bcl family, such as bax, bcl-X-long (bcl-XL), and bcl-X-short (bcl-XS). Bcl-2 functions to prolong survival of healthy and pathological cells by blocking apoptosis [65] and is opposed by bax [14, 66, 67]. The other pair of bcl-family-forming heterodimers is bcl-XL / bcl-XS. In this pair, bcl-XL promotes prolonged cell survival, while bcl-XS promotes apoptosis [14]. Endometrial epithelium is immunoreactive for bcl-2 in the follicular phase, and its strictly cyclic appearance in studies using immunohistochemical methods argues that bcl-2 is under hormonal control [52, 68, 69].

Bcl-2 may act as an oncogene [67, 70, 71], but the action of bcl-2 in human endometrium and endometrial carcinoma is complicated because of the coaction with other members of the bcl family, such as bax, bcl-XL, and bcl-XS [52, 67, 71-74].
2.3.2. *P53*

The tumor suppressor gene wild type p53 is a powerful regulator of cell proliferation and apoptosis [75, 76]. It encodes a sequence-specific DNA-binding phosphoprotein that is able to block stressed or DNA damaged cells in G1 (G0). The blocking process is even dependent on other gene expressions, such as WAF1 [77], and on growth factors [78]. After successful repair the cell is allowed to enter S-phase and replicate; otherwise, the pathway of apoptosis is chosen (Fig. 2). Wild type p53 has the capacity to protect organisms against carcinogenesis by commanding the defective cells to apoptotic pathway (if not repaired), while mutated p53 is the single genetic mutation most commonly observed in many different types of tumors [41, 76, 79-81].

Wild type p53 is a short-lived protein and has been difficult to show in immunohistochemical staining, maybe also because of its great polymorphism [82]. The mutated p53 has a longer half-life and has been widely studied in carcinomas using immunohistochemical methods. (See Section 6.2. Carcinogenesis)

3. Ovarian hormones

Estrogens and progesterone, which are ovarian hormones, control normal cyclic endometrium [83]. Like other members of the steroid hormone family, such as androgens, they elicit their genomic effects via nuclear receptors. Progesterone and androgens are bound mainly to the sex-steroid-binding globulin SHBG, and only a small free fraction of these hormones is responsible for their hormonal effects. Thus the hormonal effects can also be regulated by an excess or shortage of SHBG. The effects may be locally modulated by growth factors [84-87], but the rapid hormonal effects (via neurotransmitters) on the cellular functions of endometrium are less known [88, 89].

3.1. Estrogens

The ovarian steroidogenesis of 17β-estradiol (E2) takes place in follicular granulosa cells and depends on follicle-stimulating hormone (FSH) [35]. Estrogen has a mitotic effect on the endometrium, and it also upregulates both estrogen and progesterone receptors in the follicular phase of the normal menstrual cycle and during hormonal replacement therapy (HRT) after menopause [88, 90] [91-94]. Increasing levels of E2 are seen in the follicular phase of the menstrual cycle, and high levels of E2 together with increasing values of progesterone are observed during the luteal phase with midluteal peak 1 week from the ovulation. Decreasing values of both hormones are noted during the last 6 days of the luteal phase until the start of menstruation.

17β-estradiol (E2) is a biologically active estrogen while estrone (E1), an aromatase product, is a low-potency estrogen. The interconversion of E2 and low potency E1 by 17β-hydroxysteroid dehydrogenase (17β-HSD) isoenzymes takes place in a tissue-specific way, dependent on which type of the isoenzyme is dominant in each tissue.
17β-HSD type 2 (17 β-HSD 2) catalyzes the oxidation of E2 to E1, and 17β-HSD type 1 (17β-HSD 1) catalyzes the reduction of E1 to E2 [95] (Figure 2). In normal endometrium, progesterone acts for cell differentiation and for production of 17β-HSD 2, and thus prevents the proliferative effects of E2 [2, 3, 96-99] (Fig.3). 17β-HSD 2 is also present in endometrial hyperplasia, even if the tissue shows proliferation and the proliferative normal endometrium lacks 17β-HSD 2. Less than one half of the cases with endometrioid endometrial carcinoma show presence of 17β-HSD 2 [95, 100, 101], and these carcinomas may still have some protection against unopposed-estrogen effects. In benign and malignant breast tissue, the 17β-HSD 1 is dominant, and this difference between endometrium and breast is essential when safety aspects of hormonal therapy are discussed [5, 96, 101].

In menopause, the production of estradiol in ovaries ceases, even if some follicles can still be observed in peri- and postmenopausal ovaries, while the production of testosterone from the stroma continues during some postmenopausal years [102, 103]. Androstenedione of adrenal origin becomes the main source of estrogen products of postmenopausal women. Estriol is peripherally aromatized from androstenedione, and some organs such as the breast can further convert estrone to estradiol in the presence of 17β-HSD 1 as mentioned above [101].

Figure 3
Gonal steroid synthesis and metabolism
3.2. Progesterone

Serum progesterone levels are low in the follicular phase, but there is a rapid increase after ovulation, since progesterone synthesis occurs mainly in granulosa cells of corpus luteum during the luteal phase of the menstrual cycle. During pregnancy, the placenta is the main source of progesterone synthesis. Progesterone stops estrogen-induced proliferation [2], down-regulates both receptors [90], and enhances secretory differentiation of the epithelial cells as well as decidualization of the stroma. Progesterone is also connected with proliferation-called the second wave of proliferation-in the stroma at the end of the luteal phase[2].

Natural progesterone cannot be administered orally, and for endometrial safety synthetic progestagens are used in HRT regimens. Long term use of estrogen-only regimens, even using low-potency estrone, has been connected with clearly elevated risk of endometrial carcinoma [26, 92, 104-106]. Different doses of progesterone for continuous therapy and different lengths of progesterone therapy in sequential therapy have been tested to find the lowest possible total dose of progesterone that is able to prevent endometrial hyperplasia and cancer. Most therapies used today are reasonably safe for the endometrium when the end point of the studies has been histopathologically evaluated absence of endometrial hyperplasia and cancer [107-115].

4. Receptors ER and PR

Estrogen and progesterone receptors (ER and PR) are members of the steroid receptor family, which shares structural similarities with thyroid hormone receptors. Each receptor is loosely bound to the nuclear membrane and able to bind the respective hormone and transport it to the nucleus. This hormone-receptor complex is bound to the specific DNA sites and activates the polymerase transcription [116]. The nuclear product, messenger RNA, is produced and transported to cytoplasm for further protein production [35].

4.1. ER

Three types of ER (estrogen receptor alpha, ERα; estrogen receptor beta, ERβ; and estrogen receptor gamma, ERγ) exist in several isoforms, and the proportion of each as mediator of estrogen effects differs in organs and tissues [117, 118]. ERα, which for a long time was assumed to be the only ER, dominates normal endometrium throughout the menstrual cycle [119] and it also dominates in endometrial carcinoma [120] and endometriotic tissue [121], even if ovarian endometrioma may show more ERβ expression [122] and ERβ also exists in the endometrium. Further ERβ has been demonstrated in the oviduct, ovary, kidney, brain, and heart as well as male organs such as the prostate and testis [117]. The most recently discovered estrogen receptor, ERγ [123-125], may have some prognostic value in breast cancer [118].
The variation of the estrogen receptor types in endometrium and brain as well as in skeletal and vascular systems has also been a possibility for novel therapies with selective estrogen receptor modulators (SERM), e.g., against osteoporosis [126]. The most-used hormonal therapy for breast cancer, namely, tamoxifen, is functionally also a SERM, giving different effects for ERα and ERβ: it is an agonist for ERα and an antagonist for ERβ [127, 128]. Unfortunately, tamoxifen in long-term use has a carcinogenic effect on endometrium via ERα activation [129-132], and this risk has promoted the use of aromatase inhibitors in the therapy of breast carcinoma.

Thus, in studies of many organs such as ovaries, the evaluation of both ERα and ERβ is necessary, while ERα alone is able to illuminate the estrogen sensitivity of the endometrium.

4.2. PR

For PR, two isoforms, A and B, are known. Both homo- and heterodimers (AA, BB, and AB) are activated by the natural ligand progesterone. As well, the function of PR A and PR B differs between organs, e.g., uterus and breast: studies in knock-out mice have shown PR A as necessary for action of progesterone in the genital tract, including the uterus, while PR B is required for the normal proliferative effect of progesterone in the breast [133].

4.3. ER and PR in cyclic endometrium

Estrogen receptor (ER) increases in the endometrial epithelium and stroma during the follicular phase and decreases after ovulation to reach a low level under the late luteal phase [90, 134]. Progesterone receptor (PR) also increases during the follicular phase and decreases in the epithelium in the luteal phase, but it stays at a higher level in the stroma until menstruation [90, 135]. ERα is dominant in human endometrium [136], even if ERβ may also modulate estrogen's action, especially in the epithelial cells. In the endometrium, both PR A and PR B are known [137] with PR A as the quantitatively dominant isoform.

4.4. ER and PR in postmenopausal endometrium

There are only a few studies of sex-steroid receptors in postmenopausal endometrium using an in situ method [132, 138, 139]. One study with quantitative estimation of receptors found 92% expression of ER and 54% expression of PR [132]. Up-regulation of the receptors by estrogen is a rapid process, and in biochemical experimental studies a 3-fold increase in receptor concentration of both ER and PR was observed within 1 day and maximal increase in 3 days [140]. Progesterone in high doses is able to down-regulate the receptors in 1 to 2 days [141], and even shorter times have been observed [142].
4.5. ER and PR in endometrial carcinoma

A loss of sex steroid receptors is an early event in endometrial carcinogenesis, and endometrial carcinoma generally has a lower level of steroid receptors than does normal endometrium or endometrial hyperplasia [80, 143, 144]. There is also a great variation in receptor content, especially in PR content, in tumors of high or low differentiation grade with low progesterone content in poorly differentiated tumors [145-148], and in tumors of the subtypes with worse prognoses [149, 150]. Low PR content or absence of PR is an unfavorable prognostic factor [151], and some studies also show prognostic significance of ER [152, 153]. High receptor content is also associated with other positive prognostic factors as diploid DNA content and low S-phase fraction (SPF) [154].

4.6. Heterogeneity of receptor expression

In benign endometrial tissue, the expression of sex-steroid hormone receptors may differ between epithelium and stroma, between surface epithelium and epithelial glands, and even between superficial glands and deep glands. Meanwhile, homogeneous expression of receptors is seen mostly inside the specific tissue in specific layers of endometrium.

The density of hormone receptors may differ inside the malignant tumor in various ways: between epithelial parts and the stroma [155, 156], between different parts of the tumor, and even inside the same fraction [80, 156, 157]. Primary tumor and metastases may also have different hormone-receptor expression as the aggressive receptor-negative subpopulations may give rise to metastases more often than the relatively benign receptor-positive subpopulations [158-160]. Very little is known about the importance of the heterogeneity of sex hormone receptors in endometrial carcinoma, but theoretically hormonal therapy should give a different response in receptor-dense parts compared with parts with low receptor expression. More broadly, very little is known about the implications of receptor heterogeneity in cancer therapy in general.

5. The homeostasis of benign endometrium

The endometrium is under sex-steroid control, and its homeostasis is regulated by hormones during the menstrual cycle, which culminates in the implantation process. In a non-fertile cycle, the endometrium rapidly undergoes a remodeling process by menstruation, proliferation, and specialization of the different endometrial cells, in order to be ready for the next implantation. Every phase of endometrium has a specific balance between proliferation on one side and apoptosis and necrosis on the other side [83, 161].
5.1. Proliferation in endometrium

Proliferation of the endometrial epithelium is maximal in the proliferative and follicular phases and is stopped after ovulation. Studied with proliferation marker Ki-67, 37% to 38% of the epithelial cells were in active-cell phase in the proliferation phase [53, 162], and the corresponding mitotic index was 2.3% [162].

Stromal proliferation follows mainly that of the epithelial glands during the proliferative phase [162-166]. However, there are exceptions from the rule: stromal cells may show proliferation during the luteal phase [2] as shown, e.g., in implantation process [87, 167-169]. The basal layer of endometrium shows lower but constant proliferation throughout the menstrual cycle, and the cyclic changes are most marked in the superficial parts of the endometrium [170].

5.2. Apoptosis in endometrium during the menstrual cycle

Apoptosis is rare during the proliferation phase of endometrium even if it has been reported in some studies at the beginning of the phase [18, 171]. Increasing frequency of apoptosis has been reported during the luteal phase and during menstruation [13, 18-21, 23, 69, 162, 166, 172-174], and locally in the implantation site [167, 175-177].

Cyclic apoptosis in the endometrium provides evidence for hormonal regulation of apoptosis in endometrium [19], and this has also been demonstrated in experimental studies [178-180]. Different study animals have been used, and results may differ for that reason: in hamster epithelium, estrogen withdrawal induces apoptosis [179], while rabbit endometrium is dependent on progesterone, and, consequently, withdrawal of progesterone induces apoptosis here [178, 180]. Further, the cycle-specific apoptotic activity may provide evidence for its importance in inducing menstruation and in remodeling of the endometrium.

5.3. Paracrine mechanisms in the regulation of proliferation and apoptosis in endometrium during the menstrual cycle

In a complex interaction, growth factors, cytokines, and enzymes, as well as receptors, modulate hormonal effects in endometrium during the menstrual cycle. Epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) are seen during the late follicular and secretory phases [6] and insulin-like growth factor 1 (IGF-1) in the late secretory phase [7]. There is some evidence for the hypothesis that TGF-α is an important molecule in the pathway of estrogen-mediated cellular proliferation [27], and also, besides epidermal growth factor (EGF), important in regulation of stroma decidualization [181]. In a fertile cycle, rapid proliferation and apoptosis occur in the implantation site when levels of both estrogen and progesterone are high [167]. The paracrine signaling system is therefore important for the local regulation of tissue remodeling. As well, IGF-1 and IGF-2 have been connected with mitogenic effects and differentiation of the epithelial cells [182], and together with the interplay with the binding protein IGFBP-I they locally regulate the decidua during implantation process
and early pregnancy. Another growth factor associated with implantation is transforming growth factor beta (TGF-β) [177, 183]. Disturbances in the regulation of implantation may contribute to pregnancy loss and to pre eclampsia [182].

TGF-β has been shown to participate in apoptosis in experimental studies [184], and tumor necrosis factor (TNF-α) in the late luteal phase and during menstruation in human endometrium may have a similar role [19, 161, 185].

Changes in vascular endothelial growth factor (VEGF) and its receptor (KDR) as well as TNF-α may activate matrix metalloproteinases (MMP) in the late secretory phase of endometrium and may contribute to the menstruation process [87, 161, 186]. VEGF is an important factor in neovascularization and is present in the endometrium in the regeneration process during menstruation and in the early follicular phase [86, 168, 186], in decidualization, and in the implantation process [84, 87, 167], and in ectopic implantation of the endometrium [183], as well as in endometrial carcinoma [187-189]. The disturbances in the regulation of VEGF may contribute to intermenstrual bleedings [85]. Tissue hypoxia has been shown to be able to regulate changes in proteins coded by the VEGF family, proteins that together with angiopoietins regulate growth and death of endothelial cells [168].

5.4. Menstruation mechanisms

The vascular changes associated with menstrual bleeding were observed by Markee in the 1940s [190]. He used autotransplants of endometrium in the chamber of the eye of rhesus monkeys and observed the spiral artery spasm and subsequent necrosis of the functional endometrium during menstruation. The existence of a pressor agent or agents was proposed to be responsible for vascular stasis and also for protection against excessive blood loss. His theory came to be established as the dominant model for menstrual mechanisms. Later research connected prostaglandins (PGs) and endothelins (ETs) to the vascular changes in menstruation [191, 192]. Prostaglandin (PG) synthesis is stimulated in secretory endometrium by estrogen via cyclooxygenase [193], and prostaglandins may also have regulatory effects on angiopoietin and VEGF action. Markee also described earlier changes as thinning of the endometrium, dilatation of the arteries, and leukocytic infiltration. Only part of the findings could be interpreted by Reynolds, who was critical of Markee's studies [194] using another type of monkey (New World monkey that lacks the spiral arteries and yet menstruates), and discussion focused on the amount of endometrium shed during menstruation. Bartelmez argued that only a very small amount of endometrium was shed [15, 195], the discussion later continued by others [21, 112, 196-198].

These authors contributed greatly to the literature through their studies with electronmicroscopy, e.g., on the loss of cell-to-cell adhesion before the start of menstruation [197]. A detailed study by Christiaens also revealed the existence of defects in the vascular endothelium in superficial parts of the endometrium during menstrual spotting, hours before the menstruation starts [199]. The basophilic granules were described in endometrium and the lysosomal activity was shown. The autodigestion, together with the fact that not all primates have blood-yielding
menstruation, contributed to what was called the lysosomal concept of menstrual bleeding [200]. In any event, necrosis, vascular bleedings, and thrombosis in menstrual endometrium were also observed [112, 198, 201].

Hopwood and Lewison showed that the basophilic granules described by Bartelmez in 1933 [15] were apoptotic bodies. These and apoptotic cells could be found in normal-cycling endometrium in the proliferative phase but mainly at the end of the secretory phase in cd 24-28 [18]. Even in the ultrastructural study by Verma, apoptosis was dated to the end of the menstrual cycle [21], and Otsuki names the same results in a study that actually focuses on bcl-2 [69]. Tabibzadeh showed apoptosis in the glandular epithelium of human endometrium throughout the secretory phase, increasing toward the end of the phase [19], and Kokawa interpreted these findings but described apoptosis even in the early proliferative phase [171].

During menstruation, a part of the functional layer of the endometrium is shed, and the re-epithelization of the surface occurs from the stumps of the glands. [99, 161].

At the end of the luteal phase, infiltrating leukocytes may release many regulatory proteins such as cytokines and proteinases. Molecules such as TNF-α, interleukin (IL)-1, relaxin, and TGF-β are also locally produced in different cells of endometrium at the end of the luteal phase and during menstruation, and they may regulate MMP production [202, 203]. There is strong evidence that MMPs play a critical role in the tissue breakdown in menstruation as also described in Section 5.3. In any case, little is known about activation of leukocytes and about their interaction with endometrial cells.

The modern understanding of the menstruation process includes both apoptosis, vascular constriction, and collapse with prostaglandin action as well as MMP action with an inflammation-like process [193]. Still, there are many questions about the menstrual mechanism and about the remodeling of the remaining endometrium during menstruation.

5.5. Apoptosis and proliferation in postmenopausal endometrium: effects of HRT

There are few studies of postmenopausal endometrium, but some information can be obtained from the baseline studies of HRT. However, in most studies, only histopathological evaluation (and not immunohistology) is used, and baseline endometrial biopsy is not included routinely in all studies. Johannisson et al. reported atrophic endometrium in 76% to 90%, proliferative endometrium in 8% to 18% and occasional patients (< 2.3%) with progestational endometrium in the study groups recruited [113].

The studies using Ki-67 as an indicator of proliferative status in postmenopausal endometrium are few. Morsi showed Ki-67 positivity in 18% of the cases [22]; Mourits, in 2% [132]. An earlier morphological study indicates both low proliferation and apoptosis [18]
Histopathological evaluation during clinical studies of HRT is generally favorable to modern HRT with regard to endometrial safety [107-115]. In most epidemiological studies, therapies with monthly progesterone for > 10 days and daily progesterone in continuous combined HRT regimens also show low risk for developing endometrial carcinoma [204-206], even though there are contradictory results [207]. In clinical studies, histopathological evaluation is used, and the results in epidemiological studies are based on the registered diagnosis. However, in some studies, proliferation during HRT is investigated by proliferation markers (e.g., Ki-67) [208, 209], but the frequency of apoptosis remains unknown. High proliferation in the endometrium may provide evidence of a risk that hyperplasia will develop, but together with apoptosis it may simply be a sign of high cell turnover in a tissue.

Optimal balance between the proliferative effects of estrogen and the antiproliferative effects of progesterone is also needed for good bleeding control during HRT. Irregular bleeding during continuous HRT regimens is one of the major problems with HRT [3, 112, 210, 211] and is the most usual reason for discontinuation of the therapy. Progesterone down-regulates both estrogen and progesterone receptors and causes atrophy in the endometrium. Apoptosis appear to occur in endometrial stroma after prolonged continuous progesterone therapy and may contribute to breakthrough bleedings [3]. Decreased cell-to-cell adhesion as an effect of matrix metalloproteinases (MMP) could be involved in this mechanism of vascular and stromal breakdown, which is seen during progesterone therapy and prior to menstruation, as also discussed in Section 5.3. [212-217]. These changes occurring during the menstrual cycle or under hormonal manipulation could be expected to somehow be affected by sex steroids and mediated by receptors, even if other local regulators are of great importance. The exact mechanisms leading to vascular fragility and breakthrough bleedings remain unknown.

6. Endometrial carcinoma

6.1. Epidemiological aspects

Endometrial carcinoma is the third most common malignancy among women in Sweden, [218] and approximately 1,100 new cases are diagnosed every year. An unopposed estrogen therapy may cause pathologic changes in the endometrium, and in some cases this may lead to malignancy. Other factors related to increased risk of endometrial cancer are obesity, nulliparity, early menarche, and late menopause. Most patients are postmenopausal, and only 5% of patients are younger than 50 years of age.
6.2. Classification and prognostic factors

The majority of endometrial cancers are diagnosed at an early stage, as the main symptom of the disease is postmenopausal bleeding, which usually leads the patient to seek medical advice. In addition, doctors tend to react quickly to potential cases of endometrial cancer, as the guidelines of diagnostic procedures (ultrasonography and/or endometrial biopsy) of postmenopausal bleeding are clear.

The prognosis of endometrial carcinoma is generally good: overall 5-year survival is approximately 80%. However, endometrial carcinoma is a heterogeneous entity. Endometrioid carcinoma has a generally better prognosis, while cancers of seropapillary and clear cell types are associated with less favorable prognoses even in early stages [219, 220]. Other prognostic factors are as follows: tumor grade, stadium, S-phase fraction, ploidy, sex-steroid-receptor content, age, and invasivity of blood and lymph vessels [37, 145, 147, 151, 221, 222]. In order to find better markers of tumor aggressiveness among early stage tumors, new genetic markers such as p53 [79, 223-225] and bcl-2 [74, 226], and markers reflecting tumor growth, such as Ki-67, [37, 39, 224, 225, 227] have been tested (see Section 2).

6.3. Carcinogenesis

Many risk factors of endometrial carcinoma are associated with increased estrogen exposure, but endometrial cancer might also develop without endogenous or exogenous hormonal exposure [27]. Many tumors pass the endometrial hyperplasia [25] while others do not [28]. The heterogeneity of the molecular biology of endometrial cancer makes it difficult to find a rational stepwise model for carcinogenesis of the endometrium. At the risk of oversimplifying, endometrial carcinoma is often divided in 2 types according to the carcinogenesis: Type I is characterized as estrogen-related with a carcinogenetic pathway including hyperplasia and atypical hyperplasia [25], and type II as endometrial carcinoma independent of estrogen [26, 93]. Type II is also called atrophy-associated carcinogenesis[228]. Type I endometrial carcinoma is typically a well-differentiated carcinoma with glandular pattern, and it expresses estrogen and progesterone receptors. Patients are often near menopause, younger than the patients with type II cancer, which is associated with advanced stadium in the time of diagnosis and worse prognosis. Histopathologically, type II disease often exhibits more aggressive subtypes of endometrial carcinoma with higher incidence of oncogenes.

The histopathological subtype has great prognostic significance and has also been used to divide endometrial tumors into 2 groups: endometrioid adenocarcinoma as 1 group and together the papillary serous, clear-cell, undifferentiated, and squamous-cell subtypes as another group with less favorable prognosis [219, 229].

Additionally 1 type of endometrial cancer is associated with hereditary non-polypoid colon carcinoma (HNPCC) [230].
The 2-step model of carcinogenesis is generally well accepted and requires a series of genetic events with both oncogenes and tumor suppressor genes, as described for HNPCC [231]. Several oncogenes have been associated with endometrial carcinogenesis. The RAS family encoding p21 proteins, ERRB-2 (=HER-2 or neu) and C-MYC, are the oncogenes most studied besides the tumor-suppressor gene p53 [28, 151, 162, 229, 232-238]. Satayaswaroop has earlier presented a model where malignant transformation can occur in any differentiation level of endometrial cells [239].

Wild type p53 protein is known as a tumor-suppressor gene that leads cells to arrest with the possibility for DNA to be repaired [76]. Deranged p53 protein is the alteration most frequently documented in human tumors and is often associated with poor prognosis and advanced stadium [41, 76, 79-81]. In endometrial carcinoma, mutation of p53 is observed as a late phenomenon in carcinogenesis [234, 235, 238, 240] and it has been associated with more aggressive subtypes of carcinoma [229], being relatively rare in endometrial carcinoma of the endometrioid type [237, 241-243] and absent in premalignant endometrial hyperplasia [244]. These results may indicate that p53 mutation is a part of the pathway independent of estrogen action [27].

Microsatellite instability has been identified in sporadic endometrial cancer in 17% to 23% of cases, but is more usual in endometrial cancer associated with HNPCC syndrome [245, 246].

6.4. Homeostasis in endometrial carcinoma

Proliferation rate in endometrial carcinoma is correlated with tumor grade and with more malignant histopathological types, but not to the stadium of the tumor [36, 39, 53, 149, 224, 225, 227, 236, 247, 248].

There are only few in vivo studies of apoptosis in human endometrial carcinoma. In 2 studies there is some indication of correlation between tumor grade and apoptosis [166, 249]; others are indirect studies showing variation in the gene products of the bcl family in tumors with different prognoses [74, 250-252].

6.5. Growth factors and endometrial carcinoma

Because several growth factors such as EGF, TGF-α, and IGF-1 have been connected with the pathway of receptor mediated estrogen action in normal endometrium, they may also play a role in estrogen-dependent endometrial carcinoma [8, 27].

IGF-1 acts in regulation of several vascular endothelial growth factors (VEGF) [188, 189] that are associated with angiogenesis and metastasis of endometrial carcinoma [187].
6.6. Estrogen metabolism in endometrial carcinoma

The progesterone-induced activation of the protecting enzyme 17β-HSD 2 is altered in endometrial carcinoma, compared with the activation in the normal luteal-phase endometrium of premenopausal women [100]. In endometrioid adenocarcinoma, 17β-HSD 2 enzyme has been shown in 37% of the tumors, and it has been shown more often in tumors of younger patients [95]. In any event, the oxidation of E2 to E1 is still dominant in endometrial carcinoma compared with the opposite direction of the conversion (there is no 17β-HSD 1), at least in younger patients, and endometrial carcinoma still has some capacity to defend itself against unopposed-estrogen effects. On the other hand, more than half of the adenocarcinomas have no 17β-HSD 2. Thus, the moderate progesterone effects even in receptor-positive endometrial carcinoma may be understood as in part a result of the altered metabolism of estrogens in cancer tissue.

A worse situation is seen in breast-tissue disorders such as hyperplasia and ductal carcinoma [101], as the existence of 17β-HSD 1 in these tissues is able to stimulate the aromatization product E1 to be bioactivated to E2.

6.7. Progesterone therapy of endometrial carcinoma

Progesterone therapy has been used mostly in recurrent metastatic endometrial carcinoma and as primary treatment when surgery and radiation have been contraindicated [253]. Response rates of about 30% have been reported but vary widely according to the inclusion criteria and the tumor grade [4, 254-256]. The empirical effect of progesterone on endometrial carcinoma may consist of the receptor-mediated inhibition of estrogen-induced proliferation [257] and also of effects on growth factors [258].

Progesterone therapy has been used in treatment of metastatic endometrial carcinoma empirically since clinical studies have shown response to progesterone [253]. Different response rates of 10% to 30% have been reported, with lower rates tending to be found in later studies [4, 158, 254-256, 259]. Generally, higher response rates are observed in patient groups with PR-positive tumors [260, 261], but the response rates are not directly correlated with receptors, and variable response can be found in groups of patients with both receptor-positive and -negative tumors. One reason for the variable response rates could be the heterogeneous pattern of hormone-receptor expression [155, 156, 159]. In experimental studies, preceding estrogen therapy has been able to facilitate progesterone's effects [257], which may be mediated via the stromal cells of the tumor [5].

Even if hysterectomy with salpingo-oophorectomy is the first-choice therapy for endometrial carcinoma, progesterone therapy has been shown to be successful in some cases of young patients when an unopposed-estrogen etiology, such as PCO syndrome, has been suspected [262-264]. Although there is some conflicting evidence on adjuvant progesterone therapy [265], on balance the evidence shows that this approach has not been successful [266-268].
AIMS OF THE STUDY

This study focuses on the involvement of apoptosis and proliferation in tissue modulation, and on the importance of hormonal sensitivity for these processes, in benign endometrium and in endometrial carcinoma under different hormonal circumstances. The specific aims were as follows:

- To investigate endometrial hormone sensitivity (ER and PR), proliferation (Ki-67), and apoptotic index (Ai), as well as an antiapoptotic factor (bcl-2), during major hormonal withdrawal before and during menstruation, under the hypothesis that apoptosis is involved in the mechanisms of menstruation.

- To elucidate ER, PR, Ki-67, and Ai separately in the stroma and epithelial endometrium before and during substitution with continuous combined HRT, under the hypothesis that the ratio of proliferation to apoptosis is not increased during HRT.

- To evaluate hormonal sensitivity, apoptosis, and proliferation as well as bcl-2 and the incidence of tumor suppressor gene p53 in endometrioid endometrial carcinoma before, during, and after hormonal manipulation with progesterone, under the hypothesis that progesterone withdrawal can induce apoptosis.
METHODS

1. Ethical considerations

The Ethics Committee of Umeå University approved the 3 studies in papers I, III, and IV included in this work. The Ethics Committee of each center involved in the multicenter study, represented partly in paper II, approved the study, and the Ethics Committee of Umeå University further approved this specific study. Informed consent was obtained from all women.

2. Subjects

2.1. Paper I

Endometrial micro-biopsies were taken with a Pipelle® or Endorette® instrument from 35 regularly menstruating healthy women who were not receiving any hormonal therapy during 37 menstrual cycles from 4 days prior to the onset of menstruation (Day -4) until the second menstrual day (Day 1). The biopsies may represent any part of the superficial corpus endometrium. Altogether 75 biopsies were taken, representing 6 consecutive days and the number of biopsies varied from 10 to 15 each day (Table 1, paper I). One biopsy per day and 1 to 3 biopsies per cycle were taken from individual patients. In 2 cases, only a single biopsy was taken; in 29 cases, paired biopsies were taken; and in 5 cases, 3 biopsies were taken. The length of the menstrual cycle varied individually but the data in this study were centered on the onset of bleeding.

2.2. Paper II

The patients were recruited in a prospective multicenter study carried out in 14 centers in Sweden [210]. Out of 92 women who had not used HRT during the past 2 months, 43 women had biopsy material allowing histological evaluation in both biopsies, i.e., the biopsy obtained before HRT and the biopsy during HRT (after 1 year of HRT). The therapy consisted of either conjugated estrogen (CE) 0.625 mg + 5 mg medroxyprogesterone acetate (MPA) (= CE/MPA) or 17β-estradiol (E2) 2 mg + 1 mg norethisterone acetate (NETA) (= E2/NETA). The women included in the study were required to be in good health, with an intact uterus, = 52 years and = 2 years postmenopausal. The exclusion criteria were: adenomatous hyperplasia with or without atypia, undiagnosed vaginal bleeding, history of cancer, cardiovascular or thromboembolic disease, uncontrolled hypertension, diabetes, and long-term medication with barbiturates, psychotropics, or antiepileptic drugs. No use of steroid hormones besides the study medication was allowed during the study period.
2.3. Papers III and IV

This study included a homogenous group of 29 patients with endometrioid endometrial adenocarcinoma: 4 patients with stage IA, 20 with stage IB, and 3 with stage IC according to the FIGO criteria for surgical staging [269]. In 2 cases, no surgery was performed due to contraindications. Three samples were obtained from each patient: biopsy 1 at the diagnostic endometrial biopsy with Pipelle® (Prodimed, Neuilly-en-Thelie, France) instrument or traditional dilatation and curettage (D&C), biopsy 2 after 14 days of treatment with 20 mg medroxy-progesterone acetate per day, and in 20 cases biopsy 3 after 2 days of withdrawal of progesterone treatment. In 9 cases, biopsy 3 consisted of material obtained from the hysterectomy specimen after 6 days of withdrawal of progesterone treatment.

3. Blood samples

Paper I

Blood samples were collected immediately before or after each endometrial biopsy. Samples were centrifuged, and serum was split into small portions that were frozen and stored at -20°C until analysed in the same run by a TRFIA method (time-resolved fluoro-immunoassay, Wallac).

Duplicate analyses were performed, and the resulting mean value was used. All samples were assayed in the same run. Intra-assay coefficient of variation (CV) was 2.0% for serum progesterone (S-P) and 7.5% for serum estradiol (S-E) analysis.

Blood samples of the women in the cancer study (papers III and IV) were collected at the time of biopsy 2.

4. Tissue processing and immunohistochemistry

4.1. Processing for apoptosis

In paper I, the biopsy material from corpus endometrium was fixed in 4% formaldehyde for 4 to 6 hours and embedded in paraffin according to routine procedures. The same fixation time was applied also for biopsies 2 and 3 in the cancer material (papers III and IV) to enable the use of the in situ end labeling (ISEL) technique used and described in paper I. This technique was used in cancer material only for control and not reported because the first biopsy (diagnostic) was often fixed for longer times and the ISEL method was therefore not used. The fixation time varied for the biopsies in the multicenter study (paper II), and another TUNEL method (ApopTag®, Intergen Company, Oxford, UK), was applied for identification of apoptotic cells. For immunohistochemistry, the fixation time was not critical.
4.2. Processing for ER, PR, Ki-67, bcl-2, and p53

Immunostaining for materials used in papers I, III, and IV was carried out in the Department of Pathology in Umeå University Hospital and for paper II in the Department of Pathology in Örebro Regional Hospital as separately described in the respective papers. Monoclonal antibodies directed against ER, PR, Ki-67, bcl-2, and p53 were used. Localization of antigen-antibody complexes was performed with the avidin-biotin-peroxidase complex (ABC) technique, except localization of bcl-2, for which the APAAP method was applied. Brown nuclear color was seen in positive staining of cells, except in bcl-2 staining that indicates the cytoplasmic staining with purple color.

4.3. Evaluation of steroid receptor immunoreactivity

In normal endometrium (paper I), a semiquantitative scoring method was used for evaluation of ER and PR expression: ER and PR staining in the surface and glandular epithelium as well as in the stroma were evaluated in each section. Positive cells showed a brown reaction product in the nucleus, while the cytoplasm remained unstained. Staining was scored in 4 categories as follows: 0 = cells were negative; 1 = most cells were weakly stained or occasional cells were strongly stained; 2 = most cells were moderately stained; and 3 = most cells were strongly stained. In postmenopausal endometrium (paper II), the stained cells and the total amount of cells inside the grid were again counted in minimum 10 high power fields (hpf), and in total at least 1,000 cells were counted. The whole section was evaluated if there were < 1,000 cells in the specimen, because many postmenopausal biopsy materials were scanty.

A methodological study of ER and PR evaluation was made in cancer material before progesterone treatment (paper III). Estrogen receptor and PR staining were evaluated with a standard light microscope using 3 different methods:

(1) The counting method: A grid was mounted in 1 eyepiece of the microscope and 100 cells/field were counted in at least 10 representative fields (overall counting) of the epithelial fraction of the tumor. Positive cells stained brown in the nucleus, while the cytoplasm remained unstained. If the sections showed heterogeneous staining in areas of at least half a high-power field (hpf), the percentage of stained cells was counted in a similar way in at least 5 fields of maximal staining and in at least 5 fields of minimal staining. The percentage of cells that were stained is referred to as the staining index. Hereafter, the staining indexes for ER or PR in overall staining are abbreviated as ER and PR, respectively, the staining index in the areas of maximal staining as ER-max and PR-max, and the staining index in the areas of minimal staining as ER-min and PR-min.
(2) The mixed method: The results of the counting method were used and the intensity of nuclear staining for ER and PR was ranked separately from 0 to 3 in overall counting, in the areas of maximal staining and in the areas of minimal staining. The score was given as follows: 0 was given for absent staining; 1, for weak staining; 2, for moderate staining; and 3, for strong staining. The results were given as an index, with the percentage of stained cells multiplied by the staining intensity score (0-3).

(3) The scoring method: The whole section was evaluated according to an integrated analysis of the visual appearance of intensity and amount of stained cells. Staining was ranked on a 4-point scale, as follows: 0 was allocated if there were no stained cells; 1, if most of the cells were weakly stained or if some cells were strongly stained; 2, if most cells were moderately stained; and 3, if most cells were strongly stained.

There were good correlations between the results with all 3 methods (paper III). The counting method was used in evaluation of receptor expressions in biopsy 2 (during the progesterone therapy) and in biopsy 3 (after withdrawal) and in evaluation of the changes between biopsies 1 and 3 (paper IV).

The heterogeneity (i.e., presence of a difference between the staining indexes in the areas of maximal and minimal staining) in the staining for ER and PR was assessed in each tumor, further referred to as ER-het and PR-het. In addition, a visual estimation-based scoring of heterogeneity was performed in paper III, in which the score of 1 meant no difference; 2 meant there was a difference of 1% to 25%; 3 meant a difference of 26% to 75%; and 4 meant a difference of 76% to 100%.

4.4. Evaluation of the Ki-67 index

The Ki-67 index was evaluated as described for ER and PR in the counting method (Section 4.3) (cells stained/100 cells), and this quantitative method was used to establish the Ki-67 index in all papers. In paper I, 2 different methods were applied in microscopic evaluation of the Ki-67 index, and the results were in good accordance with each other (P = 0.001).

Because the cancer tissue showed marked heterogeneity of proliferation, the counting was interpreted separately in the areas of maximal and minimal staining for Ki-67 expression, and the heterogeneity was counted out as described for the receptors (Section 4.3) and referred to as Ki-het.

4.5. Bcl-2 and p53

The cytoplasm of bcl-2 positive cells stained purple, and in a rapid visual evaluation the intensity of bcl-2 staining was scored on a 4-point scale as absent (0), weak (1), moderate (2) or strong (3). Infiltrating granulocytes were often seen in premenstrual and menstrual endometrium as well as in cancer tissue and showed strong staining, which served as an internal positive control. The percentage of p53 positive cells in the epithelial fraction of the endometrial cancer was counted in all biopsies obtained (papers III and IV).
4.6. Evaluation of apoptosis

With TUNEL methods, the nuclear materials of apoptotic cells stain brownish, and counterstaining with a blue nuclear marker makes it easier to identify apoptotic cells among normal cells. In normal benign material (papers I and II), the TUNEL method was necessary, because the identification of apoptotic cells among stromal cells with morphological criteria alone had been difficult. The stroma was often infiltrated with granulocytes, and it could be troublesome to distinguish between apoptotic cells and granulocytes. Identification of apoptosis in epithelial cells is easier, and the evaluation of apoptosis with only morphological criteria in H&E staining correlated well with evaluation of the specimen stained with TUNEL method, in the epithelial glands of benign endometrium (Spearman's correlation analysis, r = 97; P < 0.001) (paper I).

A grid was used in 1 eyepiece of a standard light microscope. Cells inside the grid area in 20 to 40 random fields in tissue sections were evaluated, and the apoptotic index (Ai) (i.e., number of apoptotic cells per 1,000 cells) was determined after counting 2,000 to 10,000 cells in total (in the postmenopausal material, a minimum of 1,000 cells was required). The morphological criteria used for apoptotic cells were as follows: single rounded cells or fragments of cells with densely aggregated chromatin and condensed cytoplasm, often lying in a halo of extracellular space [9].

In carcinoma (papers III and IV), only the epithelial part of the tumor was evaluated for apoptosis, and only morphological criteria were used in counting the Ai in traditional hematoxylin and eosin staining. [9].

4.7. The amount of stroma in carcinoma

Evaluation of the amount of stromal tissue was made in sections of biopsy 1 (paper III) stained for ER or PR. Both the stromal and epithelial areas covering the area of the grid mounted in 1 eyepiece of the microscope were counted in 10 non-fragmented parts of the specimen according to stereological principles [62]. The areas were summarized, and the percentage of the stromal area out of the total area of the tumor in these 10 sections was used in statistical analysis. The percentage of ER- and PR-positive cells in the stroma was also counted in these 10 sections.

5. Statistical methods

Standard non-parametric methods were used to test for significant differences between the 2 groups: Wilcoxon and Mann-Whitney tests were applied for paired and independent observations, respectively. Multiple group comparisons of means were performed using 1-way and 2-way analysis of variance (ANOVA). Fisher's least significant difference (LSD) procedure was applied as a post-hoc test for conducting pairwise comparisons. In order to evaluate sex-steroid receptor expressions in different foci of high and low proliferation in the same tumor, a repeated-measures design was proposed. Repeated measures were then analyzed using a general linear model (GLM) procedure. To study the relationship between feature variables, to show the agreement
between different methods of evaluation of receptors, and to compare different methods for detection of apoptosis, correlation analysis was applied. Spearman's and Pearson's correlation coefficients were used, and regression lines were fitted for the observations. Regression analysis was also used to identify possible predictors of the effect of hormonal therapy in endometrial carcinoma. In all statistical tests used, $P < 0.05$ was considered to be statistically significant.

Plots and calculations were performed using the Statistical Package of Social Science (SPSS), version 10.1 (Scandinavia AB, Stockholm, Sweden), and by MATLAB, version 6.1, (The Math Works, Inc. Natick, Massachusetts, USA).
RESULTS AND DISCUSSION

1. Results of the methodological evaluations

1.1. Apoptotic index (Ai), morphological and ISEL methods

Comparisons were made between the apoptotic indexes (Ai) in H&E- and ISEL-stained sections (epithelium) in 23 sections. In the ISEL method, both morphological and staining criteria were used, and in the H&E-stained sections, the evaluation was based on the morphological criteria only [9]. There was a strong correlation (r = 0.97; P < 0.001) in apoptotic indexes between the ISEL and H&E methods.

1.2. ER and PR in endometrial carcinoma, 3 different methods

Estrogen receptor (ER) and PR staining was evaluated with 3 different methods: in method 1 the average percentage of stained cells was calculated, in method 2 the staining intensity was added to the staining percentage and method 3 consisted of a scoring including both frequency and intensity of staining. All 3 methods correlated well with each other (Table 1).

The counting method (method 1) and the mixed method (method 2) were both used in separate evaluation of the ER staining index in the areas of maximal staining density (ER-max) and in the areas of minimal staining density (ER-min), and the results showed good correlation (r = 0.76 and r = 0.91, respectively; P < 0.001). The scoring method (method 3) was not used for separate evaluation of the areas of maximal and minimal staining, because the areas might have been too small for correct visual analysis.

In evaluation of PR staining, there was high correlation between methods 1 and 2, methods 1 and 3, and methods 2 and 3 (Table 1). Results of methods 1 and 2 also correlated well (r = 0.86 and r = 0.93, respectively; P < 0.001) when PR-max and PR-min were evaluated.

The 3 different methods of evaluating ER and PR status can be used with equal effect in the evaluation of hormone-receptor expression. The visual scoring method is quick, but the more time-consuming counting of the percentage of stained cells is necessary if the heterogeneity of receptor status is studied. Considering the staining intensity score alongside the percentage of stained cells did not provide any further information. The counting method is used in papers II - IV, as it permits evaluation of heterogeneity without subjective scores.
Table 1

Different methods of evaluating sex steroid receptor expression in the epithelial part of endometrial carcinoma. There was a high correlation of the results established with the 3 different methods (Pearson’s correlation coefficient, r, and P-values shown).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Methods tested</th>
<th>r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td>Scoring/Counting</td>
<td>0.75</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Scoring/Mixed</td>
<td>0.78</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Counting/Mixed</td>
<td>0.88</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>PR</td>
<td>Scoring/Counting</td>
<td>0.85</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Scoring/Mixed</td>
<td>0.85</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Counting/Mixed</td>
<td>0.91</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

2. Sex steroid hormones

Both serum 17ß-estradiol (E2) and serum progesterone (paper I) were monitored in the material of 35 healthy menstruating women from the normal luteal-phase values (ca. 400 pmol/l E2, and ca. 30 nmol/l progesterone) 4 days prior to menstruation, to minimal values during the 2 menstrual days (P < 0.001) (Fig. 3; paper I). The rapid decrease in availability of estrogen and progesterone as a sign of an unsuccessful fertile cycle is the inducing factor for a series of changes in the endometrium, leading to shedding of a superficial layer and part of a functional layer of the endometrium in the process of menstruation.

Women in the cancer study (papers III and IV) were postmenopausal (range 1-44 postmenopausal years), except 1 woman, who was perimenopausal. Consequently, the E2 and progesterone levels in serum were low, as shown in table 2.

Table 2

Levels of sex steroid hormones in 28 out of 29 patients with endometrial carcinoma (papers III and IV).

<table>
<thead>
<tr>
<th>Steroid hormone</th>
<th>Mean (SEM)</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2</td>
<td>89.0 (7.6) pmol/l</td>
<td>72 pmol/l</td>
<td>265 pmol/l</td>
</tr>
<tr>
<td>Progesterone</td>
<td>1.6 (0.19) nmol/l</td>
<td>0.6 nmol/l</td>
<td>5.5 nmol/l</td>
</tr>
</tbody>
</table>
3. Tissue sensitivity to sex steroid hormones

ER and PR expression, as indicators of tissue sensitivity to sex steroid hormones, were evaluated at the end of the menstrual cycle (papers I) and in postmenopausal endometrium before and during combined continuous HRT (paper II). In malignant endometrial tumors, receptor expression was analyzed before, during, and after medroxy-progesterone therapy (papers III and IV).

3.1. ER and PR in benign endometrium

3.1.1. ER and PR in cyclic endometrium

The ER immunoreactive score decreased in the glandular epithelium of menstruating women during the period from 4 days prior to menstrual start to 2 days prior to menstrual start, at which time it began to increase again. The ER score in the stroma showed just the opposite pattern, with increasing staining during the first 2 study days and decreasing staining from the day before menstrual start to the second menstrual day (Figure 3, paper I). Throughout the study period, the surface epithelium showed little staining of ER and PR, without any obvious pattern of change. The progesterone receptor score in glands decreased from 1.5, measured 4 days prior to menstruation, to 1.0, measured 1 day later, and it stayed at a low level until the second menstruation day. In the stroma, PR scores were on a higher level 4 days before menstrual start and still increased during the first day of the study period (Figure 2, paper I), but then decreased until the second day of menstruation (Figure 3, paper I). An apparent difference was seen in the expression of sex-steroid receptors in epithelial glands and in the stroma: In the epithelium a rapid decrease was observed in receptor expression, while the stroma was still sensitive at least to the effect of progesterone. Earlier decrease of PR in the epithelium compared with endometrial stroma has been observed in a previous study, and our results concur with those results [135].

3.1.2. ER and PR in postmenopausal endometrium

A high and relatively homogenous pattern of sex-steroid receptors was observed in postmenopausal endometrium, with mean ER expression 96 (cells/100 showing staining for ER) and mean PR expression 83 before HRT. The levels of ER and PR expressions in our study were on the same level as reported earlier by 2 other groups [132, 139]. Both ER and PR expression were higher in the epithelium than in the stroma (Wilcoxon signed rank test; P < 0.001), and that difference remained in biopsy obtained during continuous combined HRT with either conjugated estrogen (CE) 0.625 mg + 5 mg medroxy-progesterone acetate (MPA) (= CE/MPA) or 17β-estradiol (E2) 2 mg + 1 mg norethisterone acetate (NETA) (= E2/NETA) (Table 3, paper II). The expression of PR was not affected by HRT, while ER expression was decreased, and this decrease was seen in the group with HRT regime E2/NETA in the glandular
epithelium but not in the CE/MPA group. A decrease in ER expression was observed also in stroma (Tables 1 and 2, paper II).

High expression of sex steroid receptors was observed both before HRT, when serum levels of E2 and progesterone were probably low, and during combined continuous HRT, when serum levels of E2 and gestagen were higher and stable. Different reactions of ER and PR were observed during HRT, with ER decreasing significantly while PR showed a non-significant tendency to increase (Tables 1 and 2, paper II). The decrease of ER expression could be an effect of gestagen and especially an effect of NETA, since the decrease was seen only in the group treated with the E2/NETA regimen, and androgens are known to be powerful down-regulators of ER [270]. Progesterone receptors may be more sensitive to the effect of estrogen when estrogen and progesterone are combined, and no difference was seen between the 2 regimens.

3.2. ER and PR in endometrial carcinoma

3.2.1. ER and PR in tumors of different grade

Great heterogeneity of both ER and PR expression was observed in the epithelial fraction of the tumors in all 3 biopsies obtained during the study period: in biopsy 1 before progesterone therapy, in biopsy 2 during the therapy, and in biopsy 3, which was obtained 2 or 6 days after progesterone withdrawal. For that reason, sex steroid expressions were separately counted in the foci of maximal and minimal expressions of receptor staining, and the overall expression (mean value from at least 10 random areas) was evaluated as well (see Methods). Results from the evaluation of the untreated endometrial carcinoma are shown in Tables 1 and 2 in paper III and the results of the composites of the mean values in all 3 biopsies are shown in Table 2. The results clearly show that ER and PR expression is higher in Grade 1 (G1) and Grade 2 (G2) tumors compared with Grade 3 (G3) tumors (Tables 1 and 2, paper III). Progesterone receptor heterogeneity varies with tumor grade, with greater heterogeneity in G1 and G2 tumors compared with G3 tumors (Table 2, paper III), while the heterogeneity of ER expression was in the same range between the tumors of different grade in biopsy 1 (Table 3, paper III) and throughout the study period.
Table 3

The composites of the mean values of ER, PR, and Ki-67 expression in endometrial carcinoma of different grades. Statistics by ANOVA (ad hoc LSD).

<table>
<thead>
<tr>
<th>Grade</th>
<th>N</th>
<th>ER</th>
<th>ER-max</th>
<th>ER-min</th>
<th>ER-het</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean (SE)</td>
<td>Mean (SE)</td>
<td>Mean (SE)</td>
<td>Mean (SE)</td>
</tr>
<tr>
<td>1</td>
<td>13</td>
<td>76.38 (3.0)</td>
<td>92.15 (2.8)</td>
<td>49.51 (4.1)</td>
<td>41.34 (5.7)</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>68.27 (6.6)</td>
<td>88.00 (4.0)</td>
<td>40.23 (6.8)</td>
<td>36.35 (5.2)</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>40.61 (14.2)</td>
<td>59.89 (11.6)</td>
<td>33.33 (14.4)</td>
<td>39.27 (6.6)</td>
</tr>
<tr>
<td>Sign.</td>
<td></td>
<td>P&lt;0.01</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Grade</th>
<th>N</th>
<th>PR</th>
<th>PR-max</th>
<th>PR-min</th>
<th>PR-het</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean (SE)</td>
<td>Mean (SE)</td>
<td>Mean (SE)</td>
<td>Mean (SE)</td>
</tr>
<tr>
<td>1</td>
<td>13</td>
<td>41.90 (4.4)</td>
<td>75.00 (4.1)</td>
<td>12.82 (2.9)</td>
<td>62.18 (5.2)</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>40.30 (6.7)</td>
<td>67.77 (6.3)</td>
<td>19.33 (5.5)</td>
<td>48.43 (5.9)</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>21.50 (9.9)</td>
<td>40.44 (13.9)</td>
<td>10.22 (5.6)</td>
<td>30.22 (7.7)</td>
</tr>
<tr>
<td>Sign.</td>
<td></td>
<td>n.s.</td>
<td>P&lt;0.05</td>
<td>n.s.</td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Grade</th>
<th>N</th>
<th>Ki</th>
<th>Ki-max</th>
<th>Ki-min</th>
<th>Ki-het</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean (SE)</td>
<td>Mean (SE)</td>
<td>Mean (SE)</td>
<td>Mean (SE)</td>
</tr>
<tr>
<td>1</td>
<td>13</td>
<td>15.18 (2.1)</td>
<td>37.05 (4.2)</td>
<td>4.67 (1.3)</td>
<td>32.39 (3.7)</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>28.10 (3.4)</td>
<td>56.63 (4.6)</td>
<td>7.40 (1.8)</td>
<td>49.23 (4.3)</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>43.83 (4.1)</td>
<td>71.11 (3.0)</td>
<td>19.28 (4.2)</td>
<td>51.83 (5.5)</td>
</tr>
<tr>
<td>Sign.</td>
<td></td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>
The heterogeneity of ER expression stayed in the same range, while the heterogeneity of PR expression decreased during the progesterone treatment from biopsy 1 to biopsy 2, and was obviously an effect of the marked decrease in PR expression in the foci of maximal expression for PR (PR-max) during the treatment.

There was a significant difference in ER expression of tumors of different grade. Higher mean values of ER were observed in G1 and G2 tumors compared with G3 tumors, which showed significantly lower ER density throughout the study period (Table 3 and Fig. 4). No change was observed during the progesterone therapy (Fig. 4). These results can be compared with the results from normal postmenopausal endometrium, where combined therapy with 2 different combined continuous HRT regimens resulted in a decrease in ER expression. Even in endometrial carcinoma, better response to progesterone therapy has been shown in cells or tissues pretreated with estrogen [257].

The mean values of PR in overall evaluation and in the foci of minimal staining for PR (PR-min) did not change during the progesterone therapy, but a decrease of PR-max was observed during the therapy (Fig. 5). The mean value of PR staining was not significantly different in tumors of different grade, but PR-max in G3 tumors was lower compared with G1 and G2 tumors before the therapy (Table 2, paper III). The decrease in the PR-max observed during the therapy was separately evaluated in tumors of different grade, and it turned out that the decrease was found for G1 and G2 tumors but not for G3 tumors (Table 3, Fig. 5).
Figure 4

ER and ER-max expression in 29 endometrial carcinomas before (biopsy 1), during (biopsy 2), and after (biopsy 3) medroxy-progesterone treatment.

A and B. Expression of ER was unchanged in the whole material as well as in tumors of different grade separately.

C and D. ER-max stayed also in the same range and there was no interaction with tumor grade.

In B and D the differences of the mean values between G3 tumors and G1–2 tumors are clearly seen.
Figure 5

A and B. The decrease in PR expression during progesterone therapy was not significant.
B. The interaction of grade was seen in PR as G1 and G2 tumors show changes in a different way, while PR in G3 tumors was unchanged.
C. PR-max was markedly decreased during the progesterone therapy and, although slightly increased (not significant) after withdrawal, was still on a lower level compared with biopsy1.

D. The interaction of tumor grade and PR-max is on a borderline level ($F_{2,52} = 2.51; P = 0.053$) but (in separate evaluation) different reaction patterns are seen in progesterone therapy in tumors of different grades.
3.2.2. **Comparison between benign and malignant endometrium**

Relatively high expression of both ER and PR was observed in endometrial carcinoma, and, as shown above, the density of sex-steroid receptors is highly dependent on tumor grade. It has previously been pointed out that ER expression is high in endometrial carcinoma [271], even if loss of receptors is a part of carcinogenesis [80, 236, 239, 271], and ER and PR are generally decreased in cancer compared with endometrial hyperplasia.

Since we had evaluated ER and PR expression in benign postmenopausal material and in endometrioid endometrial carcinoma grade by grade using the same quantitative method, we could compare these materials (Table 4).

**Table 4**

ER and PR expression, proliferation indicated as Ki-67 index and apoptotic index, Ai, in benign postmenopausal endometrial epithelium and in endometrioid endometrial carcinoma of different grades. Both ER and PR expression were higher, while the Ki-67 index and Ai were lower in benign endometrium compared with cancer of any grade (ANOVA; ad hoc LSD).

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>ER Mean (SEM)</th>
<th>PR Mean (SEM)</th>
<th>Ki-67 Mean (SEM)</th>
<th>Ai Mean (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Benign</strong></td>
<td>32</td>
<td>95.78 (2.0)</td>
<td>82.78 (4.5)</td>
<td>5.49 (0.7)</td>
<td>5.52 (0.6)</td>
</tr>
<tr>
<td><strong>Carcinoma G1</strong></td>
<td>13</td>
<td>79.85 (6.2)</td>
<td>46.08 (6.7)</td>
<td>20.23 (3.3)</td>
<td>13.92 (1.7)</td>
</tr>
<tr>
<td><strong>Carcinoma G2</strong></td>
<td>10</td>
<td>73.00 (6.3)</td>
<td>46.70 (9.0)</td>
<td>41.10 (4.0)</td>
<td>16.96 (2.2)</td>
</tr>
<tr>
<td><strong>Carcinoma G3</strong></td>
<td>6</td>
<td>39.83 (17.2)</td>
<td>21.17 (12.2)</td>
<td>44.00 (4.1)</td>
<td>14.67 (1.9)</td>
</tr>
<tr>
<td><strong>ANOVA, sign.</strong></td>
<td></td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

ER and PR in benign postmenopausal endometrium was higher than the receptor expression of endometrial carcinoma of any grade (ANOVA; P < 0.001, ad hoc LSD; P < 0.05-0.001) (Table 4). The difference increased with tumor grade (results not shown). This comparison shows that the results indicated previously (i.e., that the expression is decreased in carcinoma) could be seen in our material.
3.2.3. Random areas/specific areas

Both ER and PR were studied in at least 10 random areas of the specimen and in the areas of maximal and minimal receptor expression, as described in Methods. Further, for better evaluation of the relation between proliferation and the expression of sex-steroid-receptors, an evaluation was done in specific areas as follows. Ten specific areas were identified: 5 foci of maximal proliferation, indicated with high Ki-67 index; and 5 foci of minimal proliferation. An evaluation of ER and PR expressions in these 10 areas representing foci of maximal and minimal proliferation was established in adjacent sections for 29 cases before and during the progesterone therapy. Consequently, the changes during the therapy could be compared separately in the foci of maximal and minimal proliferation. The results from 10 areas in each section were analyzed with repeated-measures ANOVA. Evaluated in the biopsy before the treatment (biopsy 1), the areas representing the foci of high proliferation (Ki-max) expressed higher ER density in G2 tumors, and higher expression of PR in G1 tumors compared with the foci of low proliferation. The ER and PR evaluated in the biopsy during the progesterone treatment (biopsy 2) showed higher expression of PR in the foci of high proliferation compared with the foci of low proliferation in G1 and G2 tumors (F_{1,12} = 14.28; P < 0.01 and F_{2,9} = 9.00; P < 0.05, respectively), but not in G3 tumors. The difference in the expression of ER between foci of high and low proliferation was not significant. However, studied separately in G1 tumors, a difference in ER expression could be shown (F_{1,12} = 6.48; P < 0.05).

Since we had the same evaluation done before and during progesterone treatment, the changes in the mean ER and PR expression could be studied separately in the foci of maximal and minimal proliferation. The Wilcoxon signed rank test was used, and it showed that the mean PR in the areas of maximal proliferation was significantly decreased in G1 tumors (P < 0.01) during progesterone treatment, but the decrease in G2 tumors was not significant (P = 0.11), and there was no change at all in G3 tumors (P = 1.0). There was a trend toward decreased PR expression even in the areas of minimal proliferation in G1 tumors (P = 0.075), but in tumors of any grade there was no significant decrease from biopsy 1 to 2 in the mean ER expressions in the areas of either high or low proliferation (Table 3, paper IV).

The results from the evaluation of sex steroid receptors in the specific foci of maximal and minimal proliferation agree with the results from the evaluation of random foci: decrease of PR expression was revealed in both cases. The results from the study of specific foci of high and low proliferation reveal a covariation of the high expression of sex steroid receptors and high proliferation in grade 1 and 2 tumors. Decrease in PR expression could be seen in the foci of maximal proliferation, and, as will be shown in results later, a decrease of proliferation was marked in these foci. Both changes are probably effects of progesterone therapy in the same foci. The covariation of receptors and proliferation in tumors of grades 1 and 2 may help explain why these tumors respond to progesterone therapy [260, 261, 272]. High ER and PR expression could also be an estrogen effect, and pretreatment with estrogen has been
shown to facilitate the progesterone effect in experimental studies [257].
The tumors of premenopausal women are also mostly well-differentiated tumors with
sex steroid receptor expression, and unopposed-estrogen etiology such as PCO
syndrome is suspected in these cases. These tumors have shown a good progesterone
response [262-264]. Receptor responsiveness may also be an indicator of the specific
carcinogenic pathway of the tumor, and high receptor expression and good
responsiveness of the tumor to progesterone therapy may provide evidence for the
hormone-dependent pathway of carcinogenesis [25-28, 93, 104, 233] and for better
prognosis generally associated with these qualities of the tumor [4, 40, 147, 152, 153,
221, 273].

If the changes in receptor expression in this study of endometrioid carcinoma are again
compared with the effect of combined HRT in postmenopausal material, we can see
clear differences: in ca-material, ER is unchanged (↔) and PR decreased (↓) during
progesterone therapy, while in postmenopausal material, under substitution of both
estrogen and gestagen, ER was decreased (↓) and PR unchanged (↔) with tendency to
increase (↑) (see summary in Table 5). Because the changes in receptor expression
seen in postmenopausal material during combined continuous HRT could be
interpreted as evidence that this hormonal therapy is more favorable than progesterone
alone, it could be an argument for some combination of estrogen with progesterone as
a therapy against endometrial carcinoma. The increase in progesterone effect by
pretreatment with estrogen has been shown in an experimental study [257].
4. Homeostasis, indicated as proliferation and apoptosis

Proliferation and apoptosis together regulate the homeostasis of as well benign as malignant tissues. Using the same methods in evaluation of both benign and malignant tissue, the changes in tissue homeostasis can be followed during internal or external hormonal manipulation. It is, however, impossible to determine the relation indicating steady state situation by the approach we have used. Apoptosis is a quick phenomena of about 4 hours, while Ki-67 staining indicates all phases of cell cycle except G0.

4.1. Benign endometrium

4.1.1. Cyclic endometrium

Proliferation, indicated as Ki-67 index, was studied in the superficial cyclic endometrium at the end of the menstrual cycle, during decreasing E2 and progesterone serum levels. Four days prior to menstruation, about 10% of the cells were in active cell phase (Ki-67 index = 10) both in the epithelium and in the stroma. However, a different development of proliferation was seen thereafter with rapidly decreasing proliferation in the epithelium, while increasing proliferation was observed in the stroma until the first day of menstruation, followed by decrease until the second menstrual day (Fig. 1, paper I).

Apoptosis, which together with proliferation, regulates the homeostasis in the endometrium, was evaluated as well. The results showed that apoptotic cells were rare and scattered among ordinary cells both in glands and the stroma in sections from endometrium taken 3 to 4 days prior to menstruation. Two days prior to the onset of menstruation, an increasing frequency of apoptotic cells was seen in the epithelium, while apoptotic cells in the stroma were still relatively rare. The apoptotic index in epithelial endometrium increased up to the day of the menstrual start and peaked on the second day of menstruation. In the stroma an increase in the apoptotic index was seen on the day of onset of menstruation. The maximal apoptotic index in the stroma was about 50% of that observed in the glandular epithelium.

The homeostasis of the epithelium during the decline in levels of sex steroid hormones at the end of the luteal phase could be summarized as an involution process with high apoptotic activity and low proliferation. The stroma reveals a more complicated reaction, with relatively high proliferation and, after that, quick cell turnover with both high apoptotic index and high proliferation, until proliferation decreases on the second menstrual day.

The increased proliferation in the stroma in the late luteal phase has been described previously as a second wave of proliferation [2]. The reaction in the stroma is delayed compared with that in the glandular epithelium, and proliferation was still seen during the time of low serum progesterone values. If the reaction is progesterone-induced,
growth factors or other local regulators complete it. Another explanation could be that ischemia in the tissue prior to menstruation or endometrial damage during early interstitial bleeding activates the reparation mechanism where the stroma is active, and proliferation is triggered by mechanisms other than hormonal induction. Ischemia is also a possible inductor of apoptosis. In any event, apoptosis has been shown after hormonal withdrawal in experimental studies [178-180], and during the luteal phase in other studies [13, 18-21, 23, 69, 162, 166, 172-174].

The decrease of ER and PR in the epithelium prior to and at the time of increasing apoptosis provides evidence for the hormonal regulation of apoptosis in the endometrium. Further, the increase of the cycle-specific apoptotic activity near to the start of the menstrual flow shown in this study may provide evidence for its role in the induction of menstruation. However, the factors regulating the vascular changes in the stroma are probably most important for the initiation of the bleeding, and the factors giving different hormonal responses in the epithelium and stroma are outside the scope of these studies. An involution-like process in epithelial glands results in the narrow straight glands of the early follicular phase. High cell turnover, indicated as a high apoptotic index and high proliferation at the same time, suggests an active role for the stroma in remodeling of the endometrium during menstruation.

4.1.2. Postmenopausal endometrium

The Ki-67 index in the epithelial part of the endometrium was 5.5 (%), and stayed on the same level during combined continuous HRT (Tables 1 and 2, paper II). Both lower and higher rates have been reported previously [22, 132] in untreated postmenopausal endometrium. The proliferation index (Ki-67 = 5.5) in this study of postmenopausal women was higher than the Ki-67 index of 2 to 3 (%) found in epithelial glands during the 2 first days of menstruation at the time of minimal mean E2 level (123 pmol/l) of healthy menstruating women (paper I) (MWU test; P < 0.05). We have no E2 values in HRT material, but in the women with endometrial carcinoma, the mean level of E2 was 89 pmol/l (Table 2), and in the group of healthy postmenopausal women, the levels of E2 should not be higher. Thus the decreasing levels of both E2 and progesterone give lower proliferation in epithelial tissue compared with continuous low levels, or during the menstruation process there may exist other factors that counteract proliferation through different mechanisms.

The apoptotic index both in the epithelium and in the stroma was unchanged during the combined therapy. The apoptotic index of 5.2 before HRT is lower than the index in 2 other studies [22, 274] but identical with the index in a control group of 4 postmenopausal women in a third study [67]. The unchanged proliferation in the epithelium during the HRT indicates that progesterone was able to block the proliferative effects of E2 in glands. Thus, the homeostasis of the epithelial part was unaffected as well as the endometrial thickness. In the stroma the combined effect of the therapy is increased proliferation (Tables 1 and 2, paper II).

Again in postmenopausal endometrium as well as in cyclic endometrium, different responses to hormonal changes were seen in the epithelium and in the stroma. Increase
in stromal proliferation is seen during the year of HRT and a decreased incidence of breakthrough bleeding has been observed during the same period [210]. A certain volume of stroma may be needed to support the vascular network and to counteract vascular fragility, as proposed in previous studies [212, 213, 217]. The reaction of receptors, with decrease of ER expression and a non-significant tendency toward increase in PR, indicates that receptors have different sensitivity for combined therapies.

4.2. Endometrial carcinoma before, during, and after progesterone therapy

Since the tumor tissue before progesterone therapy clearly showed a heterogeneous staining pattern for Ki-67, the expression of Ki-67 was separately evaluated in the foci of maximal expression and in the foci of minimal expression as described in Methods. Only 1 tumor showed a totally homogenous staining for Ki-67 (Table 2, paper IV). The same method of evaluation was interpreted in biopsy 2 and 3. For summary, see Table 5.

Increased frequency of apoptotic cells was observed near the necrotic areas, and these areas were mostly excluded together with the necrotic foci. In vital tumor tissue, apoptotic cells were scattered among living cells. The apoptotic index (Ai) was not separately counted in the areas of higher and lower frequency because > 2000 cells were needed to state the apoptotic index and the foci of higher apoptotic activity were too small.

The Ai was the same across tumors of all grades (Table 3, paper III and Table 6), but a previous study has indicated a possible difference in the frequency of apoptosis according to tumor grade [249, 275]. One reason for this difference could be that we excluded the necrotic areas (with increased apoptosis in and around them), which were seen more frequently in tumors of high grade.

The Ki-67 index differed according to the tumor grade, with lower overall Ki-67 index and Ki-max in the tumors of G1 compared with G2 and G3, (Table III, paper III) in the biopsy before the progesterone therapy. The difference of Ki-67 index was also seen in the mean values of all 3 biopsies according to tumor grade (Table 3 and 6). A higher proliferation rate, indicated with Ki-67 index in tumors of higher grade [36, 53], more malignant phenotype [225, 236, 276] and worse prognosis [39, 224] has been observed in earlier studies, but the heterogeneity aspect has not been studied before.

Both Ki and Ki-max were decreased during the progesterone therapy, and this decrease was separately seen in tumors of G1 and G2 (Fig. 6), while tumors of grade 3 showed unchanged proliferation during the therapy. Ki-min was also unchanged in tumors of all grades. Thus the heterogeneity of Ki-67 staining was decreased during the study. A more heterogeneous pattern of Ki-67 expression was observed in biopsy 1 compared with biopsy 2 and 3 as an effect of marked decrease in Ki-max during progesterone treatment.
The AI was unchanged during the progesterone therapy and after both 2-day and 6-day withdrawal of the therapy (Fig. 7). These results were in accordance with a previous study indicating that endometrial carcinoma does not respond with increased apoptosis to progesterone [277]. Another experimental study indicates that Ishikawa cell line from a well-differentiated endometrial adenocarcinoma may respond with apoptosis at the beginning of progesterone therapy [278], but this kind of early reaction is not possible to show in this study setup.

It was interesting to see that both grade 1 and 2 tumors expressed high ER and PR intensity before the therapy, they showed coexistence of receptors and proliferation in same foci, and they also showed a response to progesterone therapy. Both G2 and G3 tumors showed higher proliferation compared with G1 tumors, but tumors of G1 and G2 both responded to the progesterone therapy, while G3 tumors stayed on a higher proliferation level during the therapy (Fig. 5 and Table 6). Higher proliferation rate has been shown in tumors of women who have not used hormonal therapy [279]. In contrast, however, lower proliferation rate, higher expression of sex steroid hormone receptors, and better response to the progesterone therapy may argue for the estrogen-dependent pathway of carcinogenesis [26, 28, 104, 244]. Great similarities were observed in G1 and G2 tumors in our material. The similar behavior of G1 and G2 tumors as well as the better prognosis in this group [39, 227, 280] compared with G3 tumors could support the previous proposal of using a 2-tiered instead of 3-tiered grading system [281] for endometrial carcinoma. In this material, both G1 and G2 tumors show changed homeostasis during progesterone therapy with decreased proliferation rate and unchanged apoptosis, while the homeostasis of G3 tumors was unaffected by progesterone (Table 5).

Apoptosis could counteract proliferation and modulate the homeostasis during the therapy trial. However, apoptosis was unchanged during the therapy and no variation was seen between the tumors of different grade. Thus, proliferation alone regulated the growth in this material and proliferation alone was responsible for the increasing aggressiveness of tumors with advancing grade.
Figure 6

A. Ki-67 expression was decreased during progesterone therapy (biopsy 2) and after withdrawal (biopsy 3) compared to biopsy 1.

B. There was an interaction of tumor grade with Ki-67 ($F_{2,52}=3.49$; $P<0.05$, repeated measurements ANOVA). G3 tumors showed a different pattern of behavior, with unchanged proliferation during the progesterone therapy and after withdrawal, while G1 and G2 tumors showed decrease of proliferation during the therapy.

C. Proliferation, measured as Ki-67 expression in the areas of maximal proliferation (Ki-max) was decreased during progesterone therapy and after withdrawal (biopsies 1 and 2).

D. Tumors of all grades show similar behavior through the biopsy series even if the decrease of Ki-max was significant only in G1 and G2 tumors. Consequently, no interaction between grade and Ki-max could be observed.
Figure 7

Apoptotic index (Ai) in biopsies before (1), during (2), and 2 or 6 days after (3) medroxyprogesterone treatment in endometrial carcinoma.

A. All tumors (29 patients).

B. Tumors grouped by grade 1, 2, and 3 (13, 10, and 6 patients). Ai was in the same range during the study period and in tumors of different grades. There was no interaction of histopathological tumor grade and Ai. (Statistics by repeated measurements ANOVA.)
4.3. Predictive factors of progesterone therapy

The decrease in proliferation was the main effect of progesterone in this study. The decrease of proliferation, further referred to as delta-Ki, was therefore studied in the scope of factors illuminating the properties of the tumors before the therapy. Previous studies have indicated the value of sex steroid receptor expression as a predictive factor of tumor responsiveness to progesterone therapy [260, 261], but the response rates are not directly correlated with receptors. Since there also are indications from experimental studies that stromal factor may mediate progesterone effect in endometrial cancer cells (Ishikawa cells) [5, 282], we studied both epithelial and stromal factors as possible predictors of progesterone effect.

4.3.1. Epithelial factors

Each of ER, PR, and bcl-2 in the epithelial part of tumors showed correlation with delta-Ki (Pearson's correlation coefficient, r = 0.61; P = 0.001 r = 0.51; P = 0.005, and r = 0.38; P = 0.045, respectively) (Figure 1, paper IV). Tumor grade also has great importance for the response. Attempts to fit a linear multiple regression test of the factors were complicated by the intrinsic correlations between the factors. Nonlinear models were also limited because of the sample size. However, the receptor expression may reflect the effect of estrogen on the tumor. The correlations may therefore support the theory that progesterone's effect consists of inhibition of estrogen-induced proliferation by activation of 17ß-HSD type 2, which converts the biologically active Estradiol (E2) to less active estriol, E1. This enzyme has been shown to be present in nearly one half of the endometrial carcinomas [95, 100, 101]. Further, the expression of 17ß-HSD type 2 is inversely correlated with the age of the patients [101], and these results contribute to the positive outcome of progesterone therapy observed in the groups of young patients with low-grade tumors [262-264].

The correlation of bcl-2 and delta-Ki was due to 4 patients who had bcl-2 negative (score 0) tumors with no response to progesterone. There has been earlier implication from a study of breast carcinoma that bcl-2 could predict effects of hormonal therapy [283], but results from the studies with bcl-2 in endometrial carcinoma have not been conclusive [73].

4.3.2. Stromal factors

The amount of stroma in tumors decreased with increasing tumor grade, as illustrated in Figure 8 (ANOVA, P = 0.003). In pairwise comparison it turned out that G3 tumors differed from G1 and G2 tumors (P =0.001 and P = 0.03 respectively). This difference was expected since the amount of stroma is indirectly included in the criteria of tumor grade in endometrial carcinoma. Both ER and PR expression in the stroma was lower than in the epithelial part of the tumors (Wilcoxon signed-rank test, P < 0.001 for both) (Figure 9) and correlated to the amount of stroma (Pearson's correlation coefficient, r = 0.63; P < 0.001 for ER and r = 0.72, P < 0.001 for PR, respectively). There was no significant correlation between ER or PR in stroma and delta-Ki. Thus, no direct
support was found in this material for the importance of stromal mediating factor for the progesterone therapy.

A. The amount of stroma in 29 tumors of endometrial carcinoma.
The amount of stroma (% of total volume) decreased with increasing tumor grade (ANOVA, \(P = 0.003\), ad hoc LSD G1/G2, ns; G1/G3, \(P < 0.01\); G2/G3, \(P < 0.05\)).

**Figure 8** – Amount of stroma.

B. Sex steroids receptor expression in 29 endometrial carcinomas.
ER and PR expression in epithelial part of the tumors was higher than in stroma (Wilcoxon signed ranks test, \(P < 0.001\) for both).

**Figure 9** – Sex steroid receptors
5. Bcl-2 and P53

5.1. Bcl-2

In cyclic endometrium, bcl-2 was scored as relatively low both in the epithelium and in the stroma, and this observation was in accordance with previous studies (Fig. 1, paper I) [52, 69]. A slight increase of bcl-2 expression in the epithelium just prior to menstruation, at the same time period with increasing apoptosis, cannot be explained in this material without knowing the possible occurrence of bax.

The bcl-2 score in endometrial carcinoma showed no significant relation to tumor grade, apoptosis, and proliferation or sex steroid receptors. Neither was there any change in expression of bcl-2 during progesterone therapy or withdrawal. The 4 tumors without any expression of bcl-2 showed no response (decrease of Ki-67 index) to progesterone therapy, in contrast to most tumors showing bcl-2 expression, which responded to progesterone with decreased proliferation.

5.2. P53

Deranged p53 gene expression in most cells of the tumor was observed in only 2 cases out of 29 tumors, and this result was expected, since a low incidence of p53 mutation in endometrioid endometrial carcinoma has been reported earlier [237, 241-243]. The expression of p53 is frequently seen in endometrial tumors of more aggressive subtypes and, probably, with carcinogenesis independent of estrogen. However, all tumors of endometrioid endometrial carcinoma were not negative according to p53 expression.
Table 5
Summary of the studies in papers I, II, and IV
ER and PR, Ki-67 index, Ai, and bcl-2 were studied during hormonal changes. The hormonal changes or provocation studied were as follows: intrinsic decrease of estradiol and progesterone in paper I, combination of estrogen and progesterone therapy in paper II and progesterone therapy and withdrawal in paper IV. The results after withdrawal are compared with the results before the therapy. Symbols used: ↓ = significantly decreased, ↑ = significantly increased, ↔ = unchanged, (↑) or (↓) = tendency to increase or decrease.

<table>
<thead>
<tr>
<th>Study tissue</th>
<th>Materials</th>
<th>Changes of steroid level</th>
<th>Epithelium.</th>
<th>Stroma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fertile endom.</td>
<td>37 women 75 biopsies</td>
<td>E↓</td>
<td>ER↓</td>
<td>ER↑↓</td>
</tr>
<tr>
<td>Paper I</td>
<td></td>
<td>P↓</td>
<td>PR↓</td>
<td>PR↑↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ki-67↓</td>
<td>Ki-67↑↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ai↑</td>
<td>Ai↑↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bcl-2↓</td>
<td>Bcl-2↑↓</td>
</tr>
<tr>
<td>PM endometrium</td>
<td>92 pm women, 2 biopsies from 43 women</td>
<td>E↑</td>
<td>ER↓</td>
<td>ER↓</td>
</tr>
<tr>
<td>Paper II</td>
<td></td>
<td>P↑</td>
<td>PR→(↑)</td>
<td>PR← (↑)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ki-67↔</td>
<td>Ki-67↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ai↔</td>
<td>Ai←</td>
</tr>
<tr>
<td>Endometrial Carcinoma</td>
<td>29 patients 3 biopsies/pat.</td>
<td>P↑</td>
<td>ER↔</td>
<td>No correl. betw.</td>
</tr>
<tr>
<td>Paper IV: Progesterone therapy</td>
<td></td>
<td></td>
<td>PR-max↓</td>
<td>stroma amount and</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ki-67↓</td>
<td>prog. effect in prolif.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ki-max↓</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ai↔</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bcl-2↔</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P52↔</td>
<td></td>
</tr>
<tr>
<td>Endom. Carcinoma</td>
<td>29 patients 20 pat. 2 days withdrawal</td>
<td>P↓</td>
<td>ER↔</td>
<td></td>
</tr>
<tr>
<td>Paper IV Progesterone withdrawal</td>
<td></td>
<td></td>
<td>PR-max↓</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ki-67↓</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ki-max↓</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Ai↔</td>
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<td></td>
<td>Bcl-2↔</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P52↔</td>
<td></td>
</tr>
</tbody>
</table>
Table 6
Summary of steroid receptor expression, proliferation, and apoptosis of endometrioid endometrial carcinoma of grades 1–3 throughout the study period of biopsies 1–3. The arrows in column biopsy 1 indicate higher (↑), lower (↓) or equal (↔) levels compared with other grades. In columns for biopsies 2 and 3 the arrows indicate a change from the previous biopsy.

<table>
<thead>
<tr>
<th>Endometrioid endometrial Carcinoma, grades 1-3</th>
<th>Biopsy 1 Before therapy</th>
<th>Biopsy 2 Changes during progesterone therapy</th>
<th>Biopsy 3 Changes during progesterone withdrawal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 1</td>
<td>ER↑, ER-max↑</td>
<td>ER↔, ER-max↔</td>
<td>ER, ER-max↔</td>
</tr>
<tr>
<td></td>
<td>PR↑, PR-max↑</td>
<td>ER-het↔, PR-het↔</td>
<td>ER-het↔</td>
</tr>
<tr>
<td></td>
<td>Ki-67↓</td>
<td>PR↓, PR-max↓</td>
<td>PR↑, PR-max↑</td>
</tr>
<tr>
<td></td>
<td>Ai↔</td>
<td>PR-het↓</td>
<td>PR-het↔</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ki-67↓</td>
<td>Ki-67↔, all variables</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ki-het↓</td>
<td></td>
</tr>
<tr>
<td>Grade 2</td>
<td>ER↑, ER-max↑</td>
<td>ER↔, ER-max↔</td>
<td>ER↔, all variables</td>
</tr>
<tr>
<td></td>
<td>PR↑, PR-max↑</td>
<td>ER-het↔, PR-het↔</td>
<td>PR↔, all variables</td>
</tr>
<tr>
<td></td>
<td>Ki-67↑</td>
<td>PR↓, PR-max↓</td>
<td>Ki-67↔, all variables</td>
</tr>
<tr>
<td></td>
<td>Ai↔</td>
<td>PR-het↔</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ki-67↓</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ki-het↓</td>
<td></td>
</tr>
<tr>
<td>Grade 3</td>
<td>ER↓, ER-max↔</td>
<td>ER↔, all variables</td>
<td>ER↔, all variables</td>
</tr>
<tr>
<td></td>
<td>PR↓</td>
<td>PR↔, all variables</td>
<td>PR↔, all variables</td>
</tr>
<tr>
<td></td>
<td>Ki-67↑</td>
<td>Ki-67↔, all variables</td>
<td>Ki-67↔, all variables</td>
</tr>
<tr>
<td></td>
<td>Ai↔</td>
<td>Ai↔</td>
<td></td>
</tr>
</tbody>
</table>
SUMMARY

Proliferation and apoptosis, the main regulators of tissue homeostasis, as well as sex steroid receptors and p53 and bcl-2, were evaluated in endometrium and in endometrial carcinoma during hormonal changes and manipulation as follows:
1. Fertile endometrium during declines in E2 and progesterone levels during the late luteal phase and beginning of menstruation (Table 5);
2. Postmenopausal endometrium during low hormonal levels of postmenopause and stable increased estrogen and gestagen levels during HRT (Table 5); and
3. Endometrial carcinoma during postmenopausal levels of estrogens and progesterone, during medroxy-progesterone therapy with 20 mg daily dose and during falling levels of medroxy-progesterone after the withdrawal of the therapy (Tables 5 and 6).

The following effects were observed during the hormonal circumstances described above (See Tables 5 and 6):

Homeostasis (apoptosis/proliferation)

The epithelial tissue showed increasing apoptosis and decreasing proliferation at the end of the luteal phase and during menstruation. This image of involution in the epithelium was in contrast with the development in the stroma, where high proliferation and increasing apoptosis were observed, indicating quick cell turnover. Combined continuous HRT for postmenopausal women induced no increase in proliferation of the endometrial epithelium, and apoptosis was unchanged as well. Increased proliferation was observed in the stroma. There was increasing proliferation in endometrial carcinoma with increasing tumor grade, while apoptosis did not vary according to the grade. Decreased proliferation and unchanged apoptosis in G1 and G2 tumors were observed during progesterone therapy. No change was seen during the therapy in G3 tumors, nor in tumors of any grade during the withdrawal of the therapy.

Hormonal sensitivity

The hormonal sensitivity illuminated as ER and PR receptors was decreased in the endometrial epithelium during decreasing serum levels of both E2 and progesterone. In the stroma an increase was observed prior to menstruation before the fall during menstruation. ER was decreased during combined continuous HRT with E2/NETA both in the epithelium and in the stroma, while PR was not significantly changed. During progesterone therapy, endometrial carcinoma showed decrease of PR, while ER was unchanged. There was a covariation of the sex steroid receptors and proliferation in the same foci of G1 and G2 tumors. P53 was rare in endometrial carcinoma of endometrioid type. This fact together with the antiproliferative hormonal effects in G1 and G2 tumors argues for the hormone-dependent pathway of carcinogenesis of most endometrioid endometrial tumors of grades 1 and 2. The changes of the antiapoptotic factor bcl-2 are not alone conclusive in cyclic endometrium or in endometrial carcinoma.
CONCLUSIONS

Apoptosis is involved in the mechanism of menstruation. Both apoptosis and proliferation collaborate in endometrial remodeling during menstruation, when the epithelium returns to the status of narrow glands and stromal damage is repaired.

The balance between proliferation and apoptosis is maintained unchanged in postmenopausal endometrial epithelium during HRT with 2 different regimens. This balance contributes to endometrial safety. Increased proliferation observed in the stroma with the same therapy may counteract breakthrough bleeding since stromal support of the vascular network was increased. It may also be a safety issue, and should be further studied.

Increasing discrepancy between proliferation and apoptosis with increasing tumor grade contributes to the more aggressive growth of grade 3 endometrial carcinomas compared with the G1 and G2 tumors.

The response to progesterone therapy in G1 and G2 tumors may be facilitated by the coexistence of sex steroid receptors and high proliferation in the same foci.

Progesterone therapy of endometrial carcinoma works via decreased proliferation, but not via increased apoptosis.

The hormonal sensitivity, illuminated as ER and PR, is regulated by estradiol and progesterone in the epithelial endometrium and in endometrial carcinoma of grades 1 and 2, but in the stroma the effect is differently modulated by local factors, and the stimulatory effect of progesterone may also be suspected.
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