CLINICAL ARTICLE

Estrogen receptor α and β in uterine fibroids: a basis for altered estrogen responsiveness

Panagiotis Bakas, M.D.,[a] *Angelos Liapis, M.D.,*[a] *Spiros Vlahopoulos, Ph.D.,*[b] *Maria Giner, M.D.,*[a] *Stella Logotheti, B.Sc.,*[b] *Georgios Creatsas, M.D.,*[a] *Aggeliki K. Meligova, B.Sc.,*[c] *Michael N. Alexis, Ph.D.,*[b,c] *and Vassilis Zoumpourlis, Ph.D.*[b]

[a] 2nd Department of Obstetrics and Gynaecology, Aretaieio Hospital, University of Athens; [b] Biomedical Application Unit; and [c] Molecular Endocrinology Programme, Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, Athens, Greece

Objective: To investigate the relative expression and the DNA-binding status of estrogen receptors α and β in fibroids and normal myometrial tissue to explore the molecular basis of altered estrogen responsiveness of leiomyomas.
Design: Biopsy samples from uterine fibroids and adjacent normal myometrial tissue at the follicular phase of the menstrual cycle.
Setting: Aretaieio University Hospital and the National Hellenic Research Foundation, Athens, Greece.
Patient(s): Thirty-five patients who underwent hysterectomy or myomectomy because of myoma symptoms.
Intervention(s): None.
Main Outcome Measure(s): Deoxyribonucleic acid–binding status of estrogen receptors α and β.
Result(s): The level of messenger RNA expression of estrogen receptor α and β and the level of estrogen receptor as a whole are increased on average to a similar extent in leiomyomas compared with normal myometrium. Occasionally, however, estrogen receptor α is disproportionately increased in leiomyomas, and this appears to increase the amount of estrogen receptor α that binds to the estrogen-responsive element of estrogen target genes as homodimer rather than as heterodimer with estrogen receptor β.
Conclusion(s): The estrogen receptor α–to–estrogen receptor β expression ratio rather than the individual expression levels determines the fraction of DNA-binding homodimers of estrogen receptor α and possibly the growth potential of myomas. (Fertil Steril® 2008;■:■–■. ©2008 by American Society for Reproductive Medicine.)

Key Words: Uterine myomas, estrogen receptor alpha, estrogen receptor beta, myometrium, fibroids

Uterine leiomyoma (also known as myoma or fibroid) is the most common benign gynecologic tumor and is present in at least 20% of women at reproductive age and in 40% to 50% of women older than 40 years of age (1). It is estimated that <50% of uterine myomas produce symptoms, and they are usually discovered by clinical examination. Although the etiology of uterine fibroids is unknown, their development is considered to be estrogen dependent, because they have the ability to enlarge during pregnancy and to shrink during menopause, ovariectomy, and other hypoestrogenic conditions (2–4). Aromatase P450 is often overexpressed in myomas, causing in situ synthesis of estrogen to increase, and this is believed to contribute to the growth of leiomyomas (5). Recent studies therefore continue to focus on the role of estrogen receptor (ER) in the pathogenesis of leiomyomas (6).

Estrogens act mainly through two ER subtypes, ERα and ERβ, which function as ligand-dependent regulators of transcription in a manner that depends on the selection of cognate cofactors provided by the cell and the structure of the ER target gene promoter, as well as that of the ligand (7–9). The two forms of ER bind to DNA enhancer elements (estrogen-responsive elements [EREs]) or to other transcription factors (e.g., Activator Protein 1 [AP1]) in the promoter of ER target genes as a heterodimer, as well as homodimers (10, 11). Estrogen receptor α and ERβ exhibit significant homology in the DNA-binding domain and the ligand-binding domain, where receptor dimerization functions are known to reside. However, the ligand-binding specificity and the transcriptional activity of the two forms of ER are known to differ substantially (8, 9). In fact, it has been reported that ERα and ERβ have opposite effects on AP1 sites and that they probably have different roles as regards regulation of AP1-dependent genes, including genes involved in the control of cell growth and viability (12). In the mammary gland, ERα and ERβ are pivotal for tissue development and terminal differentiation, respectively (13, 14). In line with

Received May 22, 2007; revised and accepted September 10, 2007.
Supported in part by grant 03E⊿644 of G.S.R.T.-Greece to M.N.A.
Reprint requests: Vassilis Zoumpourlis, Ph.D., National Hellenic Research Foundation, 48 Vas. Constantinou Ave, 116 35 Athens, Greece (FAX: 30-210-7273677; E-mail: vzub@eie.gr).

0015-0282/08/$34.00
doi:10.1016/j.fertnstert.2007.09.019

Copyright ©2008 American Society for Reproductive Medicine, Published by Elsevier Inc.

this, 17β-E_2 (hereafter referred to as E_2) has been reported to promote the growth of immortalized mammary cells through ERα and inhibit it through ERβ (15). In mammary hyperplasia of the usual type, it is the ERα-to-ERβ ratio rather than the individual receptor amounts that reportedly is associated with the risk that this lesion will develop into invasive carcinoma (16). Work with ERα-positive breast cancer cells engineered to express increasing amounts of ERβ has shown that, although ERβ could regulate genes that are not targeted by ERα alone, it increasingly affected genes regulated by ERα as well, although nearly half of ERα target genes were not affected even when the level of ERβ was set to be much higher than that of ERα (17). Notably, however, key genes involved in the hormonal control of cell proliferation and apoptosis were among those of ERα target genes that were affected by ERβ, implying that the ERα-to-ERβ expression ratio could influence tumor cell growth and consequently breast cancer prognosis and treatment as well (17).

Although endothelial and connective tissue cells of leiomyomas and normal myometrium express only ERβ, smooth muscle cells express both ER subtypes (18). Whether and how the ERα-to-ERβ expression ratio could influence the estrogen-dependent development of leiomyomas is not known presently. Several studies have demonstrated that the level of expression of ERβ messenger RNA (mRNA) in both leiomyomas and normal myometrium is lower than that of ERα (19–22). However, there is conflicting evidence as regards the level of ERα mRNA in the leiomyomas compared with the adjacent myometrium, with some studies reporting an increase in the diseased tissue compared with normal (19) and others reporting no change (20, 22). There is also conflicting evidence as regards the relative abundance of the two forms of ER in the fibroids and how this could impact fibroid response to hormonal therapy, because some studies report that the ERα-to-ERβ ratio is higher in the diseased tissue compared with normal (22), whereas others report an increase in the ERα-to-ERβ ratio after treatment with a GnRH analogue (21). In addition, it has been reported that the levels of ERα and ERβ in myometrial cells change during the menstrual cycle and that the patterns of changes are similar (4). The present study aims at estimating mRNA and protein levels as compared with the DNA-binding activities of two ER subtypes in leiomyomas and normal myometrium during the follicular phase of the menstrual cycle.

MATERIALS AND METHODS

Patient Selection and Tissue Collection

Biopsy samples of uterine leiomyomas and adjacent myometrium were taken from 35 women at the follicular phase of the menstrual cycle (between day 5 and day 9). Dating of endometrium was performed with use of Noyes criteria (23). The criterion for inclusion in the study was pending operation for hysterectomy or myomectomy because of subfertility, menorrhagia, or other bothering myoma symptoms. Criteria for exclusion from the study were adenomyosis, malignancy, or hormonal therapy given within 3 months before operation. Selected patients were subjected to a complete preoperative workup to exclude any other known possible cause of their problem. All specimens originated from 2nd Department of Obstetrics and Gynaecology, Aretaieio Hospital, School of Medicine, University of Athens, and were examined by the same histopathologists. The study took place with the permission of the local ethics committee. All patients gave informed consent for participating in the study.

Ribonucleic Acid Quantification

Ribonucleic acid extraction Total RNA was isolated from normal and pathologic (leiomyoma) myometrial samples with use of TRIzol (GIBCO BRL, Grand Island, NY) according to the manufacturer's instructions, and RNA concentration of the samples was measured with use of a U-2000 spectrophotometer (Hitachi, Tokyo, Japan).

Complementary DNA synthesis Messenger RNA transcription into complementary DNA (cDNA) was performed with use of 2 μg of total RNA and SuperScript ribonuclease (RNase) H–reverse transcriptase (Invitrogen, Carlsbad, CA), according to manufacturer's instructions.

Polymerase chain reaction Estrogen receptor α and ERβ mRNA levels were assessed with use of semiquantitative multiplex reverse transcriptase–polymerase chain reaction (RT-PCR) as previously described (24). Estrogen receptor α and ERβ cDNA fragments were coamplified with a larger reference cDNA fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (25). The ratio of the relative amounts of the amplified products in myoma samples is taken to reflect the relative amounts of ERα and ERβ mRNAs and was compared with the relative amounts in the corresponding myometrium. A 441–base pair (bp) fragment of ERα cDNA was amplified with use of primers (forward: 5′-TGC CAA GGA GAC TCG CTA CTG-3′, reverse: 5′-GGG GGC TCA GCA TCC AAC AAG-3′) corresponding to bases 896–916 and 1316–1336 of the published sequence (GenBank accession number NM 000125). A 268-bp fragment of ERβ cDNA was amplified with use of primers (forward: 5′-CGA TGC TTT GGT TTG GGT GAT-3′, reverse: 5′-GCC CTC TTT GCT TTT ACT GTC-3′) corresponding to bases 1400–1420 and 1648–1667 of the published sequence (GenBank accession number AB006590). Oligonucleotide primers and Taq polymerase were purchased from Invitrogen. Polymerase chain reaction conditions for ERα were 94°C for 5 minutes, followed by 25 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 74°C for 30 seconds, and a final step of 74°C for 10 minutes. Polymerase chain reaction conditions for ERβ were 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and a final step of 72°C for 10 minutes. The RT-PCR reaction products were analyzed by polyacrylamide gel electrophoresis (PAGE), stained with ethidium bromide, and quantified with use of a Crossfield 5400/Rip/

Laser/CMYK Densitometer (Crossfield/PS Electronic Services, Shefford Beds, UK). Normalized levels of ERα and ERβ mRNA were obtained by dividing the corresponding RT-PCR reaction product densities by the respective GAPDH product densities.

Protein Preparation and Analysis

Preparation of cytosol and nuclear extracts Frozen leiomyoma and myometrium samples were homogenized in ice-cold TEM (10 mmol/L tris[hydroxymethyl]aminomethane [Tris] pH 7.4, 1 mmol/L ethylenediaminetetraacetic acid [EDTA], 2 mmol/L Na_2MO_4, and 10% glycerol) (Sigma, St. Louis, Mo)in a tissue weight–to–buffer volume ratio of 1:4 (grams per milliliter) with use of an Ultra-Turrax T25 homogenizer (Fischer Scientific, Schwerte, Germany). The homogenate was centrifuged initially at 1,000 $\times$ g for 10 minutes, and the resulting supernatant was centrifuged at 10,000 $\times$ g for 60 minutes. The resulting cytosol (supernatant) was used to assay ER by immunoprecipitation-PAGE-immunoblotting and ligand binding. The protein concentration of the cytosol was measured by the Bradford method (26).

For the preparation of the nuclear extracts, fine slices of the leiomyoma and myometrium samples were homogenized in ice-cold TSM (25 mmol/L Tris pH 7.5, 5 mmol/L KCl, 0.5 mmol/L $MgCl_2$, 0.5 mmol/L dithiothreitol [DTT], 0.5 mmol/L phenylmethylsulfonyl fluoride [PMSF]) with use of a Teflon-glass homogenizer (Thomas, Philadelphia, PA). The nuclear fraction was pelleted, washed thoroughly with isotonic buffer (25 mmol/L Tris pH 7.5, 5 mmol/L KCl, 0.5 mmol/L $MgCl_2$, 0.5 mmol/L DTT, 1 mmol/L PMSF, and 0.2 mmol/L sucrose), and lysed with TET (25 mmol/L Tris pH 7.5, 1 mmol/L EDTA, 0.1% Triton, 0.5 mmol/L DTT, 0.5 mmol/L PMSF). Nuclear debris was removed by centrifugation at 55,000 $\times$ g for 1 hour at 4°C. The protein concentration of the resulting nuclear extract (supernatant) was measured by the method of Bradford (26).

Immunoprecipitation, PAGE, and immunoblotting Immunoprecipitation was carried out with use of 40 μg of protein A–Sepharose 6MB (Sigma, St. Louis, MO), mixed with 0.2 μg of the C-311 antibody against ERα (Santa Cruz Biotechnology, Santa Cruz, CA) in nondenaturing immunoprecipitation buffer (0.05 mol/L Tris pH 7.9, 1 mmol/L DTT, 0.1 mol/L KCl, 0.1% Nonidet P-40, 20% glycerol) (Sigma), and incubated for 2 hours at 4°C under rotary shaking. The resin with the bound antibody was collected by centrifugation (1,500 $\times$ g, 1 minute) and washed three times for 5 minutes with 1 mL of ice-cold immunoprecipitation buffer; 0.5 mg of nuclear extract protein was added, and the mixture was further incubated for 4 hours at 4°C under rotary shaking. The resin was then washed and collected by centrifugation as above. The pellet was resuspended in 2$\times$ sodium dodecyl sulfate (SDS)–PAGE sample buffer and subjected to SDS-PAGE and immunoblotting analysis, as already described (27). Immunostaining was developed by using ECLplus (Amersham Biosciences, Piscataway, NJ) and quantified by using a Storm 860 phosphorimager (Molecular Dynamics , Sunnyvale, CA).

Estrogen receptor assessment by ligand binding The assessment of ER in the cytosol of leiomyomas and adjacent myometrium was carried out with use of standard Scatchard plot analysis as previously described (28). In brief, aliquots (150 μL; 400 μg protein) of cytosol were incubated overnight at 4°C with increasing concentrations (0.1–8 nmol/L in 50 μL TEM) of tritiated 17-[2, 4, 6, 7-3H] 17β-E_2 (95 Ci/mmol; NEN) and with (50 μL of) a 1,000-fold molar excess of diethylstilbestrol in TEM (Sigma), or with (50 μL of) a 200-fold molar excess of the ERα-selective ligand propyl pyrazole triol (PPT) (29) in TEM, or with (50 μL of) TEM alone. After incubation, the samples were mixed with a dextran-coated charcoal pellet (prepared from 50 μL of 10% Norit A preincubated with 0.1% dextran T70; USB Corp., Clevelana, OH) for 20 minutes at 4°C to remove free tritiated E_2, Norit A–dextran T70 was pelleted by centrifugation at 1,500 $\times$ g for 10 minutes, and 200 μL of clear supernatant was removed and counted with use of 4.2 mL of Ultima-gold XR scintillation fluid (PerkinElmer, Waltham, MA) and a Wallac 1409 DSA scintillation counter (Wallac/PerkinElmer, Waltham, MA). The levels of total ER and ERα were obtained from the difference in tritiated E_2 binding in the absence and presence of diethylstilbestrol and PPT, respectively, and were expressed as femtomoles (of specifically bound tritiated E_2) per milligram of protein. The protein concentration was measured by the Bradford method (26).

Electrophoretic mobility shift assay Electrophoretic mobility shift assay (EMSA) was performed with use of the ERE of the *Xenopus* vitellogenin A2 gene (vitERE), as reconstituted by annealing of the following oligonucleotides: 5′-TCAAAGTCAGGTCACAGTGACCTGATCAAAGA-3′ and 5′-TCTTTGATCAGGTCACTGTGACCTGACTTTG A-3′. The reconstituted vitERE was end-labeled with γ-^{32}P-adenosine triphosphate (ATP) with use of T4 polynucleotide kinase, and the labeling reaction products were purified by PAGE with use of an 8% polyacrylamide gel. Protein DNA binding reactions were carried out by mixing 2,000 cpm of γ-^{32}P-labeled oligonucleotide with 20 μg of nuclear extract protein in binding buffer (50 mmol/L HEPES pH 8.0, 500 mmol/L NaCl, 0.5 mol/L PMSF, 0.5 mg/mL bovine serum albumin, 1 mmol/L EDTA, 20% glycerol) plus 1 mmol/L DTT and 150 μg/mL poly(2′-deoxyinosic-2′-deoxycytidilic acid) sodium salt (poly(dI-dC)) (Sigma). The reaction mixture was left at room temperature for 30 minutes, and the samples were subsequently subjected to electrophoresis on a 6% polyacrylamide gel at 150 V for 90 minutes, dried, and visualized by autoradiography. For the supershift assay, the reaction mixture was incubated with antibodies to ERα or ERβ (Santa Cruz Biotechnology) for 30 minutes at 4°C.

Statistical Analysis

Statistical analysis was performed with use of MedCalc software version 7.6.0.0 (MedCalc Software, Mariakerke, Belgium). The levels for ERα and ERβ mRNA relative to

GAPDH mRNA in leiomyomas and the corresponding normal myometrium were compared with use of the paired samples *t*-test (and confirmed by the Wilcoxon paired samples test, i.e., the nonparametric equivalent of the paired samples *t*-test). For comparing the ER status of leiomyomas and normal myometrium, the nonparametric Wilcoxon paired samples test was used, because the data were not normally distributed, and results were plotted with use of a box-and-whisker graph.

RESULTS

Patient Characteristics

Biopsy samples of uterine leiomyomas and adjacent myometrium during the follicular phase of their cycle from 35 women who underwent myomectomy or total abdominal hysterectomy for uterine fibroids were included in this study. The mean age of the patients was 45.7 ± 2.9 years (95% confidence interval 44.7–46.7 years), the mean body mass index was 28.1 ± 1.3 kg/m^2 (95% confidence interval 27.7–28.6 kg/m^2), and mean parity was 1.9 ± 0.7 (95% confidence interval 1.7–2.2).

Estrogen Receptor Status of Leiomyomas and Normal Myometrium

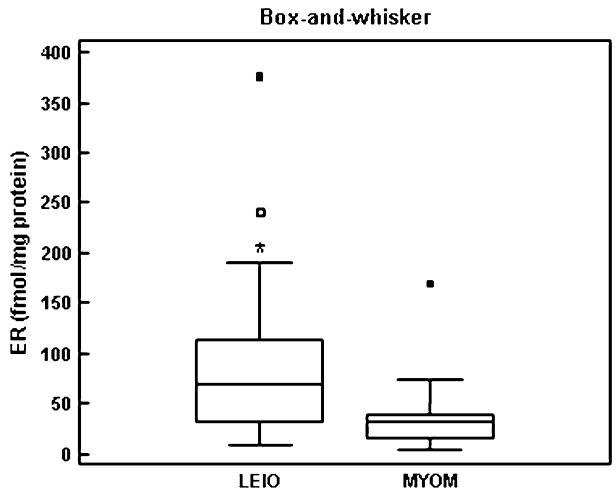
Initially we studied tritiated E_2 binding of the ER in the cytosol of leiomyomas and the adjacent myometrium with use of Scatchard plot analysis. We found that the level of ER in the cytosol of leiomyomas (median level, 67 fmol/mg protein) was significantly higher (Wilcoxon paired samples test; $P<.002$) compared with that of myometrium (median level, 30 fmol/mg protein) (Fig. 1). Then we tried to use PPT, which reportedly exhibits a relative (to E_2) binding affinity for ERα (RBAα) that is much higher than that (RBAβ) for ERβ (29), to estimate the fraction of E_2 binding of ER that could be attributed to ERα alone, with inconsistent results. The reason for this could be that the ERα-to-ERβ binding selectivity of PPT, as determined with use of purified ERα and ERβ and a fluorescence polarization assay previously described (27, 30), was found not higher than 100 (RBAα = 50.2 ± 2.4, RBAβ = 1.4 ± 0.3, RBAα/RBAβ = 36; RBAα and RBAβ of E_2 are set equal to 100). This caused substantial inhibition of E_2 binding of purified ERβ by PPT at concentrations of the ligand capable of inhibiting E_2 binding of purified ERα only partially (data not shown), which rendered the estimation of the fraction of ERα in mixtures with ERβ with use of PPT fairly inaccurate.

Estrogen Receptor α and ERβ Gene Expression of Leiomyomas and Normal Myometrium

Next we examined whether the higher level of ER in the myoma cytosol was due to an increase in the level of gene expression of ERα, ERβ, or both. We therefore determined the levels of mRNA of ERα and ERβ in the myomas and the adjacent myometrium using semiquantitative multiplex RT-PCR. To account for differences during the reverse transcription (RT) step, GAPDH cDNA was also amplified and used as a reference standard in all the samples (Fig. 2). Unlike the levels of cytosolic ER we determined by tritiated E_2 binding, the levels of mRNA of ERα and ERβ relative to those of GAPDH, deduced as described in Materials and Methods, were distributed quite normally. The mean ratio of mRNA levels of ERα relative to GAPDH was 1.7 ± 1.7 (95% confidence interval 1.1–2.3) in the myometrium and 3.2 ± 2.4 (95% confidence interval 2.4–4.1) in the myomas. The mean level of ERα mRNA in the myomas was found 1.9 times higher (paired samples *t*–test; $P<.0001$) than in the adjacent myometrium. Using a similar approach we found that the mean ratio of mRNA levels of ERβ relative to GAPDH was 1.0 ± 0.5 (95% confidence interval 0.8–1.3) in the myometrium and 1.8 ± 1.2 (95% confidence interval 1.4–2.3) in the myomas. The mean level of ERβ mRNA in the myomas was found 1.8 times higher (paired samples *t*–test; $P<.0025$) than in the myometrium. Thus, it appears that

FIGURE 1

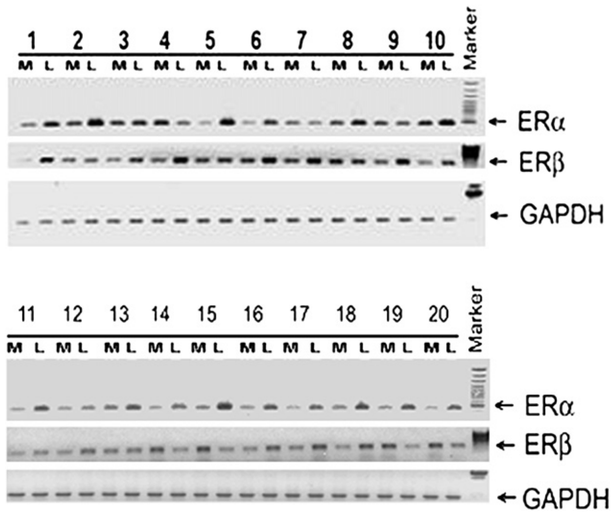
Estrogen receptor levels (median values) in the cytosol of leiomyomas (LEIO) compared with normal myometrium (MYOM) from 35 patients operated on for symptomatic fibroids. Median values of ER levels in leiomyoma and the corresponding myometrium samples, as assessed by Scatchard plot analysis with use of tritiated E_2, were calculated by using the nonparametric Wilcoxon paired samples test, expressed as femtomoles of ER per milligram of cytosol protein and plotted with use of a box-and-whisker graph. The *boxes* contain the values between the upper (75th percentile) and lower (25th percentile) quartiles, the *lines* across the boxes correspond to the medians, and the *whiskers* extend to the highest and lowest values, excluding outliers (*open square*) and extreme values (*filled squares*). *Significantly different compared with myometrium ($P<.05$).



Bakas. Estrogen receptors α and β in fibroids. Fertil Steril 2008.

FIGURE 2

Estrogen receptor α and ERβ mRNA levels in leiomyomas and the corresponding normal myometrium, as compared with the respective GAPDH mRNA levels. Estrogen receptor α, ERβ, and GAPDH mRNA levels in the myometrium (M) and the corresponding leiomyoma samples (L) from each individual patient were obtained by semiquantitative multiplex RT-PCR. Typical ethidium bromide–stained gels of the RT-PCR reaction products from the samples of 20 patients are shown.



Bakas. Estrogen receptors α and β in fibroids. Fertil Steril 2008.

the above reported increase in the level of ER in the cytosol of myomas compared with myometrium is due to similar increases in the level of mRNA of ERα and ERβ.

Deoxyribonucleic Acid–binding Activity of Leiomyoma and Myometrial ER

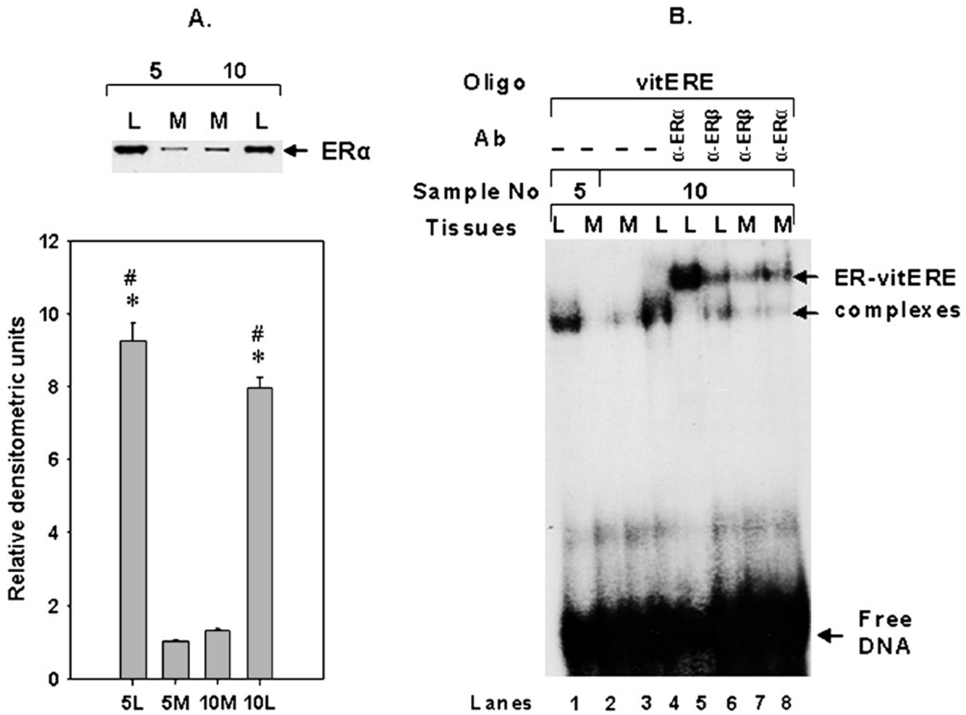
To find whether and how up-regulation of myoma ER affects the DNA-binding activity of the receptor we carried out comparative EMSA analysis of the ER of myomas and the respective myometrial samples using the vitERE as bait. We invariably observed that vitERE binding of protein in nuclear extracts from leiomyoma samples was higher as compared with normal myometrium. Then we focused on the samples in which up-regulation of ERα mRNA relative to ERβ mRNA was most noticeable (e.g., samples 5 and 10 of Fig. 2). Initially we examined whether the higher level of ERα mRNA expression of these leiomyomas was accompanied by a higher level of the receptor in the nuclear extract. Immunoprecipitation and immunoblotting analysis using ERα-specific antibodies and quantification of ERα by phosphorimaging revealed that leiomyoma levels of ERα from, for example, samples 5 and 10 were 9.3-fold and 6.2-fold higher, respectively, as compared with the respective myometrial levels (Fig. 3A). Next we compared vitERE binding of proteins in the nuclear extracts of leiomyoma and the respective adjacent myometrium. Quantification of the vitERE-protein complexes using phosphorimaging detected 11.2-fold and 7.8-fold higher vitERE binding activity in leiomyoma samples 5 and 10, respectively, compared with the adjacent myometrium (Fig. 3B; lanes 1–4). Supershifting analysis of the ERE-binding activity of leiomyoma sample 10 and the respective myometrium using antibodies specific for ERα and ERβ followed by phosphorimaging revealed that the participation of ERα in the ER-vitERE complexes exceeded that of ERβ by 9.4-fold (Fig. 3B; compare the supershifted bands in lanes 5 and 6), as compared with 2.2-fold in the adjacent myometrium (Fig. 3B; compare the supershifted bands in lanes 7 and 8). Thus, the 6.2-fold increase in the level of ERα in myoma sample 10 compared with the adjacent myometrium (Fig. 3A) is faithfully reflected by a comparable increase in the participation of ERα in ER-vitERE complexes (Fig. 3B; compare the supershifted bands in lanes 5 and 8). Similarly, the increased participation of ERβ in the ER-vitERE complexes in myoma sample 10 compared with the adjacent myometrium (Fig. 3B; compare the supershifted bands in lanes 6 and 7) faithfully reflects the mean 1.8-fold increase in the level of ERβ mRNA in the myomas compared with the myometrium reported above. Interestingly, Fig. 3B shows, in addition, that ER-vitERE complexes containing ERα alone occurred to a much higher extent with myoma than with myometrial ERα (Fig. 3B; compare the nonsupershifted bands in lanes 6 and 7).

DISCUSSION

Estrogens regulate a variety of physiologic processes in many different tissues and organs. They act predominantly through the two hormone-binding forms of ER, ERα and ERβ, both of which function as ligand-dependent regulators of transcription by binding to enhancer elements in the promoter of estrogen target genes in heterodimeric or homodimeric form (9–11). Uterine leiomyomas express ERα and ERβ, and their development is known to be estrogen dependent (4–6), but the molecular basis of this dependency is not clearly understood. In the present study we used myomas and adjacent myometrium from 35 women at the follicular phase of their menstrual cycle to examine whether ERα mRNA and protein levels are up-regulated in the myomas compared with the adjacent myometrium and how this up-regulation might impact the ERE-binding activities of ERα and ERβ. We found that the level of expression of both ERα and ERβ mRNA is by and large higher in leiomyomas compared with normal myometrium (Fig. 2), causing the level of ER to increase accordingly (Fig. 1), and that the abundance of ERα relative to ERβ can increase in leiomyomas compared with the myometrium to a level that is high enough to allow leiomyoma ERα to bind to the vitERE as a homodimer, as well as a heterodimer with ERβ (Fig. 3). Specifically, the data of Figure 3B suggest that up-regulation of ERα in myoma sample 10 (Fig. 3A) caused the receptor to bind to

FIGURE 3

Protein levels and DNA-binding status of ERα in leiomyomas and the corresponding normal myometrium. (**A**) Representative immunoprecipitation, PAGE, and immunoblotting analysis of ERα in nuclear extracts from normal myometrium (M) and leiomyoma (L) samples no. 5 (5M and 5L) and no. 10 (10M and 10L), selected for their high ERα-to-ERβ mRNA expression ratio (see samples numbered in Fig. 2). (**B**) vitERE binding of protein in the nuclear extracts from normal myometrium and leiomyoma samples no. 5 and no. 10, as assessed by EMSA (lanes 1 vs. 2 and 3 vs. 4, respectively). Supershifting of vitERE-binding of protein in the nuclear extracts from normal myometrium and leiomyoma sample no. 10, as assessed by EMSA in the presence of antibodies to ERα (a-ERα, lanes 5 and 8) and ERβ (a-ERβ, lanes 6 and 7). *Significantly different compared with 5M ($P<.05$; t-test). #Significantly different compared with 10M ($P<.05$; t-test). Ab = antibodies.



Bakas. Estrogen receptors α and β in fibroids. Fertil Steril 2008.

the ERE as a homodimer (not supershifted with antibodies to ERβ), as well as a heterodimer with ERβ, and that this is not the case with ERα in the corresponding normal myometrium. Notably, formation of an ERE-binding homodimer of ERα occurred in spite of the participation of leiomyoma ERβ in ER-vitERE complexes being nearly twice as high as that of myometrial ERβ, suggesting that the formation of ERE-binding homodimers of ERα depends on the ERα-to-ERβ ratio rather than the individual amounts of the two ER subtypes. That ERβ is usually up-regulated in the myomas compared with the myometrium is further substantiated by the data of Figure 2. Thus, it appears that the formation of ERE-binding homodimers of ERα is most likely the result of higher up-regulation of ERα compared with ERβ rather than the result of down-regulation of ERβ during the myometrium-to-myoma transition.

Estrogen receptor α is considered to be a key player in the development of uterine fibroids, because ERα mRNA and protein levels are often up-regulated in fibroids compared with myometrium (19, 22). In line with this notion, adenovirus-mediated expression of a dominant negative ERα was recently shown to inhibit tumor growth in nude mice (6). In addition, ERα is considered to be a key player during progression of benign mammary proliferative disorders to invasive carcinomas. Recent evidence suggests, however, that a high ERα-to-ERβ ratio rather than the level of ERα alone is what characterizes those cases of mammary hyperplasia of the usual type that are likely to progress to breast cancer (16). In fact, there is ample evidence that, although ERα expression is maintained in breast cancer compared with benign tumors or normal mammary tissue, ERβ expression is decreased (31). Interestingly, work with HC11 immortalized mammary epithelial cells has revealed that, although E_2 promotes cell growth through ERα, at the same time it causes cells to undergo apoptosis through ERβ (15). Thus, it appears that the establishment of a high ERα-to-ERβ ratio, as the result of down-regulation of the expression of ERβ during progression of benign mammary tumors to breast cancer, is somehow associated with enhancement of tumor growth, and this could reflect stimulation of ERα-dependent cell proliferation, suppression of ERβ-dependent cell death, or both. The physiologic significance of the disproportionate up-regulation of the ERα-to-ERβ mRNA ratio during uterine fibroid development (e.g., Fig. 2, sample 5), and the relative abundance of ERα over ERβ that is likely to result, as regards proliferation and survival of myoma smooth muscle cells, is, however, unexplored.

It has been reported that ERα and ERβ can form heterodimers that bind DNA with an affinity similar to that of ERα homodimers and greater than that of ERβ homodimers (10); that ERβ can regulate gene expression in a ligand-independent manner (17, 32); and that the ERE-dependent transcriptional activity of ERα in the presence of E_2 is negatively modulated by ERβ, which thus appears to act as a dominant suppressor of ERα (30, 32). The transcriptional activity of ERα through other transcription factors in the promoter of estrogen target genes also is modulated negatively by ERβ. Most important, E_2 activation of cyclin D1 gene expression through ERα is completely inhibited by ERβ (33). The opposing action and dominance of ERβ over ERα in E_2 activation of both ERE-dependent and AP1-dependent gene expression supports a role for ERβ as a dominant negative inhibitor of the proliferative and antiapoptotic effects of E_2 through ERα. In line with this notion, it has been proposed that the modulation of ERα-dependent cyclin D1 expression by ERβ could be defective in leiomyomas (34). Our data provide a mechanistic basis as to how this might come about, because they show that in leiomyomas with a high ERα-to-ERβ ratio, formation of ERα homodimers is increased substantially, and this may cause the opposing action of ERβ on, for example, ERα-mediated activation of cyclin D1 gene expression to decrease accordingly, with potentially pronounced implications as regards tumor growth. In addition, our data indicate that a substantial increase in the fraction of ERα homodimers, as detected with use of vitERE as bait, could be the molecular determinant that differentiates fast-growing myomas from stationary ones. A study is currently in progress to test this hypothesis.

No effective medical treatment is available presently to women who have symptomatic fibroids and want to avoid surgery (35). Because leiomyomas may depend on an ERα-to-ERβ ratio higher than normal for growth in the presence of estrogen, agents causing down-regulation of ERα and/or up-regulation of ERβ could be used against fibroids. Interestingly, indole-3-carbinol, a constituent of *Brassica* vegetables reportedly endowed with potent anticancer activity in rodent models of carcinogenesis (36), was recently shown to down-regulate ERα expression without altering ERβ expression of MCF-7 breast cancer cells and to cause the levels of ERE-bound ERα and ERβ to decrease and increase, respectively, and the stimulation of the proliferation of the cells by E_2 to drop as a consequence (37). Thus, agents that can decrease the inherently high ERα-to-ERβ ratio of MCF-7 cells may also inhibit the estrogen-dependent proliferation of these cells, apparently by improving promoter occupancy of ER target genes by ERβ relative to ERα. Whether such agents could have an impact on the growth of fibroid smooth muscle cells, which are known to express ERβ as well as ERα (18), is presently a matter of conjecture.

REFERENCES

1. Buttram VC, Reiter RC. Uterine leiomyomata: etiology, symptomatology and management. Fertil Steril 1981;36:433–45.
2. Parker WH. Etiology, symptomatology, and diagnosis of uterine myomas. Fertil Steril 2007;87:725–36.
3. Stewart EA. Uterine fibroids. Lancet 2001;357:293–8.
4. Noe M, Kunz G, Herbertz M, Mall G, Leyendecker G. The cyclic pattern of the immunocytochemical expression of estrogen and progesterone receptors in human and endometrial layers: characterization of endometrial-subendometrial unit. Hum Reprod 1999;14:190–7.
5. Shozu M, Sumitani H, Segawa T, Yang HJ, Murakami K, Inoue M. Inhibition of in situ expression of aromatase P450 in leiomyoma of the uterus by leuprorelin acetate. J Clin Endocrinol Metab 2001;86:5405–11.
6. Al-Hendy A, Lee EJ, Wang HQ, Copland JA. Gene therapy of uterine leiomyomas: adenovirus-mediated expression of dominant negative estrogen receptor inhibits tumor growth in nude mice. Am J Obstet Gynecol 2004;191:1621–31.
7. Shang Y, Brown M. Molecular determinants for the tissue specificity of SERMs. Science 2002;295:2465–8.
8. Hall JM, Korach KS. Analysis of the molecular mechanisms of human estrogen receptors α and β reveals differential specificity in target promoter regulation by xenoestrogens. J Biol Chem 2002;277:44455–61.
9. Dahlman-Wright K, Cavailles V, Fuqua SA, Jordan VC, Katzenellenbogen JA, Korach KS, et al. International Union of Pharmacology. LXIV. Estrogen receptors. Pharmacol Rev 2006;58:773–81.
10. Cowley SM, Hoare S, Mosselman S, Parker MG. Estrogen receptors alpha and beta form heterodimers on DNA. J Biol Chem 1997;272: 19858–62.
11. Osborne CK, Schiff R. Estrogen-receptor biology: continuing progress and therapeutic implications. J Clin Oncol 2005;23(Suppl 8):1616–22.
12. Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ, et al. Differential ligand activation of estrogen receptors ERα and ERβ at AP1 sites. Science 1997;277:1508–10.
13. Mueller SO, Clark JA, Myers PH, Korach KS. Mammary gland development in adult mice requires epithelial and stromal estrogen receptor alpha. Endocrinology 2002;143:2357–65.
14. Forster C, Makela S, Warri A, Kietz S, Becker D, Hultenby K, et al. Involvement of estrogen receptor beta in terminal differentiation of mammary gland epithelium. Proc Natl Acad Sci USA 2002;99: 15578–83.
15. Helguero LA, Faulds MH, Gustafsson JA, Haldosen LA. Estrogen receptors alfa (ERalpha) and beta (ERbeta) differentially regulate proliferation and apoptosis of the normal murine mammary epithelial cell line HC11. Oncogene 2005;24:6605–16.
16. Shaaban AM, Jarvis C, Moore F, West C, Dodson A, Foster CS. Prognostic significance of estrogen receptor beta in epithelial hyperplasia of usual type with known outcome. Am J Surg Pathol 2005;29:1593–9.
17. Chang EC, Frasor J, Komm B, Katzenellenbogen BS. Impact of estrogen receptor beta on gene networks regulated by estrogen receptor alpha in breast cancer cells. Endocrinology 2006;147:4831–42.
18. Valladares F, Frías I, Báez D, García C, López FJ, Fraser JD, et al. Characterization of estrogen receptors alpha and beta in uterine leiomyoma cells. Fertil Steril 2006;86:1736–43.
19. Matsuzaki S, Fukaya T, Uehara S, Murakami T, Sasano H, Yajima A. Characterization of messenger RNA expression of estrogen receptor-alpha and -beta in patients with ovarian endometriosis. Fertil Steril 2000;73:1219–25.
20. Jakimiuk AJ, Bogusiewicz M, Tarkowski R, Dziduch P, Adamiak A, Wrobel A, et al. Estrogen receptor alpha and beta expression in uterine leiomyomas from premenopausal women. Fertil Steril 2004;82(Suppl 3): 1244–9.

21. Wang H, Wu X, Englund K, Blanck A, Lindblom B, Sahlin M. Different expression of estrogen receptors α and β in human myometrium and leiomyoma during the proliferative phase of the menstrual cycle and after GnRHa treatment. Gynecol Endocrinol 2001;15:443–52.
22. Roan CJ, Chuang JH, Hsu TY, Tsai HY, Pan LL, Cheng JT. Estrogen receptor beta is not increasingly expressed in leiomyoma nodules which show no progressive enlargement in premenopausal women. J Formos Med Assoc 2005;104:920–6.
23. Noyes RW, Hertig AT, Rock J. Dating the endometrial biopsy. Am J Obstet Gynecol 1975;122:262–3.
24. Gorgoulis VG, Zacharatos P, Mariatos G, Liloglou T, Kokotas S, Kastrinakis N, et al. Deregulated expression of c-mos in non-small cell lung carcinomas: relationship with p53 status, genomic instability, and tumor kinetics. Cancer Res 2001;61:538–49.
25. Karakaidos P, Taraviras S, Vassiliou LV, Zacharatos P, Kastrinakis NG, Kougiou D, et al. Overexpression of the replication licensing regulators hCdt1 and hCdc6 characterizes a subset of non-small-cell lung carcinomas: synergistic effect with mutant p53 on tumor growth and chromosomal instability—evidence of E2F-1 transcriptional control over hCdt1. Am J Pathol 2004;165:1351–65.
26. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
27. Katsanou ES, Halabalaki M, Aligiannis N, Mitakou S, Skaltsounis AL, Alexi X, et al. Cytotoxic effects of 2-arylbenzofuran phytoestrogens on human cancer cells: modulation by adrenal and gonadal steroids. J Steroid Biochem Mol Biol 2007;104:228–36.
28. Alexis MN, Stylianopoulou F, Kitraki E, Sekeris CE. The distribution and properties of the glucocorticoid receptor from rat brain and pituitary. J Biol Chem 1983;258:4710–4.
29. Stauffer SR, Coletta CJ, Tedesco R, Nishiguchi G, Carlson K, Sun J, et al. Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-alpha–selective agonists. J Med Chem 2000;43:4934–47.
30. Fokialakis N, Lambrinidis G, Mitsiou DJ, Aligiannis N, Mitakou S, Skaltsounis AL, et al. A new class of phytoestrogens; evaluation of the estrogenic activity of deoxybenzoins. Chem Biol 2004;11:397–406.
31. Roger P, Sahla ME, Makela S, Gustafsson JA, Baldet P, Rochefort H. Decreased expression of estrogen receptor beta protein in proliferative preinvasive mammary tumors. Cancer Res 2001;61:2537–41.
32. Hall JM, McDonnell DP. The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology 1999;140:5566–78.
33. Liu MM, Albanese C, Anderson CM, Hilty K, Webb P, Uht RM, et al. Opposing action of estrogen receptors alpha and beta on cyclin D1 gene expression. J Biol Chem 2002;277:24353–60.
34. Kovacs KA, Oszter A, Gocze PM, Kornyei JL, Szabo L. Comparative analysis of cyclin D1 and estrogen receptor (α and β) levels in human leiomyoma and adjacent myometrium. Mol Hum Reprod 2001;7:1085–91.
35. Kolankaya A, Arici A. Myomas and assisted reproductive technologies: when and how to act? Obstet Gynecol Clin North Am 2006;33:145–52.
36. Bradlow HL, Michnovicz J, Telang NT, Osborne MP. Effects of dietary indole-3-carbinol on estradiol metabolism and spontaneous mammary tumors in mice. Carcinogenesis 1991;12:1571–4.
37. Sundar SN, Kerekatte V, Equinozio CN, Doan VB, Bjeldanes LF, Firestone GL. Indole-3-carbinol selectively uncouples expression and activity of estrogen receptor subtypes in human breast cancer cells. Mol Endocrinol 2006;20:3070–82.