Progesterone Is Essential for Maintenance and Growth of Uterine Leiomyoma

Hiroshi Ishikawa, Kazutomo Ishi, Vanida Ann Serna, Rafael Kakazu, Serdar E. Bulun, and Takeshi Kurita

Division of Reproductive Biology Research, Feinberg School of Medicine at Northwestern University, Chicago, Illinois 60611

Uterine leiomyomata (ULs) represent the most common tumor in women and can cause abnormal uterine bleeding, large pelvic masses, and recurrent pregnancy loss. Although the dependency of UL growth on ovarian steroids is well established, the relative contributions of 17β-estradiol and progesterone are yet to be clarified. Conventionally, estradiol has been considered the primary stimulus for UL growth, and studies with cell culture and animal models support this concept. In contrast, no research model has clearly demonstrated a requirement of progesterone in UL growth despite accumulating clinical evidence for the essential role of progesterone in this tumor. To elucidate the functions of ovarian steroids in UL, we established a xenograft model reflecting characteristics of these tumors by grafting human UL tissue beneath the renal capsule of immunodeficient mice. Leiomyoma xenografts increased in size in response to estradiol plus progesterone through cell proliferation and volume increase in cellular and extracellular components. The xenograft growth induced by estradiol plus progesterone was blocked by the antiprogestin RU486. Furthermore, the volume of established UL xenografts decreased significantly after progesterone withdrawal. Surprisingly, treatment with estradiol alone neither increased nor maintained the tumor size. Although not mitogenic by itself, estradiol induced expression of progesterone receptor and supported progesterone action on leiomyoma xenografts. Taken together, our findings define that volume maintenance and growth of human UL are progesterone dependent. (Endocrinology 151: 2433–2442, 2010)
coadministration of P₄ (15). In vitro, progestin can show similar growth-promoting effects as estrogen on primary culture of human UL cells by inhibiting apoptosis and stimulating proliferation (10, 16, 17). However, progestin can also inhibit UL growth under certain culture conditions (18, 19).

In contrast to these research models, clinical studies have suggested that P₄ plays an equally important role as E₂ in the growth of ULs. Proliferation markers such as Ki67 and proliferating cell nuclear antigen (PCNA) are highest in UL in the luteal/secretory phase (20–22). Moreover, quantitative proliferation indices of ULs in postmenopausal women increased significantly with combined estrogen plus progestin replacement but not with estrogen replacement alone (20). These authors have even suggested that P₄ may be the primary hormone driving the growth of UL. However, systemic hormone-levels in patients cannot be manipulated experimentally, and thus, the effects of estrogen vs. progestin on UL growth cannot be delineated solely by analysis of clinical specimens.

In total, there remains significant conflict regarding the role of P₄ on human UL. Within human subjects, manipulation of systemic hormone levels beyond routine administration of estrogen/progesterone-containing medications is unethical, and thus, study findings are hindered by factors such as dosing and patient compliance. Furthermore, most UL-related procedures are scheduled within the first half of the menstrual cycle to avoid the possibility of pregnancy, thereby limiting the number of UL specimens obtained within the luteal/secretory phase. Thus, to advance research on UL, development of a novel model system that incorporates characteristics of original human tumors is essential.

In human ULs, cells are embedded in extracellular matrix (ECM), which is largely composed of collagens and glycosaminoglycans. ECM is known to influence several critical cellular functions, including apoptosis and cell proliferation (23, 24). Indeed, myometrial and UL cells alter their gene expression pattern significantly in the organotypic three-dimensional environment in vitro vs. an artificial, two-dimensional culture within plastic dishes (25, 26). In addition, accumulation of ECM contributes significantly to the growth of the tumor (27). These observations raise additional questions regarding the correlation between in vitro studies to human in situ tumors.

We report a method of xenografting and growing human ULs in immunodeficient mice. Xenografting into an immunodeficient mouse host is a standard approach in studying human tissues in vivo. Previously UL tissues with ECM were implanted under the skin of severe combined immunodeficiency mice (28). However, the UL xenografts were maintained only when their original phenotypes were altered by transduction of proangiogenic genes, PTGS2 (COX2) and VEGFA (28). These findings imply that the limiting factor for UL xenograft survival within subcutaneous (sc) tissue with low intrinsic vascular density was angiogenesis. Thus, we chose to use a subrenal capsule graft site because of its superior blood supply (29).

On the kidney of nontobese diabetic (NOD)-scid IL2Rγnull mice (30), the survival rate of UL and normal myometrial tissue xenografts was nearly 100%, and the xenografts retained the histological characteristics of original tissue. Using this model, the objective of this study was to address the effects of E₂ vs. P₄ on UL growth.

Materials and Methods

Subrenal grafting of myometrial and UL tissues

All procedures involving animals in this study were approved by Northwestern University’s Animal Care and Use Committee. Protocol for the acquisition of surgical specimens was approved by Northwestern University’s Institutional Review Board. Surgically removed UL and normal myometrial tissue from the same patient were cut into small pieces (~1 × 2 × 2 mm) and grafted onto opposing kidneys of adult female nonobese diabetic-scid IL2Rγnull mouse hosts (Jackson Laboratory, Bar Harbor, ME). The tissue pieces were high in water content, and thus, they became smaller under the pressure of subrenal capsule. The estimated starting volume of tissue grafts under the renal capsule was approximately 1 mm³. The growth of UL in intact female hosts without hormone treatment was minimum and inconsistent. Therefore, all hosts were ovariecotomized (OVX) and supplemented with sc implantation of 50 mg P₄ plus 50 µg E₂ 60-d slow-release pellets (Innovative Research of America Inc., Sarasota, Fl). Stable dosages of E₂ (50 µg per 60 d) and P₄ (50 mg per 60 d) were used throughout the study. These dosages were chosen because previous studies demonstrated that they were able to sustain systemic E₂ and P₄ levels within cycling women (31–35). Additionally, if required by the experimental design of the specific study, RU486, a progesterone receptor (PR) antagonist, was also given as sc implant (slow release pellet containing 25 mg per 60 d; Innovative Research of America). The effects of ovarectomy and hormone treatments were then confirmed by gross appearance and histology of host female reproductive tracts. The presence of hormone pellets was also confirmed at the time of termination of the host.

Preparation of cell graft

Fresh surgical specimens of human myometrial/UL tissues were digested into single cells by type I collagenase (Sigma, St. Louis, MO) and cultured for 2–3 d as previously described (36). Cells were collected from the culture plates by trypsin digestion and suspended into rat-tail collagen (type I) solution (BD Bioscience, San Jose, CA) at 10⁶ cells per 10 µl. The method has been successfully used to study hormonal response of human endometrial tissue in our previous report (37). The low-density collagen gel consisted mostly of water, and thus, the pellet volume (10 µl) did not reflect the starting volume of tumor. When cell pellets were incubated at 37°C overnight as floating-culture, they became smaller than 1 mm in diameter by contraction of collagen.
by UL cells. Therefore, the estimated starting volume of cell graft was smaller than 0.6 mm³.

Data analysis

The tumor volume was estimated using the formula: volume (cubic millimeters) = 0.52 (derived from π/6 × width × height (millimeters) (38). When tumor volume of the E₂ + P₄ group was less than 1.0 mm³ at 8 wk after grafting, the UL was categorized as growth negative. In the statistical analysis, average value (e.g. tumor volume, cell density, labeling index) of three to six xenografts per group in each experiment was considered as a single measurement. Only the experiments with the complete set of hormone treatment groups (e.g. OVX, E₂, P₄, E₂ + P₄, and E₂ + P₄ + RU486) were included in the analyses of tumor volume, Ki67 labeling, cell size index, and cell density.

Histological analysis

The following histological analyses were performed routinely for all xenografts and the original tissues: hematoxylin and eosin (H&E) staining, immunohistochemistry (IHC) for estrogen receptor (ER)-α (LabVision, Fremont, CA), PR (Dako, Carpinteria, CA), Ki67 (proliferation marker; Novocastra Laboratories, Burlingame, CA) and active caspase-3 (apoptosis marker; Cell Signaling, Danvers, MA) (37, 39). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed using Apo-Brd UTM DNA fragmentation assay kit (BioVision, Mountain View, CA). The cellular component was detected by IHC for β-actin (Abcam, Cambridge, MA). The cell density was determined on H&E sections by counting the number of nuclei per optical field with a ×20 objective lens. Morphometric analysis of immunohistochemical staining has been described previously (39). Images of β-actin-stained tissues were captured with a Leica microscope imaging system (Leica Microsystems Inc., Bannockburn, IL). Total and β-actin-positive tissue areas were measured with ImageJ (National Institutes of Health, http://rsbweb.nih.gov/ij/index.html). The area with OD higher than 180 [from 255 (white) to 0 (black)] in blue channel of RGB mode was considered negative. Relative cell size was calculated by β-actin-positive area per cell number and expressed as relative value to that of 2 wk after grafting.

Results

Growth of UL tissue in mouse hosts

All myometrial grafts (74 grafts) survived for 8 wk but did not show detectable growth with any hormone treatments (Fig. 1), indicating the presence of an intrinsic growth-suppressive mechanism within normal adult myometrium. Due to small tissue size, further analysis was difficult for myometrial xenografts. Thus, we focused on UL in the following analyses. In contrast, UL xenografts increased their size in response to E₂ + P₄ but not to either E₂ or P₄ alone. Among a total of 29 UL cases, xenografts of 14 cases (48.2%) increased in size in response to E₂ + P₄ treatment (growth-positive UL), whereas there was no detectable growth (tumor size <1 mm³) in the other 15 cases (growth-negative UL). The most noticeable difference between growth-positive and -negative ULs was the cell density in the original patient tissue. Specifically, growth-positive xenografts arose from patient tissue in which the original cell density was significantly higher (831 ± 113 cells/area, n = 12), whereas growth-negative UL cases demonstrated fewer cells within the original patient tissue (425 ± 155 cells/area, n = 12) (Student’s t test, P < 0.05). Thus, the number of cells/xenograft significantly affected growth of UL xenografts. This conclusion was further confirmed by cell-grafting experiments in the following section. Because the absence of growth was simply due to the quality of the original tissue, we excluded the growth-negative UL cases from the following analyses.

Tumor volume was significantly higher in the E₂ + P₄ group than the other four groups (Fig. 2, A and B). The growth-promoting effect of E₂ + P₄ was completely blocked by coadministration of RU486, a PR antagonist (Fig. 2). Therefore, P₄ induced tumor growth via PR. There was no significant difference in the tumor volume (P < 0.05) between E₂ + P₄ + RU486, E₂ alone, P₄ alone, and untreated control groups, confirming the requirement of both E₂ and P₄ for human UL growth. In fact, E₂ alone failed to increase tumor size, even when a supraphysiological dose was administered (~10 µg/d) (n = 4).

The Ki67 index was significantly higher (Fig. 2C) in the E₂ + P₄ group than the other four groups, indicating that E₂ + P₄-stimulated tumor growth via proliferation of UL cells. At the same time, cell density was significantly lower in the E₂ + P₄-treated group (Fig. 2D), suggesting that an increase in ECM volume and/or cell size also contributed to the enlargement of tumor. This issue was further assessed in the following section.
As assessed by IHC, ERα was expressed in the xenografts of all five groups (Fig. 3). In contrast, expression of PR in UL xenografts was totally dependent on E2. The level of PR was very low to undetectable in the untreated-control (OVX) and P4 groups but high in the E2, E2/P4 and E2/P4/RU486 groups (Fig. 3). These results indicate that E2 is essential for P4 to act on ULs in vivo.

Growth of established tumors

In our xenograft model, tissue grafts must first establish a blood vessel network to survive and subsequently grow. Although the kidney is highly vascular and thus capable of supporting survival of tissues grafted onto it, efficiency in blood vessel recruitment can be a limiting factor for subsequent growth of xenograft (29). Therefore, lack of growth within xenografts treated with E2 alone may be explained by a requirement of P4 for UL xenografts to sufficiently establish an adequate vascular network. Thus, E2 may still be a mitogen for established ULs. To examine this possibility, xenografts were established first in the hosts with the optimum hormone treatment (E2+P4) and then subjected to different hormone treatments. All hosts were ovariectomized and supplemented with E2 and P4 at the time of grafting. Two weeks later, to allow establishment of vascularization, the hosts were divided into four groups, and hormone pellets were changed as indicated (Fig. 4A). After the hormone pellet change, established UL xenografts increased their size further only with E2+P4 treatment (Fig. 4B). In contrast, E2 or P4 alone failed to maintain tumor size. All xenografts of groups in which E2 and/or P4 was withdrawn exhibited significant reduction in tumor volume and Ki67 labeling index (Figs. 4 and 5). These results confirm the essential role of E2 and P4 in growth and maintenance of ULs. All xenografts were negative for apoptotic cells as assessed by active caspase-3 IHC and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Absence of apoptotic cells at 8 wk after hormone withdrawal (data not shown) does not necessarily mean that UL cells do not die via apoptosis during regression of tumor. Nevertheless, UL cells appeared healthy and showed no signs of tissue degradation as shown in Fig. 5. Thus, loss of cells via apoptosis does not appear to play a major role in tumor regression. Instead, UL cells reduced their size significantly after withdrawal of E2 and/or P4 (Fig. 4D). Accordingly, cell density increased significantly in these groups (Figs. 4E and 5). This result indicates that cell size reduction is one of the mechanisms via which UL shrinks.

In the E2+P4-treated group, cell size remained the same (Fig. 4D), but the cell density significantly decreased from 2 to 10 wk after grafting (Fig. 4E), suggesting an enlargement of ECM. Indeed, β-actin-negative areas significantly increased from 2 to 10 wk (Fig. 4F). Therefore, E2 and P4 stimulate UL growth via increase in cell number and ECM volume. Ki67 was coexpressed with ERα and PR (Supplemental Fig. 1 published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org), suggesting that Ki67-positive cells were the direct target of E2 and P4. As tumor volume increased, the Ki67 labeling index decreased significantly from 9.4 ± 3.4% at 2 wk to 3.1 ± 1.6% at 10 wk (mean ± SD) (Fig. 4C). This result indicates that the peak for cell proliferation is an early event in the UL growth, whereas increase of ECM appears to occur at a constant rate.
Although it was not statistically significant, cell size appeared smaller (Fig. 4D) and ECM volume appeared higher (Fig. 4E) in the P₄-alone group than control and E₂-alone groups (Fig. 5). It suggests that P₄ may have some effects on UL cell, even in the absence of E₂.

Reconstruction of UL tissues from cultured UL cells

Because of the significant heterogeneity that exists within human tissue, comparison between subjects is difficult. Factors including cell density of the original surgical specimen and the distribution of UL cells within the sampled portion of the tumor can affect growth of the xenograft. To address this issue, tumor tissue was digested into single cells, and UL tissue was reconstructed by suspending the isolated UL cells in type I collagen. After 8 wk of in vivo growth with E₂ + P₄ treatment, UL cell grafts containing 10⁶ cells formed uniform tissues with histology comparable with the original tumor (Fig. 6). For efficient tumor growth, a concentration of 10⁶ cells/graft was the optimum. With lower cell concentrations (5.0 × 10⁵ cell/graft and lower), growth of xenografts was inconsistent.

With higher cell concentration (5.0 × 10⁶ UL cells/graft), the collagen gel became too soft to graft. To test whether normal myometrium was able to grow as cell grafts, the same cell-graft protocol was applied to the normal human myometrium. Xenografts of reconstructed myometrium (10⁶ cells/graft) formed a tissue with typical histology of myometrium, but graft volume did not increase with E₂, P₄, or E₂ + P₄ treatment for 8 wk (Fig. 1, n = 12).

Previous studies demonstrated that UL cells alter gene expression profile in vitro and lose expression of ERα and PR rapidly (26). Therefore, expression of these receptors in cell grafts was assessed by IHC. As in the tissue xeno-
grafts, ER\textalpha was expressed in all xenografts of UL cells, whereas PR was expressed only in the presence of E2 (Fig. 6). The growth response of UL cell grafts to E2 and P4 was identical with that of tissue grafts. The growth of UL cell grafts was stimulated by E2 and P4 and inhibited by RU486 (Fig. 7). Moreover, E2 and P4 treatment increased Ki67 labeling index (Fig. 7B) and reduced cell density significantly (Fig. 7C).

The efficacy of the cell graft method was experimentally tested by comparing growth of the UL cell graft vs. that of the UL tissue xenograft in tissue/cells obtained from the same subject. Among three UL cases, two cases with relatively low cell density (cases 128 and 135) did not show detectable growth in response to E2 and/or P4 as tissue xenografts (Table 1). However, when cell grafts were generated from these same UL tissues, the volume of all three cases increased significantly in response to E2 and P4 treatment (Table 1 and Fig. 7). The overall rate of growth-positive tumors in the entire experiment improved from 48.2% (14 of 29) in tissue grafts to 76.4% (13 of 17) in cell grafts. This result further confirmed that the absence of growth as tissue xenograft was mostly due to the low cell number and/or thick ECM layer in the original tissues from which the tissue xenografts were generated. The growth-positive and -negative ULs do not represent two different entities with different hormone requirement. Our study suggests that all human ULs require E2 and P4 for growth and volume maintenance.

**Discussion**

There has been conflicting evidence for the role of P4 in the regulation of UL growth. This is the first study to definitively demonstrate that both growth and maintenance of human UL in vivo are dependent on P4 action via PR. Our conclusion agrees with the results of recent clinical trials of selective progesterone receptor modulators for ULs (40–44). This study also defined for the first time that estrogen by itself is not a mitogen for human UL in vivo. Nonetheless, our findings do not discount the importance of estrogen in UL. PR is widely recognized as a marker for estrogen action, and regulation of the PR gene (PGR) by estrogen/ER has been well defined (45). Because the PR protein level is thought to be a critical determinant of sensitivity to P4, almost all hormone treatment protocols designed to elicit effects of P4 involve priming with estrogen. This was also true for human UL in vivo, and the expression of PR was dependent on E2 in UL xenografts. Based on the results of the current study, we propose the following model of UL growth control. P4 action via PR increases tumor volume through cell proliferation and ECM accumulation. E2 is not a mitogen but is required for the growth and maintenance of ULs to sensitize cells to P4 by inducing PR. This model is currently being tested by whether PR overexpression replaces E2 action in P4-dependent UL growth.

Because coadministration of E2 with P4 was essential for growth and maintenance, inhibition of ER should also be an effective treatment for UL. However, efficacy of selective ER modulators in clinical trials for UL has been inconsistent (46). This may be due to the technical difficulty in blockade of ER signaling in vivo (47). In addition, our study suggests that E2 is required only for up-regulation of PR, which should be achieved with a relatively low...
level of E₂. In reality, effective blockade of E₂/ER signaling is difficult to achieve and should cause severe adverse side effects such as hot flashes and osteoporosis. Hence, the systemic inhibition of E₂/ER by antiestrogens may not be a practical option for treatment of symptomatic ULs.

Previously clinical observations associated with pregnancy have raised questions about the growth-promoting effect of P₄ on human UL. The major criticism is a lack of conclusive evidence for the enlargement of UL during pregnancy despite the elevated systemic P₄ level (48–50). Furthermore, epidemiological evidence indicates that parity is protective, rather than promoting, for UL development (51, 52). However, there are several reasons these findings may not necessarily reflect the role of P₄ on ULs. First, the majority of longitudinal studies examining the natural history of UL in pregnancy enrolled patients that were found to have UL on their obstetrical ultrasounds after pregnancy was well established. It is possible that early exposure to elevated P₄ levels induced UL growth that was not detected given the timing of enrollment of the subjects. This is supported by the fact that when ULs do increase in size during pregnancy, the majority of growth occurs by the 10th week of gestation (49). Furthermore, pregnancy-associated stretching and hypertrophy of myometrium may affect tumor volume. Lastly, pregnancy physiology affects multiple systems including immunological and vascular systems within the uterus (53). In total, multiple factors likely contribute to UL size in pregnancy, and the phenotype of UL in pregnancy is not necessarily representative of increased systemic E₂ and P₄. Indeed, data on miscarriage or induced abortion suggest that the protective effects of parity for UL may be associated with an event at delivery or during the postpartum process (52, 54–56). Therefore, the absence of detectable growth in ULs during pregnancy does not necessarily contradict with our findings of UL P₄ dependency.

Our study also clearly demonstrated that E₂ was not mitogenic for human ULs in vivo. In contrast, studies with cell culture and animal models repeats
edly demonstrated mitogenic effects of estrogens on UL cells (4–14). Therefore, estrogen/ER signal transduction in cell culture and animal models should significantly differ from that in human UL in vivo. In the case of UL cell culture, the reduced levels of ERα and PR may be the reason for the aberrant actions of E2 and P4 in vitro (26). The difference in hormone responsiveness between human and rodent leiomyoma may reflect differences in the intrinsic growth control mechanism of human and rodent myometrial cells. Previously we demonstrated fundamental differences of human vs. mouse endometrial epithelial cells in the estrogen-regulated proliferation by xenograft experiments (37). Considering fundamental differences in cellular kinetics of menstrual vs. estrous cycles and the duration of cycles, growth control of uterine cells should be significantly different in human vs. rodent. Rodent UL models may have some limitation in the studies of growth control of human UL.

Within the clinical realm, tumor growth can be assessed only by gross volume change. However, the volume of UL reflects multiple factors such as cell number/size, ECM, and water content. Therefore, tumor growth is not necessarily due to the proliferation of UL cells. To envision therapeutic strategies, it is essential to understand how ovarian steroids regulate the tumor volume. For example, if tumors grow solely by accumulation of ECM, inhibition of cell proliferation should not be an effective treatment. In such a case, inhibition of PR is only a temporary suppression of tumor and not a cure for UL. It agrees with the clinical observation that regrowth of shrunken UL occurs slowly after cessation of RU486 treatment for premenopausal woman (57).

To improve the efficiency of UL xenograft growth, we developed the cell graft system in which UL tissues were reconstructed with dissociated single UL cells. The phenotype and hormone responsiveness of cell grafts were identical with those of tissue xenografts. The advantages of cell graft over tissue graft were reproducibility of cell number in each xenograft and improved success rates in xenograft growth. In addition, transgenic human ULs can be generated from UL cells transduced with genes of interest. In total, the cell graft model is a novel and relevant tool for basic research on UL growth. The knowledge gained from these studies is critical if effective treatments are to be discovered.

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Address all correspondence and requests for reprints to: Takeshi Kurita, Ph.D., Division of Reproductive Biology Research, Northwestern University Feinberg School of Medicine, Department of Obstetrics and Gynecology, 4th Floor, Suite 4-127, 303 East Superior Street, Chicago, Illinois 60611. E-mail: t-kurita@northwestern.edu.

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References


TABLE 1. Efficacy comparison between tissue and cell grafts

<table>
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<tr>
<th>Case no.</th>
<th>Age (yr)</th>
<th>Phase</th>
<th>Nodule size (cm)</th>
<th>Cell density</th>
<th>Xenograft size (mm³)</th>
<th>Tissue graft</th>
<th>Cell graft</th>
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<td>128</td>
<td>31</td>
<td>Follicular</td>
<td>12 x 12 x 10</td>
<td>513</td>
<td>Growth negative (&lt;0.5)</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>130</td>
<td>34</td>
<td>Follicular</td>
<td>8 x 6 x 6</td>
<td>901</td>
<td>4.3</td>
<td>9.6</td>
<td></td>
</tr>
<tr>
<td>135</td>
<td>37</td>
<td>Luteal</td>
<td>8 x 8 x 6</td>
<td>373</td>
<td>Growth negative (&lt;0.5)</td>
<td>1.5</td>
<td></td>
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