From the Department of Clinical Science, Department of Woman and Child Health and Department of Molecular Medicine, Karolinska Institutet, Stockholm, Sweden

Molecular Mechanisms Involved In the Growth of Human Uterine Leiomyomas

Xuxia Wu

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To my parents

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications and manuscripts, which are referred to in the text by their Roman numerals:

I **Xuxia Wu**, Agneta Blanck, Matts Olovsson, Björn Möller, Robert Favini, Bo Lindblom. 2000 Apoptosis, cellular proliferation and expression of p53 in human uterine leiomyomas and myometrium during the menstrual cycle and after menopause. *Acta Obst Gynecol Scand*; 79(5):397-404.

II **Xuxia Wu**, Agneta Blanck, Matts Olovsson, Rudi Henriksen, Bo Lindblom. 2002 Expression of Bcl-2, Bax, Bak, Bcl-x and Mcl-1 in human uterine leiomyomas and myometrium during the menstrual cycle and after menopause. *J Steroid Biochem & Mol Biol*, 80(1):77-83.

III **Xuxia Wu**, Hong Wang, Katarina Englund, Agneta Blanck, Bo Lindblom, Lena Sahlin. 2002 Different expression of the progesterone receptor (PR-A and PR-B) and insulin-like growth factor-I in human myometrium and fibroids after GnRHa treatment. *Fertil & Steril,* 78(5): 985-993.

IV **Xuxia Wu**, Agneta Blanck, Gunnar Norstedt, Lena Sahlin, Amilcar Flores-Morales. 2002 Differential cloning of genes with a higher expression in human uterine leiomyomas than in the corresponding myometrium. *Mol Hum Reprod*, 8(3):246-254.

V **Xuxia Wu**, Amilcar Flores-Morales, See-Tong Pang, Lena Sahlin, Agneta Blanck, Gunnar Norstedt. 2002 Detection of differentially expressed genes in rat uterus after ovariectomy and estrogen treatment using microarray hybridization. *Manuscript*.

TABLE OF CONTENTS

ABBREVIATIONS

ABC method	avidin-biotin-peroxidase complex method
AP-1	activating protein-1
AR	androgen receptor
bFGF	basic fibroblast growth factor
Bcl-2	B-cell leukaemia/lymphomas-2 protein
CDK	cyclin-dependent kinase
CL	corpus luteum
DAB	diamino-benzidine
DHA	dehydroepiandrosterone
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	estrogen receptor
ERE	estrogen response element
EST	expressed sequenced tags
Fas	FS-7 associating surface antigen
FasL	FS-7 associating surface antigen ligand
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FSH	follicle-stimulating hormone
GnRH	Gonadotropin releasing hormone
GnRHa	Gonadotropin releasing hormone agonist
HB-EGF	heparin-binding EGF-like growth factor
HMG	high-mobility-group protein
HREs	hormone response elements
IGF	insulin-like growth factor
IGFBPs	IGF high-affinity binding proteins
kDa	kilodalton
LH	luteinizing hormone
MMP	matrix metalloproteinase
MPA	progestin medroxyprogesterone acetate
NBT/BCIP	4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate
NGF	nerve growth factor
OVX	ovariectomy
PBS	phosphate-buffered saline
PR	progesterone receptor
RDA	representational difference analysis
TGFα	transforming growth factor alpha
TGFβ	transforming growth factor beta
TIMP	tissue inhibitor of metalloproteinase
TNF	tumor necrosis factor
TUNEL	terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick-end labeling method

GENERAL INTRODUCTION

Background

Human uterine leiomyomas (fibroids) are benign neoplasms of monoclonal origin that arise from uterine smooth muscle cells (Mashal et al., 1994). They represent the most common gynecological tumors in women of reproductive age, and constitute the primary indication for hysterectomy in the USA (Wilcox et al., 1994). They are clinically apparent in up to 25% of women and cause significant morbidity (Wallach et al., 1992; Cramer, 1992), including prolonged or heavy menstrual bleeding, pelvic pressure and pain, and reproductive dysfunction in rare cases. Therefore, both the economic cost and the effect on quality of life are substantial. Leiomyomas are classified according to the tumors **(Figure 1)**. For example, subserosal leiomyomas protrude into the uterine serosal membrane, intramural leiomyomas are within the smooth muscle layer, and submucosal leiomyomas protrude into the endometrium with a mucosal membrane covering.

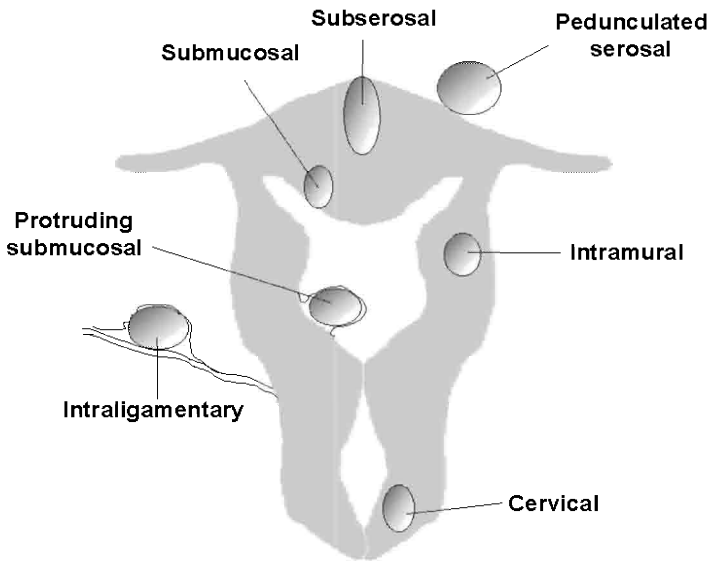

Figure 1. Location of human uterine leiomyomas.

Menstrual cycle

The menstrual cycle is divided into the proliferative (days 7-14 of the cycle), secretory (days 15-28 of the cycle) and menstrual phases (days 1-7 of the cycle). A standardized cycle, set to 28 days, is morphologically dated according to Noye *et al.,* 1951 (Noyes et al., 1951). The secretory profile of the gonadotropins, LH, FSH, and the ovarian steroids such as estrogen and progesterone, is charactistic for each phase of the menstrual cycle.

Most women (90%) have menstrual cycles with an interval of 24 to 35 days (Treloar et al., 1970). The normal volume of menstrual blood loss is 30mL; more than 80mL is abnormal. The menopause is that point in time when permanent cessation of menstruation occurs following the loss of ovarian activity.

Epidemiology

1) Ethnic group

Ethnicity has been suggested as an important risk factor for uterine leiomyomas. The incidence of leiomyomas has been shown to be higher in African-American women than in Caucasian women (Faerstein et al., 2001), whilst the incidence rates among Hispanic and Asian women appear to be similar to those among Caucasians (Marshall et al., 1997).

2) Parity

The incidence of leiomyomas was shown to decrease consistently with an increasing number of term pregnancies (Ross et al., 1986). Women with five term pregnancies or more had only a 25% risk of those women who never carried a pregnancy.

3) Family

The existence of a heritability component of leiomyomas has been implicated by twin-pair studies and the existence of familial forms of leiomyomas, both of which suggest an inherited diathesis for leiomyoma formation (Garcia Muret et al., 1988; Kjerulff et al., 1993; Treloar et al., 1992).

4) Contraceptive use

The published data on the relationship between contraceptives uses and leiomyoma incidence is controversial. As yet, there is no conclusive data on whether combined estrogen and progestin therapy is a risk factor for leiomyomas (Schwartz, 2001).

5) Smoking

No evident relationship exists between smoking and risk of leiomyomas (Marshall et al., 1998).

6) Dietary factors

Leiomyomas are associated with beef and ham consumption, whereas a high intake of green vegetables appears to have a protective effect (Chiaffarino et al., 1999).

7) Hypertension

In logistic regression analysis, leiomyomas were significantly associated with hypertension (Luoto et al., 2001).

Symptoms

The symptoms related to leiomyomas are variable, depending on the size and location of the tumors. The most common problems associated with leiomyomas are:

1) Pelvic pressure, pain and/or bloating due to uterine and leiomyoma enlargement.

2) Abnormal uterine bleeding, particularly menorrhagia and hypermenorrhea. This is most common when leiomyomas are submucosal.

3) Reproductive dysfunction including infertility, recurrent miscarriage or labor complications.

Treatment

1) *Surgical therapy*

Surgery has long been the main mode of therapy for leiomyomas.

a) Hysterectomy

Hysterectomy eliminates both the symptoms and the possibility of recurrence. For many women who have completed childbearing, this therapy can relieve them from the symptoms and improve their quality of life (Kjerulff et al., 2000).

b) Myomectomy

Myomectomy (removal of the leiomyomas with uterine conservation) is widely accepted for women who desire future pregnancies or wish to retain the uterus. The disadvantage of myomectomy is the risk that new leiomyomas will form. The rate of recurrence of leiomyomas after abdominal myomectomy as detected by ultrasonography is approximately 50% within 5 years (Fedele et al., 1995). The prognosis is better however in women with solitary leiomyomas.

c) Endometrial ablation

For women who have completed childbearing and for whom bleeding is the primary problem, endometrial ablation may abolish or decrease the bleeding (Derman et al., 1991).

d) Uterine-artery embolisation

This is a novel technique for treatment of leiomyomas, which is useful in controlling menorrhagia but provides a more variable response in reducing uterine volume (Pelage et al., 2000).

2) *Non-surgical therapy*

Medical therapy is intended to reduce or eliminate the symptoms of leiomyomas, such as decreasing the size of leiomyomas, amount of bleeding or other symptoms. It may also be used as an adjunct to surgical therapy, as a preoperative measure to control tumor size, bleeding or to facilitate surgery (Chavez et al., 2001).

a) Gonadotropin-releasing hormone agonist (GnRHa) therapy

GnRH plays a key role in mammalian reproduction. By binding to gonadotropic cells in the pituitary, GnRH elicits the synthesis and release of gonadotropins, i.e., luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Conn et al., 1994). LH and FSH stimulate gonadal production of sex steroids, which in turn influence the growth and function of a variety of steroid-dependent tissues. Administration of GnRH analogs induces a pseudomenopausal hypoestrogenic state as a result of desensitization of the gonadotropin receptors in the anterior pituitary. Following an initial increase in the release of LH and FSH, desensitization leads to decreased levels of these hormone and consequently decreased levels of estrogen and progesterone. Identification of GnRH and GnRH receptor mRNAs in both myometrium and leiomyoma tissues indicates the possibility of GnRHa alone inducing cellular and molecular changes in the uterus, independent of ovarian hormones (Chegini et al., 1996). Many studies of GnRHa therapy in women with uterine leiomyomas have reported between 35-61% reduction of both leiomyomas and uterine volume after a relatively short treatment period of 3 to 6 months

(Friedman et al., 1991; Schlaff et al., 1989). The administration of GnRHa alone to perimenopausal women with symptomatic or large leiomyomas is a reasonable choice in the hope that during this treatment natural menopause will begin, thus reducing the probability of leiomyoma growth. In young women with symptomatic leiomyomas, GnRHa is primarily used for preoperative therapy.

b) Gonadotropin-releasing hormone agonist and add-back therapy

The adverse effects of GnRHa therapy, especially bone loss, make its prolonged use difficult. With the aim of minimizing the adverse effects of prolonged GnRHa therapy, "add-back" regimens were developed to counteract the hypoestrogenic effect of GnRHa and permit extension of treatment. Most add-back regimens use GnRHa first, to achieve uterine shrinkage and amenorrhea, followed by the additional of estrogen and progestin in either a cyclic, or a continuous fashion known as, sequential method (Friedman et al., 1993; Friedman et al., 1994). Tibolone is a synthetic gonadomimetic steroid structurally related to the progestogens norethynodrel and norethisterone, exhibiting estrogenic and, to a lesser extent, progestational and androgenic actions in animal studies (de Visser et al., 1984; van der Vies, 1987). Tibolone may therefore offer many of the advantages of conventional add-back therapy in a single agent (Palomba et al., 1999).

c) Gonadotropin-releasing hormone (GnRH) antagonists

GnRH antagonists act via a classic competitive blockade of the GnRH receptors on the cell membrane of the gonadotropic cells, inhibiting the micro-aggregation of these receptors. For uterine leiomyomas, pilot studies have been conducted with both the Nal-Glu antagonist and both daily and depot formulations of Cetrorelix (ASTA Medica, Frankfurt, Germany). These produced significant uterine shrinkage within 2 to 4 weeks, similar to that seen with a somewhat longer GnRHa treatment (Gonzalez-Barcena et al., 1997; Felberbaum et al., 1998).

d) Androgen therapy

The primary action of danazol is an androgenic effect, increasing free serum testosterone by decreasing serum sex hormone-binding globulin production, and by displacing testosterone from sex hormone-binding globulin (Barbieri et al., 1979). Vaginal administration of danazol has been suggested as an alternative treatment for leiomyomas with fewer side effects (Mizutani et al., 1995). Another androgenic steroid, gestrinone, has been studied for the treatment of leiomyomas (Coutinho et al., 1989; Coutinho, 1990). The major advantage of this therapy is lack of rebound effects after discontinuation of therapy.

e) Progesterone antagonist

Mifepristone (RU486) is a derivative of norethindrone with both anti-progesterone and anti-glucocorticoid activity (Baulieu, 1989). RU486 is primarily a progesterone antagonist but can also act as a progesterone agonist in the absence of progesterone, a glucocorticoid antagonist, and exert an anti-estrogenic effect (Wolf et al., 1989). However, it is believed in leiomyomas that its primary action is as an antiprogestin. In general, RU486 has mild side effects compared with GnRHa. No report has yet been published regarding long-term treatment with RU486 for uterine leiomyomas.

Cytogenetics aspects

Leiomyomas are monoclonal tumors with independent origins for multiple tumors within individual uteri (Linder et al., 1965; Mashal et al., 1994).

Approximately 40-50% of leiomyomas show karyotypically detectable chromosomal abnormalities that are both non-random and tumor-specific (Rein et al., 1991). A correlation exists between leiomyoma size and the presence of cytogenetic abnormalities (Nibert et al., 1990; Rein et al., 1998), suggesting that rearrangements occur in existing tumors and are secondary events in leiomyoma tumor progression. Cytogenetic abnormalities are classified into several categories based upon the chromosome aberrations present, and include the following subgroups: t(12;14)(q14-q15;q23-q24), del(7)(q22q32), rearrangements involving 6p21, 10q, trisomy 12, and deletions of 3q. A recent study showed a positive correlation between the presence of a cytogenetic abnormality and the anatomic location of uterine leiomyomas (Brosens et al., 1998). *HMGIC*, a high-mobility-group protein (HMG) gene encoding an architectural transcription factor, was recently identified as the target of gene fusion in a variety of human benign mesenchymal tumors. Some of these events were chromosomal translocations involving 12q13-15. *HMGIC* consists of three DNA-binding domains (encoded by exons 1-3), a spacer, and an acidic carboxyl-terminal regulatory domain (exons 4-5). It has been postulated, that HMG-1 enhances DNA binding of steroid hormone receptors, resulting in a more stable interaction of the receptor with DNA. DNA bending could also play a structural role in altering protein-DNA contacts, or alternatively it could be directly involved in transcriptional activation (Grosschedl et al., 1994).

Leiomyomas with t(12;14) rearrangements, but not matched myometrium, express *HMGIC*, providing evidence for dysregulation of *HMGIC* in this subgroup of leiomyomas at least (Gattas et al., 1999). Interestingly, there is a gene in the *HMGIC* family known as *HMGIY* that has been mapped to 6p21, another site of chromosomal rearrangement in mesenchymal tumors including leiomyomas. *HMGIY* has been shown however to be expressed in tumors that were either karyotypically normal or with chromosome rearrangements other than those involving 6p21. Thus there is not a simple correlation of *HMGIY* expression with a particular karyotype, like that observed for *HMGIC* (Sornberger et al., 1999).

ASPECTS OF SEX STEROID HORMONES INVOLVED IN THE GROWTH OF LEIOMYOMAS

Background

The occurrence of leiomyomas during reproductive age, and the reduction following menopause and with GnRHa treatment indicates that sex steroids are important for the development of leiomyomas (Kawaguchi et al., 1985; Friedman et al., 1990; Lemay et al., 1994). Sex steroid hormones are derived from cholesterol, a highly conserved compound that is fundamental in the structure and function of biological membranes in addition to the biosynthesis of sex steroid hormones (Bloch, 1983). Although the estrogen responsiveness of uterine leiomyomas is well established, the impact of environmental estrogens and their contribution to the development of these tumors is currently unknown. The Eker rat is the only model where the animal develops spontaneous uterine leiomyomas, which share many characteristics with those found in humans (Houston et al., 2001).

1) Estrogen and the estrogen receptors (ER)

Estrogens function through their interactions with specific high affinity intracellular receptors (Sarff et al., 1971; Mester et al., 1975). The classical ERα was cloned in 1986 (Green et al., 1986) and a second estrogen receptor, ERβ, was cloned from rat prostate and ovary in 1996 (Kuiper et al., 1996). Both ERs are members of the steroid receptor superfamily and consist of six regions, A-F, of which the DNA-binding domain (C) and ligand-binding domain (E) are highly conserved, with 96% and 58% amino acid sequence homology, respectively **(Figure 2)**.

Figure 2. Schematic structure of the nuclear receptor domains.

Estrogen enters the cell by passive diffusion across the cellular membrane and further into the nucleus, where it binds to unoccupied receptors. Following binding of the ligand to the ER and release from the heat shock protein complex, two ER molecules bind as either homo- or heterodimers to the estrogen responsive element (ERE) present in the promoter regions, follows binding of ligand to the ER and the release from the heat shock protein complex (Smith, 1993). Once bound to an ERE in a target gene, the ER dimer directly or indirectly interacts with the basal transcription machinery to modulate transcription, leading to the biological response to hormone stimulation. The estrogen receptors can be activated both ligand independent **(Figure 3 path A)** and ligand dependent **(Figure 3 path B)** (Osborne et al., 2001).

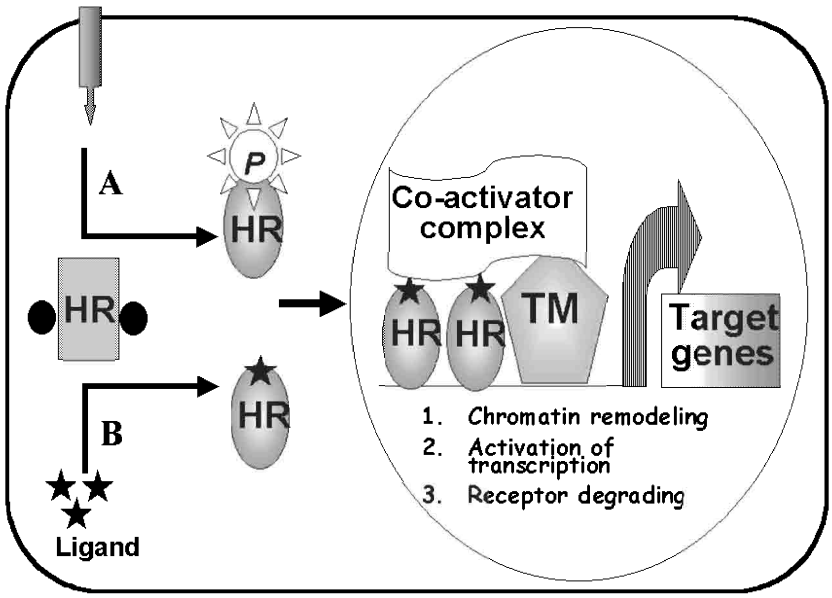

Figure 3. A schematic presentation of the mechanism of action of nuclear receptor regulation of gene transcription. HR, hormone receptors; TM, transcription machinery; *P*, phosphorylation; ●, chaperone proteins.

The concentration of estradiol is significantly higher in leiomyomas compared with normal myometrium (Otubu et al., 1982) and the conversion of estradiol to estrone is significantly lower in leiomyomas when compared with the corresponding myometrium (Yamamoto et al., 1984). In the guinea pig animal model, leiomyomas can be induced with long-term estrogen treatment for 70 to 100 days where estradiol benzoate and estrone were the most effective when given as a series of weekly injections or implants (Lipschütz, 1942). However estrogen alone is unlike to be responsible for tumorigenesis as the myometrium is cyclically exposed to relatively high levels of estrogen during the reproductive years yet most women do not develop leiomyomas. The heterogeneity of leiomyoma size within an individual uterus also suggests that leiomyoma growth is involves by additional factors as well.

Previously published results concerning the expression of ERα and ERβ in leiomyomas are contradictory. Englund and coworkers showed that during all phases of the menstrual cycle, ERα expression was elevated in comparison with adjacent myometrium and two reports demonstrated that ERβ expression was higher in leiomyomas only during the proliferative phase (Englund et al., 1998; Kovacs et al., 2001; Wang et al., 2001). Following menopause, ERβ expression was shown to increase in leiomyomas, while the ERα expression was not significantly altered and was similar to that in myometrium (Kovacs et al., 2001). In contrast, Nisolle *et al.* reported that the ER

content was significantly higher in leiomyomas than in myometrium only during the proliferative phase of the cycle, and that after GnRHa therapy, the ER content in leiomyomas was not significantly altered when compared to that during the menstrual cycle (Nisolle et al., 1999). In GnRHa treated women an increased ratio of ERα/ERβ proteins was found as compared to the proliferative phase (Wang et al., 2001). In another study, the ER levels during GnRHa treatment in both myometrial and leiomyoma tissues were reported to be similar to those in the proliferative phase, but significantly higher than in the secretory phase (Englund et al., 1998). It was postulated from this study that high progesterone levels down-regulate ER in both leiomyomas and myometrium. Furthermore, binding kinetics revealed two specific binding sites (with high or low affinity) for 17β-estradiol, which were present in the normal myometrium, yet only the low-affinity binding sites were detectable in leiomyomas (Benassayag et al., 1999).

2) Progesterone and progesterone receptor (PR)

The action of progesterone is mediated by specific intracellular receptors, PR, binding to hormone response elements (HREs), which activate or repress signals to the transcriptional machinery of target genes. The PR has two isoforms, PR-A, with a size of 94 kDa, and PR-B with a size of 120 kDa (Horwitz et al., 1983). They result from translation of two different mRNA populations, created by transcription from alternate initiation sites within the same gene.

In vitro the PR-A and PR-B promoters act independently, suggesting that they are regulated in a tissue-specific manner. Since there is differential gene regulation of PR-A and PR-B, tissue-specific gene expression may be established by altering the balance between the PR-A and PR-B isoforms, leading to variations in tissue-specific responses to progestins (Mulac-Jericevic et al., 2000). The function of human PR-B is recognized as a transcriptional activator of progesterone-responsive genes, whereas PR-A is associated with a trans-dominant repressor function of other steroid hormone receptor genes (Mulac-Jericevic et al., 2000). During the different developmental stages in the corpus luteum (CL), the ratio of PR-A and PR-B is altered and the total luteal PR-A/B concentration is inversely correlated to CL age (Ottander et al., 2000). In general, estrogen can increase and progesterone decrease expression of PRs (Katzenellenbogen, 1980; Englund et al., 1998). PR-A can also antagonizes ER action (McDonnell et al., 1994), and has the ability to suppress the activity of PR-B (Vegeto et al., 1993; Tung et al., 1993).

Segaloff *et al.* reported increased cellularity and mitotic activity in uterine leiomyomas obtained from six patients treated with 20 mg progesterone daily for 30-189 days (Segaloff et al., 1949). Increased mitotic activity has been reported in leiomyomas during the secretory phase of the menstrual cycle, and also in leiomyomas obtained from patients treated with the progestin medroxyprogesterone acetate (MPA) alone (Kawaguchi et al., 1989; Tiltman, 1985). Murphy and co-workers presented a pilot study demonstrating a significant reduction in leiomyoma volume among patients treated with the progesterone antagonist RU-486 (Murphy et al., 1991). In a further study, there was no significant change in uterine or leiomyoma volume during simultaneous treatment with GnRHa (leuprolide) plus medroxyprogesterone acetate (MPA) (Friedman et al., 1988). These

findings support the hypothesis that progesterone plays a role in leiomyoma growth (Rein, 2000).

Contents of PR in the cytosol of the tissue determined by enzyme immunoassay were shown to be significantly higher in leiomyomas than in myometrium in different endocrine conditions (Englund et al., 1998). However, in myometrial and leiomyoma tissues, the PR levels were lower in the secretory phase and during GnRHa treatment when compared with the proliferative phase (Englund et al., 1998). In the proliferative phase of the menstrual cycle, estrogen has been suggested to mediate the up-regulation of the PR in both myometrium and leiomyomas (Englund et al., 1998). Moreover, Nisolle and co-workers reported that the PR-AB and PR-B content were higher in leiomyomas than in adjacent myometrium, with a statistically significant dominance of PR-AB over PR-B. During GnRHa therapy, a dramatic decrease was observed in PR-AB and PR-B content when compared to levels seen during the menstrual cycle (Nisolle et al., 1999).

3) Androgen and androgen receptor (AR)

The major androgenic products of the ovary are dehydroepiandrosterone (DHA) and 4-androstene-3,17-dione (androstenedione) with only a small amount of testosterone produce. These androgens are mainly secreted by theca cells derived from stromal tissue. Two forms of the androgen receptor protein have been demonstrated in human genital skin fibroblasts with molecular masses of approximately 110 kD and 87 kD (Wilson et al., 1994). The 87-kD isoform (AR-A) contains an intact C terminus but lacks the normal N terminus found in the 110-kD isoform (AR-B). This is created by initiation of AR synthesis at the first internal met188 residue of AR-B, and has also been observed in a mutant form of AR produced in fibroblasts from an androgen-resistant individual (Zoppi et al., 1993). These two AR isoforms also serve different functions aside from interactions with androgen (Zoppi et al., 1993). Using a well-differentiated adenocarcinoma endometrial cell line (Ishikawa) model, it has been demonstrated that AR is up-regulated by estrogen and androgen, but down-regulated by progesterone and EGF (Lovely et al., 2000; Apparao et al., 2002). 17β-estradiol induced the expression of AR mRNA by predominantly increasing the level of testosterone-binding sites (TBS) in uterine stroma (Fujimoto et al., 1995). In women with polycystic ovarian syndrome (PCOS) higher levels of both serum androgen and AR have been observed in the endometrium (Apparao et al., 2002). The estrogen-induced AR has been localized exclusively in the stroma, mainly in the myometrium where it co-localizes with ERα but not with ERβ (Weihua et al., 2002). Similar findings have been reported in human myometrium and leiomyomas (Fujimoto et al., 1994).

CELLULAR PROLIFERATION AND APOPTOSIS

Background

The balance between tumor cell proliferation and cell death determines tumor growth. Cell death occurs by two major mechanisms, necrosis and apoptosis (programmed cell death). Classical necrotic cell death occurs due to injury or trauma whilst apoptosis takes place during normal cell development, regulating cellular differentiation and number.

1) Cellular proliferation

Cell proliferation plays a fundamental role in neoplasia. Increases in cell proliferation and changes in the cell cycle are essential features during many different stages of carcinogenesis (Grisham et al., 1983).

Cellular kinetics involve the duration of the cell cycle, the fraction of cells in the cycle, and the growth fraction. The phases of the cell cycle include G1 (before DNA synthesis), S (synthesis of DNA), G2 (before mitosis) and M (mitosis). G0 is the resting phase of cells outside the cell cycle **(Figure 4)**.

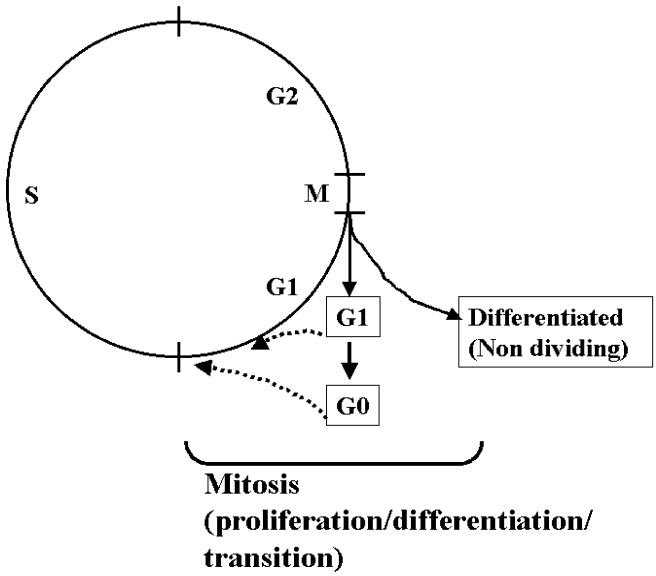

Figure 4. Schematic view of the mammalian cell cycle.

Cell proliferation in normal mammalian cells is regulated by growth factors at two levels in the cell cycle. The first cell cycle checkpoint is at the G1-S transition, a point with the highest sensitivity to injury and toxicity. During the G1 phase, growth factors exert their influence. The second checkpoint is at the G2-M transition. During the G2 phase, immune surveillance and DNA damage repair mechanisms are operating, with

modulation of DNA repair by gene amplification, proto-oncogene activation or tumor suppressor gene activation.

Cell cycle progression is regulated by a family of protein kinases termed cyclin-dependent kinases (CDKs). CDKs are serine/threonine protein kinases controlling the transition between successive phases of the cell cycle. They require binding of regulatory subunits, named cyclins, as an initial step in the activation process. To date, 12 different cyclins have been described in vertebrates and at least 9 CDKs in mammalian cells (Nigg, 1995; Bagella et al., 1998). Cyclins can form catalytically active complexes with different types of CDKs in mammalian cells, and different cyclin/CDK complexes are assembled and activated at specific points in the cell cycle. Passage from one stage of the cell cycle to another is regulated not only by the presence of cyclins but also by modifications of the kinase subunits. Multiple phosphorylation and dephosphorylation events occur on both the cyclins and the CDKs. Phosphorylation controls the activity of CDKs both positively and negatively, depending on the phosphorylation sites and may also serve in the stabilization of the cyclin/CDK complex. Besides phosphorylation, the presence of cyclin-dependent kinase inhibitors (CKIs) may regulate CDK activity. CKIs inhibit the activity of CDKs by binding *in vivo* with the CDK subunit, the cyclin or the cyclin/CDK complex (Dirks et al., 1997; Reed, 1997). In mammalian cells, two families of CKIs have been described, based on protein sequence similarity: the Cip/Kip family and the INK family (Reed, 1997; Sherr et al., 1995).

Studies in ovariectomized ewes demonstrated that administration of estradiol increased uterine weight 2.3-fold by 24 hours with further increases after 48 hours (Reynolds et al., 1998). A similar finding in rats was reported by Sahlin and co-workers (Sahlin et al., 1994). It is well known that estrogens stimulate DNA synthesis and cell proliferation in steroid responsive tissues of female reproductive organs (Sutherland et al., 1983). Previous data also demonstrated that long-term treatment with estradiol in female non-human primates induced a marked glandular proliferation seen by increased ki-67 labeling and thickening of the endometrium (Cline et al., 2001). In cultures of leiomyoma cells, the addition of either estradiol (10 ng/ml) or progesterone (100 ng/ml) resulted in an increase in proliferating cell nuclear antigen (PCNA) expression in the cells. In cultures of normal myometrial cells, the only addition of estradiol augmented PCNA expression, whereas progesterone did not (Matsuo et al., 1999). Furthermore, in luminal and glandular epithelia in the uterus of ovariectomized rats, estradiol treatment leads to the appearance of mitoses that are oriented perpendicularly to the basement membrane of the epithelium (Gunin, 2001). Estradiol effect on mitosis orientation is considered specific (Gunin, 2001). In ovariectomized adult mice, estradiol treatment induces a re-localization of cyclin D1 and, to a lesser extent, CDK4 from the cytoplasm into the nucleus. This results in the orderly activation of cyclin E- and cyclin A-CDK2 kinases and hyperphosphorylation of pRb and p107 (Tong et al., 1999). Conversely, progesterone pretreatment abrogated estradiol-induced cyclin E-CDK2 activation by dephosphorylation of CDK2, followed by inhibition of cyclin A expression. This consequently inhibits cyclin A-CDK2 kinase activity and further phosphorylation of pRb and p107 (Tong et al., 1999).

2) Apoptosis

Apoptosis is a physiological form of cell death that plays a critical role in development, tissue homeostasis, and immune defense in multi-cellular organisms (Kerr et al., 1972; Raff, 1998). The common phenomena of apoptosis include loss of viability accompanied by membrane blebbing, chromatin condensation, cytoplasm shrinkage and DNA fragmentation (White, 1996). Apoptosis is selectively triggered in cells by a variety of stimuli. Inappropriate regulation of apoptosis may result in acute damage or degenerative diseases from excessive apoptosis, or cancer and autoimmune disease due to impaired apoptosis (Barr et al., 1994; Thompson, 1995). The genes associated with apoptosis include *Fas* and *FasL*, the *bcl-2* family, *p53*, *Rb-1* and several others (Wyllie, 1993).

Steroids regulate both induction and inhibition of cell death, along with many other cellular functions. They are also involved in the regulation of cellular composition of organs throughout the body together with other hormones and growth factors. Steroids are potent regulators of apoptosis in many mammalian steroid-dependent cell types and tissues such as the mammary gland, prostate, ovary, uterus and testis where they can impede or facilitate the apoptotic process by their presence or absence. In addition to direct steroid hormone action on target cells, steroid facilitated apoptotic events can also be initiated indirectly by altering expression of paracrine effectors in the supporting stromal cells (Kucharova et al., 2002).

In the primate endometrium, progesterone and estrogen may have proliferative, anti-apoptotic effects or both. This control of endometrial differentiation and apoptosis is believed to be regulated by controlling Bcl-2 gene family expression (Vaskivuo et al., 2000).

Fas and *FasL*

Fas in humans is located on the long arm of chromosome 10 (Inazawa et al., 1992; Lichter et al., 1992) and encodes a type I membrane protein of approximately 45kDa belonging to the tumor necrosis factor/nerve growth factor (TNF/NGF) family of receptors. Upon treatment with anti-human Fas antibody, murine T-cell lymphoma WR19L cells and fibroblast L929 cells suffered apoptosis, indicating that Fas is a "death receptor" which can transduce an apoptotic signal (Itoh et al., 1991) **(Figure 5)**. Fas is highly expressed in activated B and T lymphocytes and in tumor cells of hematopoietic origin, with a lower expression in non-lymphoid cell lines (Leithäuser et al., 1993). After the initial discovery of the Fas molecule, the endogenous ligand for this receptor was rapidly cloned and sequenced (Suda et al., 1993). The FasL gene is located on human chromosome 1 (Takahashi et al., 1994), belonging to the TNF family (Ashkenazi et al., 1998). FasL is a death factor, as Fas, its receptor (Nagata et al., 1995). FasL mRNA expression is detected in both lymphoid and non-lymphoid cells (Suda et al., 1993), however FasL expression is more restricted in contrast to the widespread expression of Fas.

FasL expression increases both at the RNA and protein levels in hormone-sensitive breast cancer cells treated with estradiol, suggesting direct estrogenic effects on FasL expression (Mor et al., 2000). Estradiol can induce apoptosis, but estrogen-induced

apoptosis through ERβ requires the expression of Fas and FasL proteins during rat brain development (Nilsen et al., 2000). In addition, estradiol and progesterone regulate maternal immunotolerance for the implantation process by inducing FasL expression to may mediate apoptosis of endometrial cells during trophoblast invasion (Selam et al., 2001).

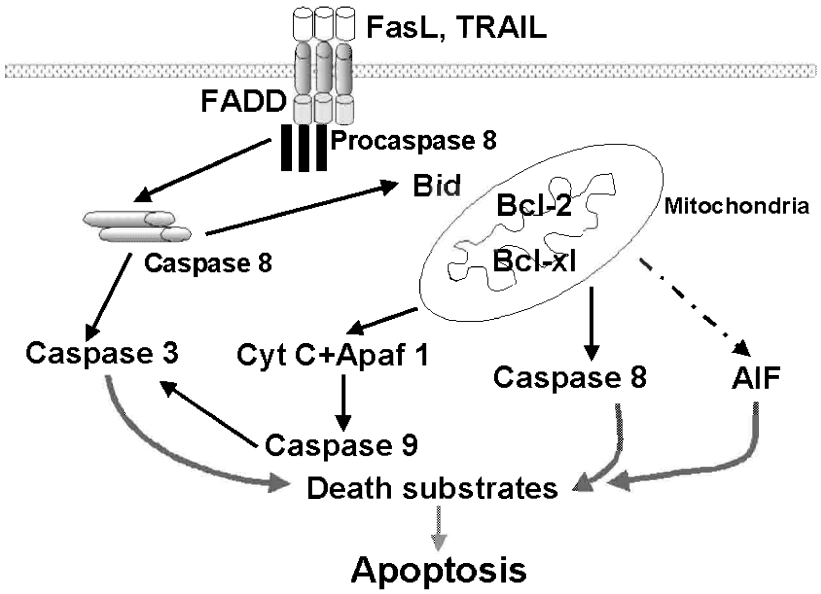

Figure 5. Schematic diagram of the Fas/Fas-L signaling pathway.

Bcl-2 family

The Bcl-2 protein family regulates a distal step in an evolutionarily conserved pathway for apoptosis (Reed, 1994; Yang et al., 1996). On the basis of their functions, these proteins can be divided into two broad categories: those that inhibit apoptosis, such as Bcl-2, Bcl-xl, Bcl-w, and Mcl-1, and those that promote apoptosis, such as Bax, Bcl-xs, Bak, Bok and Bad. They can physically interact with each other in a network of homo- and heterodimers in which the relative proportions of the anti-apoptotic and pro-apoptotic members of this family determine the ultimate sensitivity of cells to cell death stimuli (Sato et al., 1994; Oltvai et al., 1994). Moreover, bcl-2 is a key protein since it is capable of counteracting most proapoptotic stimuli (Herrmann et al., 1996). It has been revealed that overproduction of Bcl-2 significantly enhances the longevity of cells in the surface of classical apoptotic stimuli, including interaction between Fas and FasL, survival factor deprivation and various types of irradiation (Weller et al., 1995; Garcia et al., 1992; Kyprianou et al., 1997). In addition to these proteins, p53 is also a crucial player in the

regulation of cellular homeostasis; its importance is demonstrated by the high mutation rate of this gene in most cancers (Agarwal et al., 1998) **(Figure 6)**.

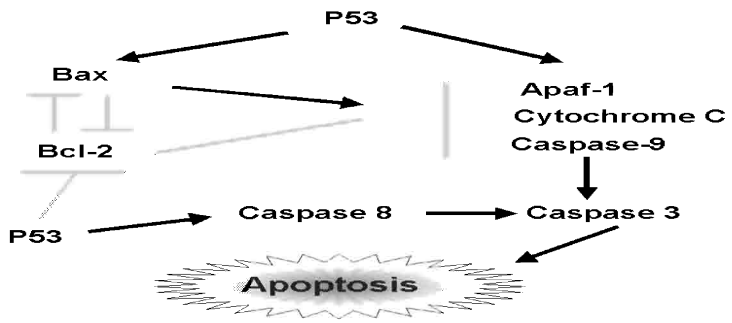

Figure 6. The regulation of apoptosis by Bcl-2 and P53.

All members of the Bcl-2 family contain at least one of four conserved Bcl-2 homology domains, designated Bcl-2 homology (BH)1, BH2, BH3 and BH4 (Gross et al., 1999; Kelekar et al., 1998). Many of the anti-apoptotic members contain at least BH1 and BH2, and those most similar to Bcl-2 display all four BH domains. The pro-apoptotic members display less structural homology, with some possessing all four BH domains, and some with only the short BH3 domain. BH3 is presumed to serve as a critical death domain among the pro-apoptotic members, based upon deletion and mutagenesis data. Many proteins of the Bcl-2 family have a hydrophobic tail forming the transmembrane domain, enabling them to bind to the outside surface of mitochondria, the endoplasmic reticulum and the nucleus, and therefore function mainly on these organelles (Gross et al., 1999; Dragovich et al., 1998) **(Figure 7)**.

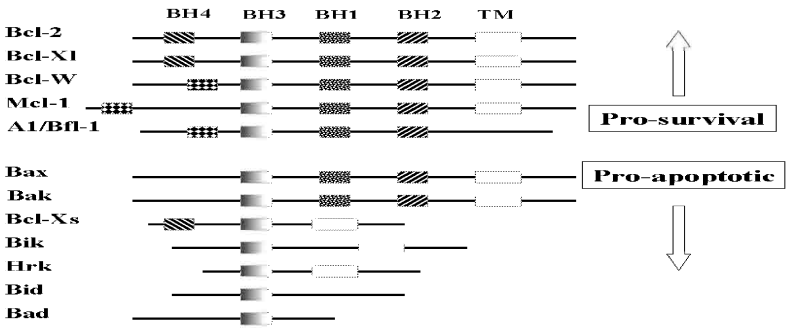

Figure 7. A schematic drawing indicating structures of Bcl-2 family proteins. BH, Bcl-2 homology; TM, Transmembrane domain.

bcl-2 is involved in the t(14;18) translocation that is characteristic of follicular lymphoma, a human B cell malignancy (Tsujimoto et al., 1984). In breast cancer, *bcl-2* expression has been demonstrated to correlate with the expression of ER and PR (Gee et al., 1994). High Bcl-2 protein expression would allow both cell proliferation and progression by blocking apoptosis in human well-differentiated ER-positive breast carcinomas (Diaz-Cano et al., 1997). These findings suggest a role for hormonal factors in the regulation of *bcl-2* expression, and indicate that the role of *bcl-2* in human cancer is much more complex than can be explained by direct apoptosis regulation.

bcl-x has two forms. The most abundant form, called *bcl-xl*, is a blocker of apoptosis whereas the other named *bcl-xs*, is a promoter of apoptosis (Boise et al., 1993). In an endometrial cell line progesterone was shown to increase the ratio of *bcl-xl* to *bcl-xs* (Pecci et al., 1997).

mcl-1 functions as an anti-apoptotic factor, which can inhibit apoptosis e.g. induced by *c-myc* overexpression (Reynolds et al., 1994).

bax encodes a protein with approximately 21% amino acid sequence homology with Bcl-2, such that the two proteins are capable of forming heterodimers (Oltvai et al., 1993). Therefore, the ratio between *bcl-2* and *bax* determines cell survival or death following an apoptotic signal. *bax* has also been suggested to be a common partner involved in heterodimerization and regulation of the function of other *bcl-2* family members (Sedlak et al., 1995).

bak can accelerate apoptosis in mammalian cells, at least in part, by interacting with *bcl-2* and *bcl-x* (Chittenden et al., 1995; Sattler et al., 1997). The expression of *bak* was shown to correlate with estradiol (Leung et al., 1998).

bok has been isolated from a rat ovarian fusion cDNA library as a pro-apoptotic *bcl-2* gene. Bok interacts strongly with some (Mcl-1, BHRF-1 and Bfl-1) but not other (Bcl-2, Bcl-xL, and Bcl-w) anti-apoptotic members, directly contrasting the ability of other pro-apoptotic members (Hsu et al., 1997). As Bok is highly expressed in the ovary, testis and uterus, further studies of this protein could facilitate elucidation of apoptosis mechanisms in reproductive tissues undergoing hormone-regulated cyclic cell turnover.

EXTRACELLULAR MATRIX (ECM) REMODELING, GROWTH FACTORS AND GROWTH FACTOR RECEPTORS

Background

1) Extracellular Matrix

Female reproductive tissues possess a unique ability to accommodate a remarkable amount of cell turnover and extracellular matrix (ECM) remodeling following puberty. Cellular structures within ovarian, uterine, and mammary tissue not only change cyclically in response to ovarian hormones but also undergo differentiation during pregnancy, with eventual return to structures resembling the pre-pregnant stage. ECM components include e.g. fibronectin, vitronectin, laminin and collagen. Myometrial smooth muscle cells have a rich cytoskeletal structure, and agonists which stimulate myometrial activation provoke measurable changes in actin fibres that, may be important for efficient contractility (Yu et al., 1998). This is a fundamental feature of leiomyomas; called fibroids due to their fibrotic nature which develops from deposition of abundant ECM. Leiomyomas contain 50% more ECM than the corresponding myometrium (Fujita, 1985). The basis for tissue fibrosis involves both increased connective tissue deposition and decreased ECM degradation. Matrix metalloproteinases (MMPs) are the enzymes implied to be the primary contributors to the degradation process of ECM. Degradation regulated by a balanced activity of these enzymes and their endogenous inhibitors, tissue inhibitor of metalloproteinases (TIMPs). Expression and hormonal regulation of MMPs and TIMPs has been a recent investigation of interest (Dou et al., 1997; Palmer et al., 1998). Dou et al analyzed the expression of MMP-1, 2, 3, and 9 in both myometrium and leiomyomas, finding their expression to be lower in leiomyomas (Dou et al., 1997). However, Palmer and co-workers demonstrated higher expression of the fibronectin-degrader MMP-11 in leiomyomas than in myometrium (Palmer et al., 1998). It would be expected to find a lower level of MMPs in leiomyomas, yet this finding may indicate a unique role for this particular MMP in leiomyomas.

Although the growth of leiomyomas is believed to be sex steroid dependent, *in vitro* studies in human tissues have shown inconsistent results when aiming to demonstrate a direct growth-promoting action of ovarian hormones. This suggests the presence of intermediate elements, such as cytokines and growth factors, through which the ovarian hormones may be exerting their growth-stimulatory effects on leiomyomas. Estrogen and progesterone may regulate gene expression of these cytokines and growth factors, which in turn modify other genes´ transcription.

2) Growth factors and growth factor receptors

A number of cytokines and growth factors have been investigated in leiomyomas to determine if and how they may be responsible for mediating the growth-promoting effects of ovarian hormones.

IGF family

IGFs are small polypeptides that are structurally similar to proinsulin, and can promote cell proliferation and differentiation. The two well-characterized IGFs are the 70-amino

acid IGF-I and the 67-amino-acid IGF-II. These two growth factors are produced by uterine cells in a number of animal species, and their expression is controlled by ovarian steroid hormones (Boehm et al., 1990). IGFs action is mediated by interaction with specific cell surface receptors, and interruption of these signaling pathways may result in inhibition of cellular growth, differentiation and metabolism (Yee, 1994). IGFs are associated with six soluble, high-affinity binding proteins (IGFBPs), which can bind the IGFs and prevent receptor activation (Rechler, 1993; Yee, 1994).

In human endometrium, IGF-I mRNA expression is estrogen dependent and is also regulated by progesterone (Giudice et al., 1993a). Estrogen regulation of IGF-I gene transcription is mediated through an activating protein-1 (AP-1) site in the IGF-I gene promoter (Umayahara et al., 1994). IGFs have been shown to be expressed in both leiomyomas and myometrium regulated by estrogen (Rein et al., 1990; Giudice et al., 1993b; Englund et al., 2000). A higher IGF-I receptor content in leiomyomas when compared with myometrium suggests that it may play a role in the growth of leiomyomas (van der Ven et al., 1994). IGF-II gene expression does not appear to be dependent on endogenous steroid concentrations or the menstrual cycle phase in either myometrium or leiomyomas (Giudice et al., 1993b). Both IGF-I and IGF-II were shown to be down-regulated in leiomyomas from GnRHa treated patients (Rein et al., 1990, Giudice et al., 1993b).

FGF family

The FGF family presently consists of at least 20 different members, including the well-characterized acidic FGF and basic FGF. These heparin-binding polypeptides share 30-70% amino acid sequence homology, are mitogenic and angiogenic, and are involved in cell differentiation and tissue development and repair (Tanaka et al., 1992; Mason, 1994). Their actions are dependent on their ability to bind and activate a family of cell surface receptors with intrinsic protein tyrosine kinase activity (Duan et al., 1992; Johnson et al., 1993). Four distinct genes encoding high affinity FGF receptors designated as FGFR-1, -2, -3 and -4 have been identified. These receptors possess an extracellular cytoplasmic ligand-binding domain containing three immunoglobulin-like regions, a hydrophobic transmembrane domain, and a discontinuous intracellular tyrosine kinase domain exhibiting a short intervening sequence (Jaye et al., 1992).

Members of this family can influence numerous cellular processes, including cell proliferation, differentiation, and motility (Basilico et al., 1992). *In vivo* and *in vitro*, bFGF induces both mitogenic and non-mitogenic responses in different cell types (Folkman et al., 1987; Rifkin et al., 1989).

Accordingly, bFGF gene expression in the rat uterus is stimulated by estradiol (Cullinan et al., 1991). On the other hand, bFGF influences uterine stromal cells in pregnant rats via an activation of FGFR1, suggesting that both bFGF and FGFR1 expression correlate with the sex steroid environment (Rider et al., 1995).

TGF-β family

TGF-βs are multifunctional peptides that occur as five similar isoforms, each encoded by a distinct gene, yet only TGF-β1, 2 and 3 are identified in mammalian cells and tissues (Massague, 1990; Barnard et al., 1990). TGF-βs are considered to be the prototype of

multifunctional cytokines, their primary role modulating cell development. Consequently they influence cell proliferation, acting both as inhibitors and stimulators depending on the type of tissues. Moreover, TGF-βs up-regulate the synthesis of many ECM components leading to fibrosis (Massague, 1990; Ignotz et al., 1986). The biological activities of TGF-βs in their target tissues are mediated through three specific cell surface receptors, designated receptor types I-III.

TGF-βs are located in the female reproductive tract in the oviduct, endometrium and myometrium. They exhibit diverse biological activities, including stimulation or inhibition of cell growth, differentiation, regulation of ECM production and chemotaxis (Massague, 1990; Barnard et al., 1990).

TGF-βs are response for important functions involved in uterine endometrium apoptosis. In ovariectomized mice, increased sensitivity of uterine epithelial cells to TGF-βs, as demonstrated by an increase in TGF-β type II receptor mRNA, is involved in the induction of apoptosis after estrogen deprivation (Wada et al., 1996). However, the signals produced by TGF-βs do not appear sufficient to induce apoptosis, suggesting that the apoptotic functions of TGF-βs involve other factors. Pretreatment of the human endometrial epithelial cell line HHUA with either TGF-β1 or EGF enhanced Fas-mediated growth suppression and Fas-mediated DNA fragmentation in the cells. This suggests that TGF-β1 and EGF enhance apoptotic susceptibility of the cells (Tanaka et al., 2000). In addition, pretreatment with TGF-β1 in endometrial stromal cell cultures induced a dose-dependent up-regulation of FasL expression, which was specifically inhibited by its antibody (Garcia-Velasco et al., 1999). The effect on ECM turnover by TGF-βs has recently been implicated in the development of endometriosis. In an established experimental model of endometriosis, blocking the action of TGF-βs opposed progesterone-mediated suppression of MMPs and blocked the ability of this steroid to prevent endometriosis (Bruner et al., 1999). In *in vitro* studies, MMPs appeared to be over-expressed in endometriotic lesions, with expression levels decreasing following successful medical therapy (Osteen et al., 1999; Bruner et al., 1999). More recently, TGF-β1 was shown to exert a similar effect in myometrial cells, increasing TIMP-1 and decreasing both MMP-1 and MMP-3 (Ma et al., 1999).

So far, TGF-β is the only growth factor shown to be not only over-expressed in leiomyomas compared with myometrium, and be both mitogenic and fibrogenic in these tissues, but also be hormonally regulated *in vivo* and *in vitro* (Arici et al., 2000; Chegini et al., 1996; Chegini et al., 1994; Tang et al., 1997; Ma et al., 1999). The mRNA and protein for TGF-β1-3 and all three receptors have been detected in human myometrium and leiomyomas (Tang et al., 1997; Murphy et al., 1994). Interestingly, although the proliferation of normal myometrial smooth muscle cells is inhibited by TGF-β1 and TGF-β3, leiomyoma smooth muscle cells are not growth-inhibited by TGF-β1 and even show an increase in proliferation in response to TGF-β3 (Lee et al., 2001).

EGF family

EGF is a pleiotropic polypeptide of 53 amimo acids (Cohen, 1962), generated by proteolytic processing of a larger molecular precursor, a 133kD pro-EGF. Structurally homologous EGF-related growth factors include heparin-binding EGF-like growth factor

(HB-EGF) and transforming growth factor α (TGFα). EGF has a profound effect on the differentiation of specific cells *in vivo* and is a potent mitogenic factor for a variety of cultured cells of both ectodermal and mesodermal origin (Carpenter et al., 1979; Goustin et al., 1986). The EGF receptor is a tyrosine protein kinase of three regions (Aden et al., 1976). One region projects outside the cell surface and contains the binding site EGF, the second is embedded in the membrane, and the third projects into the cytoplasm of the cell's interior. EGFR functions as a kinase, attaching phosphate groups to tyrosine residues in proteins (Haley et al., 1987).

Both EGF and EGFR are expressed in leiomyomas and myometrium with progesterone up-regulating EGF, and estrogen up-regulating EGFR in leiomyomas (Shimomura et al., 1998).

AIMS OF THE PRESENT INVESTIGATION

The general aim of this research project was to investigate growth mechanisms of uterine leiomyomas influenced by sex steroid hormones. The specific aims were to:

- Investigate the balance between cell proliferation and apoptosis in leiomyomas (Paper I).
- Investigate the expression of the apoptosis-associated Bcl-2 gene family in leiomyomas (Paper II).
- Investigate the expression of progesterone receptors (PR-AB, PR-B) and IGF-I in leiomyomas under different endocrine conditions, and their possible influence on leiomyoma growth (Paper III).
- Identify new genes influenced by estrogen, with higher expression in leiomyomas than in the adjacent myometrium (Paper IV).
- Screen for genes influenced by estrogen in the rat uterus using advanced gene expression profiling (Paper V).

RESULTS AND DISCUSSION

Apoptosis, cellular proliferation and expression of p53 in human uterine leiomyomas and myometrium (Paper I)

Uterine leiomyomas are the most common tumors in the female genital tract. Sex steroid hormones are considered to be important in the development and growth of leiomyomas. Generally, the balance between cell proliferation and cell death determines tumor growth. It is well known that estrogens stimulate DNA synthesis and cell proliferation in steroid-responsive tissues of female reproductive organs (Sutherland et al., 1983). In the absence of appropriate hormone signals, apoptosis can be induced in terminally differentiated cells of hormone-dependent tissues, such as the prostate and mammary gland, resulting in tissue regression (Kyprianou et al., 1990; Lund et al., 1996).

We have measured the cell proliferation marker Ki-67 using immunostaining, and apoptosis using the TUNEL method in leiomyomas and in the corresponding myometrium.

Leiomyoma cells had significantly higher mitotic activity than myometrial cells, with a higher Ki-67 index in the secretory phase compared with the proliferative phase in both tissues. These findings suggest that progesterone is important for cell proliferation in leiomyoma growth as the secretory phase corresponds with the greatest concentration of progesterone during the menstrual cycle. This hypothesis is consistent with clinical observations that treatment with low doses of progesterone antagonist RU-486 can substantially reduce the size of leiomyomas (Murphy et al., 1993). Together, this data suggests that the function of estrogen and progesterone may be complementary, with estrogen priming a prerequisite for optimal stimulation of proliferation by progesterone.

In contrast to the proliferation results, no difference in apoptotic indices could be observed either between the proliferative and secretory phases, or between leiomyomas and myometrium. In several tissues, induction of apoptosis appears dependent upon the levels of sex steroids, as seen in, the rabbit endometrium after removal of progesterone (Rotello et al., 1989). During the human menstrual cycle however, the altered ratio of estrogen to progesterone represents a less dramatic change than in reports using animal models. Moreover, the marked differences in biological characteristics between uterine smooth muscle cells and epithelial cells may also contribute to the different responses to variable sex steroid levels. Interestingly, a previous study of leiomyomas found that GnRHa therapy suppressed cell proliferation and caused a transient increase in apoptosis in uterine leiomyomas at an extremely high level only at the 4th week after this treatment (about 5 times more than that of control patients) suggested that apoptosis is responsible for the regression (Mizutani et al., 1998). It also implied that the process of apoptosis is very transient at the exact time point when the leiomyomas regress.

All leiomyoma samples studied to date are p53 negative, differing from the findings of malignant smooth muscle cell tumors. This negative expression of p53 in leiomyomas is in agreement with the benign character of the tumors (Zhai et al., 1999).

In conclusion, during stimulation with sex steroid hormones, uterine leiomyomas have a selective growth advantage when compared to the corresponding myometrium, with a higher rate of proliferation and a similar rate of apoptosis.

Expression of Bcl-2, Bax, Bak, Bcl-x and Mcl-1 in human uterine leiomyomas and myometrium (Paper II)

As mentioned above, it is known that apoptosis takes place at a transient time period (Mizutani et al., 1998). Together with our own finding that the apoptotic index decreases in both myometrium and leiomyomas after menopause (Paper I), we still hypothesize that apoptosis in both myometrium and leiomyomas is controlled by estrogen and progesterone. Therefore, we investigated the expression of proteins belonging to the apoptosis-related Bcl-2 family. These proteins can induce apoptosis and are also components of the cell cycle (Huang et al., 1997).

Tissue samples were collected from 18 pre-menopausal women and 6 menopausal women. The protein expression of Bcl-2, Bcl-x, Mcl-1, Bax and Bak in leiomyomas, together with the corresponding myometrium, was measured by immunohistochemistry and confirmed by Western blot.

The expression of Bcl-2 in leiomyomas was stronger than in myometrium both in the proliferative and secretory phases. Following menopause, Bcl-2 expression was reduced with no significant difference between two tissue types. It has been shown previously that the content of both ER and PR is higher in leiomyomas than in myometrium (Englund et al., 1998), raising the question of a functional interaction between sex steroids and Bcl-2 in leiomyomas. A similar observation was found in human endometrial glands where the expression of Bcl-2 and ER was strongly correlated (Otsuki et al., 1994). More direct evidence to prove the connection between Bcl-2 and estrogen has been done in nude mice with MCF-7 tumors untransfected or transfected with a *bcl-2* cDNA sense or antisense expression vector (Pratt et al., 1998). In this experiment, estrogen withdrawal resulted in regression of untransfected and *bcl-2* antisense tumors, whilst *bcl-2* sense tumors were unaffected. However in an *in vitro* study, the Bcl-2 protein was shown to be up-regulated by progesterone but not estrogen in leiomyomas (Matsuo et al., 1997). This does not necessarily counteract the concept that estrogen and ER may be involved in the regulation of Bcl-2 in leiomyomas.

The expression of Bcl-x, another apoptotic blocker, was higher in leiomyomas than in myometrium with the strongest expression in the secretory phase. However, Bcl-x expression was lower in both tissues after menopause with no tissue difference observed. Evidence for relationship between sex steroids and Bcl-x has been found in the hippocampus area, an effect thought to be mediated through a putative ERE in the *bcl-x* gene (Pike, 1999). In addition, a study in normal and neoplastic ovarian tissues showed a negative correlation between gene expression of *bcl-x* and the level of PRs (Marone et al., 1998). Our observations suggest the possibility of an interaction with progesterone, and perhaps estrogen, in the secretory phase when concentrations of both hormones are relatively high.

The immunostaining of Mcl-1 in samples from fertile women was more pronounced in myometrium than in leiomyomas. After menopause, Mcl-1 expression became more abundant in leiomyomas, yet no alterations were observed in myometrium.

Overexpression of Mcl-1 in Chinese hamster ovary cells has been shown to delay apoptosis (Reynolds et al., 1994). The genes of *mcl-1* and *bcl-2* often exhibit a converse pattern of expression implying that the Mcl-1 and Bcl-2 proteins fulfil different roles in the overall physiology of cell death regulation and that differential regulation of the protein suggests a unique role for Mcl-1 in control of apoptosis *in vivo* (Krajewski et al., 1995b). In contrast to *bcl-2*, up-regulation of *mcl-1* represents a relatively early event associated with malignant transformation (Krajewska et al., 1996; Soini et al., 1998). This leads one to speculate that the lower expression of Mcl-1 in leiomyomas is related to the benign leiomyoma phenotype.

The expression of Bax was also higher in leiomyomas than in myometrium, and decreased after menopause. In a study on breast tissue, Bax was shown to be independent with respect to estrogen and ER (Reed, 1996), whereas the presence of Bcl-2 and Bax had a strong positive correlation (Krajewski et al., 1995a; Binder et al., 1996). The convert correlation between *bax* and *bcl-x* mRNA expression has also been observed in rabbit corpus luteum, where *bax* increased and *bcl-x* decreased in parallel with apoptosis induced by estrogen withdrawal (Goodman et al., 1998).

In myometrium, the staining for Bak protein showed a stronger immunoreactivity in the secretory phase than in the proliferative phase, whilst the expression in leiomyomas was similar for the two phases. Comparing tissue types, Bak staining was more intense in secretory phase myometrium than in the corresponding leiomyomas. Bak expression in human endometrium has been found to correlate with sex steroid hormones serum levels, and is localized to endometrial glandular epithelial cells detected only in the secretory phase (Tao et al., 1998). Leiomyomas have less expression of Bak compared to myometrium, which theoretically could prevent the leiomyoma cells undergoing apoptosis during the menstrual cycle and allow them to develop further. However the consequences of this tissue difference expression in response to the fluctuating endocrine condition requires further clarification.

In summary, Bcl-2, Bcl-x, Mcl-1, Bax and Bak are expressed in myometrium and leiomyomas with tissue differences that appear to be influenced, at least in part, by the levels of estradiol and progesterone during different endocrine conditions.

Expression of the progesterone receptor and insulin-like growth factor-I in human uterine leiomyomas and myometrium (Paper III)

To determine the levels of the PR and insulin-like growth factor-I (IGF-I) in human myometrium and leiomyomas, and the relationship between their levels and the growth advantage of leiomyomas, we measured their mRNA and protein expression during the proliferative phase of the menstrual cycle and after treatment with a gonadotropin releasing hormone analogue (GnRHa).

The PR mRNA level, as measured by solution hybridization, did not differ significantly between myometrium and leiomyomas in any of the two groups. However,

significant differences existed between women in the proliferative phase as compared to the respective tissue in GnRHa treated women of both tissue types. Both myometrium and leiomyomas exhibited moderate and similar staining for PR protein, yet a significantly reduced signaling following GnRHa treatment. Furthermore, a positive correlation was found between the serum estradiol concentration and PR mRNA levels in fibroids from women in the proliferative phase. Although no tissue differences were observed, myometrium and leiomyomas in the proliferative phase showed more positive immunostaining of both PR-AB and PR-B when compared to those collected after GnRHa treatment. The results of the present study suggest that PR-B is the dominant form of PR in human myometrium and leiomyomas. Leiomyoma growth has been associated with progesterone, and a 1985 study revealed that women treated with progesterone before hysterectomy demonstrated significantly higher mitotic activity in their tumors than women untreated or pretreated with a combined estrogen–progesterone preparation (Tiltman, 1985). Several studies have also strongly suggested that the proliferative activity of leiomyomata is under the dual control of both estradiol and progesterone (Kawaguchi et al., 1985; Nisolle et al., 1999).

PR-B expression in endometrium appears to be dependent on circulating estrogens in women during the normal menstrual cycle, with low levels of expression during GnRHa therapy (Mangal et al., 1997). Different ratios of PR-A and PR-B can result in different responses to progesterone (Vegeto et al., 1993). In our study, both PR-AB and PR-B decreased after GnRHa treatment with no difference observed between tissues. Exogenous estrogen administration in the form of oral contraceptives during the follicular phase of the menstrual cycle has been shown to increase the protein level of PR-B in the uterus of cycling women who have undergone endometrial biopsy (Mangal et al., 1997). This finding suggests that estrogen induces the expression of PR-B. Although both PR-A and PR-B levels could be increased by estrogen (Okulicz et al., 1989), there is evidence in the literature for a preferential up-regulation of PR-B by estrogen. In some circumstances, PR-B may be the more sensitive isoform to this hormone (Vegeto et al., 1993; Graham et al., 1995). Estradiol treatment can increase the activity of PR-B but not PR-A, whereas progestins decrease the levels of all PR transcripts causing a reduction in the PR-A/B ratio (Graham et al., 1995). Altering the PR-A/B ratio results in a different response to progesterone in the cells.

Although it is generally assumed that expression of sex steroids hormone receptors is crucial in the regulation of leiomyoma growth, Viville and co-workers were not able to correlate PR positive cells with a marker of proliferation in leiomyoma cells by double immunohistochemical staining (Viville et al., 1997). This can be explained if the action of progesterone is mediated by subsequent activation of growth factors, growth factor receptors and oncogenes. A previous finding, demonstrating that IGF-I may be of importance, was performed in primary monolayer cell cultures of leiomyoma and myometrial cells with different concentrations of IGF-I or IGF-II treatment. IGF-I but not IGF-II, preferentially stimulated the leiomyoma cells in monolayer culture, yet neither factor exerted a stimulatory effect on myometrial cells (Strawn et al., 1995).

A significant positive correlation observed between the serum estradiol concentration and the IGF-I mRNA levels in leiomyomas from women in the proliferative phase is in concordance with previously published data (Englund et al., 2000; Giudice et al., 1993b). These data imply that IGF-I gene expression is regulated by estrogen in fibroids. Furthermore, *in situ* hybridization showed that IGF-I mRNA in the myometrium and leiomyomas is located where the smooth muscle cells are dominant. This supports the possibility that IGF-I acts as a stimulator of muscle cell growth. In addition, fibroids and myometrium expressed significantly less IGF-I mRNA after GnRHa therapy than those obtained from women in the proliferative phase. We suggest that the difference in IGF-I mRNA levels between treated and untreated patients, is at least partly due to the hypoestrogenic environment. The expression of IGF-I appears to be regulated not only by estrogen but also by non-steroid modulation of ERα (Fournier et al., 2001), a receptor critical for IGF signaling (Oesterreich et al., 2001). Furthermore, IGF-I has been proposed as a "G1-progression factor", and as a mediator of the mitogenic effects of estrogen on the uterus, including a critical role in G2 progression (Adesanya et al., 1999). Selective estrogen receptor modulators (SERM) have been shown to positively regulate the IGF-I gene through ERα (Fournier et al., 2001). Both estrogen and progesterone are involved in this regulation. Progesterone has been suggested to enhance the estrogen mediated increase in IGF-I mRNA (Adesanya et al., 1996a). The observation that IGF-I is dependent on estradiol for significantly increased expression in uterine smooth muscle cells, and is augmented by the addition of P4, provides evidence for complex local interactions between estrogen and progesterone regulated components of the IGF system (Adesanya et al., 1996b).

Our results have demonstrated a significant decrease in PR-AB and B after GnRHa treatment, possibly associated with shrinkage of leiomyomas and the uterus, influenced by both estrogen and progesterone. Moreover, our data support the view that IGF-I is involved in sex steroid-mediated growth regulation of leiomyomas.

Screening for the genes involved in the development and growth of leiomyomas by representational difference analysis of cDNA (Paper IV)

In order to identify genes differentially expressed in leiomyomas when compared with normal myometrium, we have used an un-biased approach, representational difference analysis of cDNA (cDNA-RDA). The advantage of this new approach is based on the fact that the method allows us to simultaneously identify a number of genes with higher expression in leiomyomas than in the corresponding myometrium.

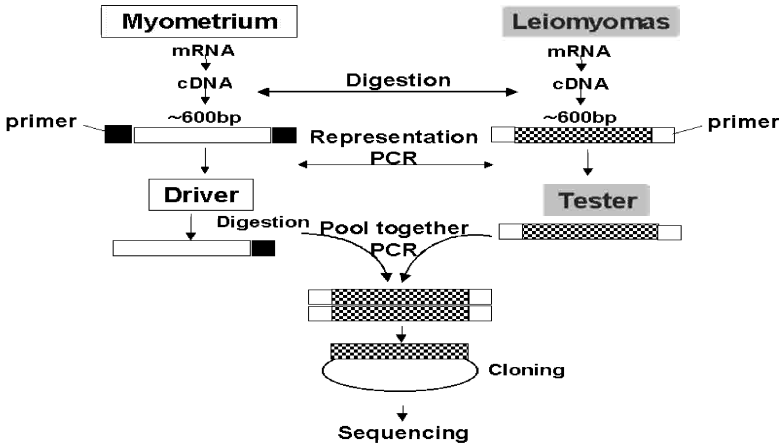

Figure 8. Schematic presentation of the cDNA-RDA method.

The experiment was designed as follows:

1) For the basal RDA analysis a single leiomyoma and corresponding myometrium were collected from an individual in the proliferative phase of the menstrual cycle. The tissue had a significantly higher number of ER and PR in the leiomyomas than in the surrounding myometrium, and was denoted "the first patient". Sequence analysis of differentially expressed cDNA products was lated performed and analyzed for homologies with published sequences in the non-redundant and expressed sequenced tags (EST) divisions of public databases (GenBank, EMBL, DOBJ, and PDP) using BlastN software. RDA was performed to identify genes over-expressed in leiomyomas. Following extensive sequencing of the RDA clone products, 34 different cDNA clones were identified, 29 corresponding to genes of assigned function whilst the remaining had high homology to EST found in public databases. It is possible that additional gene products are missing from the list of putative up-regulated clones due to the limitation of all differential cloning methods (See review by Carulli et al., 1998).
2) To rule out artifacts during the RDA procedure, we analyzed the expression levels of a subset of genes in samples from "the first patient" by solution hybridization with ^{35}S-labeled cRNA probes. The cRNA probes were prepared from 26 cDNA clones identified by the RDA product. Seven genes with higher expression in leiomyomas than in myometrium were identified (>130%), including tomoregulin, cellular retinoid acid binding protein 1 (CRABP1), zinc finger protein 185 (ZFP 185), pregnancy associated plasma protein A (PAPPA), regulator of G protein signaling 12, placental bikunin and latent TGF-β binding protein 2 (LTBP2).

3) To identify genes of general importance for tumor growth, the expression of a subset of candidate genes (defined above) were measured by solution hybridization in paired and pooled leiomyoma and myometrial samples, respectively, collected from 11 patients in the proliferative phase and 4 patients in the secretory phase of the menstrual cycles. The list of genes that were differentially expressed in the pooled samples included PAPPA, tomoregulin, CRABP1, ZFP 185, regulator of G protein signaling 12, placental bikunin and LTBP2.
4) Taking into account the putative cellular function as well as the previous expression data, a more detailed study of selected gene products was performed using individual samples. Six paired leiomyoma and myometrial samples collected from individual patients during the proliferative phase of the menstrual cycle were measured for the expression of the candidate genes. The expression transcripts coding for LTBP2, ZFP 185, tomoregulin, CRABP1 and PAPPA were significantly higher in leiomyomas compared to the adjacent myometrium when mRNA levels were normalized to β-actin.
5) To understand the biological function of one of the candidate genes, we performed immunohistochemistry in matched leiomyoma and myometrial samples to see the localization and expression of the identified gene protein product. We were able to obtain an antibody for LTBP2 among the five identified genes. LTBP2 was primarily detected in the cytoplasm of cells, with mild specific LTBP2 immunostaining observed in the myometrium, leiomyoma and vascular smooth muscle cells. Weak to strong immunostaining of LTBP2 was found in vascular endothelium. Due to the limited sample sizes and the non-quantitative method used in this study, no significant difference in LTBP2 protein expression could be discerned.

Subsequently we have shown that PAPPA, tomoregulin, CRABP1, ZFP 185 and LTBP2 have a significantly higher expression in leiomyomas than in myometrium when analyzed by solution hybridization in the first patient, in the pooled samples of multiple patients during the menstrual cycle and in the individual paired samples of leiomyomas and myometrium.

PAPPA is a glycoprotein present in the serum of pregnant women in increasing concentrations throughout pregnancy (Lin et al., 1974). PAPPA also has a function outside pregnancy since its mRNA can be synthesized in non-reproductive tissues (Overgaard et al., 1999). PAPPA can specifically cleave IGF binding protein (BP)-4 (IGFBP4), which results in release of IGF bound to IGFBP-4. In leiomyomas IGFBP-4 is the most abundant of the IGFBPs (IGFBP-4>>IGFBP-3>>IGFBP-2) (Giudice et al., 1993b). PAPPA cleavage of IGFBP-4 may therefore lead to increased bioavailability of IGF in leiomyomas (Lawrence et al., 1999; Conover et al., 1999; Mazerbourg et al., 2000). The IGF family has previously been proposed as being involved in the growth of leiomyomas as exogenous IGF-I stimulates proliferation in leiomyoma-derived cell lines (Howe et al., 1996).

In the uterus, retinoic acid (RA) can inhibit estrogen-induced uterine smooth muscle cell proliferation (Boettger-Tong et al., 1995; Boettger-Tong et al., 1997; Benson et al., 2000), and has been used in the treatment of hyperproliferative disorders (Fenaux et al., 2000; Recchia et al., 2000). RA actions appear to be mediated by two distinct classes of proteins: a family of nuclear receptors that regulates gene transcription in a ligand-dependent fashion and a group of cellular RA-binding proteins (CRABPs). CRABP can compete with RA binding nuclear receptors to bind to intracellular RA (Napoli, 1993; Napoli, 1996; Ong, 1994). The higher expression of CRABP detected in leiomyomas may limit the amount of RA available for the genomic actions of RAR and/or RXR resulting in promotion of cell proliferation. Several previous studies support the importance of CRABP in protecting smooth muscle cells and inducing cell proliferation in the uterus (Bucco et al., 1996; Wardlaw et al., 1997).

In animal models the sex steroids, especially estrogen, have been shown to modulate the expression of TGF-β (Takahashi et al., 1994). The expression of TGF-β is higher in leiomyomas than in myometrium (Dou et al., 1996). In addition, leiomyoma smooth muscle cells are not growth-inhibited but instead growth-stimulated by TGF-β (Arici et al., 2000; Lee et al., 2001). LTBP can positively regulate the bioactivity of TGF-β (Mecham et al., 1994) by controlling its deposition and targeting the latent and active form of TGF-β to the ECM and connective tissues (Saharinen et al., 1999). In the reproductive systems, LTBP2 has been implicated in implantation and early development (Shipley et al., 2000). We saw that LTBP2 has higher mRNA expression in leiomyomas when compared with matched myometrium, yet no any significant difference was detected in the protein level. However it is possible that LTBP2 has an important function in uterus and leiomyomas since LTBP2 interacts closely with TGF-β, important for both the fibrogenic process and the induction of cell proliferation in these tumors.

Tomoregulin is a newly identified transmembrane protein with a single epidermal growth factor (EGF)-like domain closely related to the EGF/neuregulin (NRG) family of growth factors (Uchida et al., 1999). The exact function of tomoregulin in the uterus is unknown. It may be a potential modulator of extracellular signaling acting to regulate cell growth, differentiation or apoptosis (Uchida et al., 1999).

The significance of the over-expression of LIM ZFP 185 in leiomyomas may only be speculated upon due to the scarcity of functional information regarding this gene. The LIM domains family of ZFP has previously been identified to play an important role in transcriptional regulation and cellular differentiation, appearing to be involved in a wide range of cellular functions (Schmeichel et al., 1997).

In conclusion, we used RDA to clone five candidate genes with higher expression in leiomyomas than in myometrium for investigation of their potential growth-promoting effects in leiomyomas. The changes in gene expression reported above are of small magnitude, and the biological significance of these alterations requires further investigation using special experimental strategies for individual genes.

Analyzing gene expression profiles of estrogen deficiency (Paper V)

To obtain more information concerning estrogen-regulated genes in the uterus, we studied ovariectomized rats with or without estrogen replacement. This allowed for correlation of gene expression pattern variations to estrogen related physiological changes in the uterus.

Two weeks after ovariectomy, the numbers of PCNA positive cells decreased both in endometrium and myometrium, whereas the numbers of apoptotic positive cells increased in comparison to intact rats in the estrus phase. After 7-days treatment with estrogen, the PCNA positive cell number increased whilst the number of apoptotic cells decreased.

Using DNA microarrays containing 3000 to 4000 different rat complementary DNA (cDNA) clones, approximately 1456 clones were detectable (signal 1.6-fold above background) in the uterus. Of the total detectable elements, 88 clones were over-expressed (at least 1.7-fold increase), and 103 clones were under-expressed (at least 1.7-fold decrease) in the ovariectomized rat uterus compared with uterus from intact rats. This represents approximately 10% of the detectable transcripts in the tissue. Eighteen genes were shown to be putatively estrogen-responsive, including: collagen α, secreted apoptosis related protein 1 (SFRP2), peptidylglycine α-amidating monooxygenase (PAM), non-erythroid α-spectrin, ubiquitin-homology domain protein (SUMO-1), carbonic anhydrase 3 (CA3), glucuronyltransferase 1, urinary protein 2 precursor/ATPase inhibitor, kinesin heavy chain, glutathione peroxidase, calreticulin, 3-hydroxyacyl-coA dehydrogenase, CD24, syndecan, hemoglobin β, follistatin-related protein (FSRP), thy-1 protein and polyadenylate-binding protein. Among these genes, several have been reported in other uterine studies such as: collagen α (Yokogawa et al., 2001), SFRP2 (Das et al., 2000), PAM (El Meskini et al., 1998), SUMO-1 (Abdel-Hafiz et al., 2002), CA3 (Hodgen et al., 1971), 3-hydroxyacyl-coA dehydrogenase (Khan et al., 1990), glutathione peroxidase (Serviddio et al., 2002) and syndecan 3 (Russo et al., 2001). We selected Fas, Fas-L and CD24 from the estrogen-related genes that had not been previously described, for immunohistochemical analyses when the respective antibodies were available. Fas and Fas-L stained mainly in the cytoplasm of endometrial epithelial cells and myometrial cells. In the estrogen-treated of rats, the immunostaining of Fas and Fas-L in the uterus decreased compared to that in intact rats, however the strongest immunostaining of Fas and Fas-L was observed in untreated ovariectomized rats. The typical membrane staining of CD24 was found to decrease in the epithelial cells of the intact rats, while it could also be observed in the stroma following estrogen replacement of ovariectomized rats.

We observed estradiol-dependent differential expression of thymosin β4, CD24, FSRP, Thy-1 protein and FAST.

Thymosin β4 is a polar 5kDa peptide identified as a G-actin sequestering peptide with the capacity to inhibit actin polymerization. Estradiol can reduce both apical cell-cell contacts and basolateral cell-substrate adhesion, which is accompanied by rearrangement of F-actin and plakoglobin (DePasquale et al., 1994). This has been found to be associated with enhanced levels of thymosin β4 in human breast cancer cells (Otto et al., 2002).

The expression of the CD24 gene decreased following ovariectomy and increased after estradiol treatment. CD24 mediates intracellular signaling via a glycolipid-enriched membrane (GEM)-dependent mechanism associated with intracellular tyrosine kinase (Suzuki *et al.*, 2001), and is related to the process of apoptosis in Burkitt's lymphoma cells (Sammar et al., 1997). A possible relation between steroid hormones and CD24 was observed in the ER-positive cell lines (MCF7 and T47D) where CD24 was also over-expressed (Yang *et al.*, 1999).

FRSP can bind to activin A and members of the transforming growth factor β family (Tsuchida et al., 2000; Tanaka et al., 1998). Using an osteoblastic cell line (CDO7F), it has been demonstrated that FRSP is one of the estrogen-regulated genes (Ohashi et al., 1997).

Thy-1 protein is a major glycoprotein in rodent thymocytes and in adult neuronal cells in cell-cell interaction (Barclay, 1979; Campbell et al., 1979) and has later been shown to be expressed in a variety of tissues (Mansour et al., 1987). The detailed function of this protein in uterus requires further investigation.

The Fas-activated serine/threonine kinase (FAST) gene is a potent trigger of lymphocyte apoptosis, and is influenced by estrogen. FAST is a serine/threonine kinase that is activated during FAS-mediated apoptosis (Tian et al., 1995). It is reasonable to hypothesize that Fas and Fas-L may be important for hormonal regulation of the uterus considering that both Fas and Fas-L appear to be regulated by estrogen. Increasing evidence point towards an involvement of these two proteins in the regulation of uterine function (Yamashita *et al.*, 1999), leading to our investigation of their expression following ovariectomy and estradiol treatment. The differential protein expression of Fas and Fas-L in the rat uterus was similar to FAST expression, and also mimicked endometrial apoptosis occurring at the same time point and cellular localization. The predominant type of cell death observed in rabbit uterus under ovarian hormonal control is apoptosis (97.5%) as opposed to necrosis (2.5%) (Nawaz et al., 1987). The Fas signaling pathway has been suggested to be involved in necrotic cell death (Holler et al., 2000). In contrast to the results from the Fas-deficient lpr and lpr(cg) mice who demonstrated similar responses in the uterus as the intact mice, it is possible that the Fas/Fas-L pathway may mediate some estrogen actions in normal animals with some compensatory mechanisms developing in lpr mice. Another interpretation, supported by our data is that several mechanisms may co-exist, constituting estrogen regulation of the uterus.

The present study deals with several genes that are oppositely regulated by ovariectomy and estrogen treatment, and may constitute novel regulators of apoptosis and proliferation in the uterus. Further analyses of these genes may reveal central aspects regarding growth regulation in sex steroid regulated tissues.

CONCLUSIONS

The growth of uterine leiomyomas is believed to be dependent upon sex steroids, especially estrogen and progesterone. However, one should keep in mind that leiomyomas are individual tumors and that no clear pattern exists between levels of gene expression. This implies the importance of analyzing more than one leiomyoma from each patient whenever possible. It is also difficult to conclude exactly regarding which alterations that lead to a growth advantage of the leiomyomas when compared with myometrium. It is unlikely that only one or two growth factors or pathways are responsible for the enlargement of leiomyomas and it is reasonable to assume that other mechanisms than described here are also of importance.

- The different status of cell proliferation and apoptosis between leiomyomas and myometrium is closely related to the effects of estrogen and progesterone. The functions of estrogen and progesterone may be complementary, with estrogen priming a prerequisite for optimal stimulation of cell proliferation by progesterone.
- The expression of Bcl-2 in leiomyomas tends to be stronger than in myometrium both in the proliferative and secretory phases. It raises the question of a functional interaction between sex steroids and Bcl-2 in leiomyomas. The expression of Bax is shown to be significantly higher in leiomyomas than in myometrium only in the secretory phase of the menstrual cycle and significantly decreases after menopause.
- In both leiomyomas and myometrium there were significant differences in the level of PR and IGF-I mRNA and proteins, in the proliferative phase as compared to that in the respective tissue after GnRHa treatment. *In situ* hybridization showed that IGF-I mRNA in the myometrium and leiomyomas was located where the smooth muscle cells are dominant.
- Five candidate genes were found to have higher expression in leiomyomas than in myometrium assessed using the representational difference analysis (RDA) technique of cDNA. These genes are latent transforming growth factor binding protein 2 (LTBP2), zinc finger protein (ZFP 185), tomoregulin, cellular retinoid acid binding protein 1 (CRABP1) and pregnancy-associated plasma protein A (PAPPA), and may possibly have growth-promoting effects in leiomyomas.
- Using ovariectomized rat models with or without estrogen replacement, five genes were identified to be putatively estrogen-responsive in the uterus. These include Fas-activated serine/threonine kinase (FAST), CD24, thymosin β4, follistatin-related protein (FSRP) and thy-1 protein. We hypothesize that the Fas/Fas-L pathway and CD24 may be of importance during the estrogen influenced cellular processes in the uterus. Gene expression profiling provides a unique opportunity for understanding the molecular mechanisms of estrogen action in the uterus.

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ABSTRACT

Human uterine leiomyomas (fibroids) are benign neoplasms arising from uterine smooth muscle cells, and are suggested to be sex steroid dependent. The aim of this study was to investigate the molecular mechanisms by which sex steroids, mainly estrogen and progesterone, influence the growth of leiomyomas.

The first study investigated cell proliferation and apoptosis in both leiomyoma and myometrial cells during the menstrual cycle to determine if a growth advantage existed for leiomyomas. Leiomyoma cells had significantly higher mitotic activity than myometrial cells in the secretory phase compared with the proliferative phase of the menstrual cycle, suggesting that progesterone is important for cell proliferation in leiomyomas.

Apoptosis in leiomyomas is related to the function of Bcl-2 family proteins and was explored in the second study. The expression of Bcl-2 in leiomyomas was stronger than in myometrium both in the proliferative and secretory phases of the menstrual cycle. Bax immunostaining was also higher in leiomyomas than in myometrium and decreased after menopause, however the expression of other proteins in this family (Bak, Bcl-x and Mcl-1) was very diverse.

In the third study we investigated expression of the progesterone receptor (PR) and local growth factor insulin-like growth factor (IGF-I). There were significant differences in mRNA and protein expression of PR and IGF-I in both myometrium and leiomyomas proliferative phase tissue compared with tissue after gonadotropin releasing hormone agonist (GnRHa) treatment. *In situ* hybridization revealed that IGF-I mRNA in the myometrium and leiomyomas was located in areas where the smooth muscle cells were dominant.

Using representational difference analysis (RDA) of cDNA in the fourth study, we identified five candidate genes, latent transforming growth factor binding protein 2 (LTBP2), zinc finger protein (ZFP 185), tomoregulin, cellular retinoid acid binding protein 1 (CRABP1) and pregnancy-associated plasma protein A (PAPPA) with higher expression in leiomyomas than in myometrium, suggesting a potential role as growth promoters leiomyomas.

In the final study we analyzed the expression of approximately 3000 genes affected by estrogen in the rat uterus by using cDNA-microarray. Five interesting genes were putatively estrogen-responsive in the uterus, including Fas-activated serine/threonine kinase (FAST), CD24, thymosin β4, follistatin-related protein (FSRP) and thy-1 protein. We hypothesize that the Fas/Fas-L pathway and CD24 may be important during the cellular processes in the uterus influenced by estrogen. Gene expression profiling provides a unique opportunity for understanding the molecular mechanisms of estrogen actions in uterus.

In conclusion, there exists a selective growth advantage for leiomyomas involving a complex of factors regulated by the sex steroid hormones whose actions are very universe.

Key words: human uterine leiomyomas, myometrium, the menstrual cycle, estrogen, progesterone, cellular proliferation, apoptosis, oncogene, growth factor, growth factor receptor.

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