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Progesterone Receptor Regulates Bcl-2 Gene Expression through Direct Binding to Its Promoter Region in Uterine Leiomyoma Cells

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Context: Uterine leiomyomas are smooth muscle cell tumors that cause irregular uterine bleeding and pregnancy loss in many reproductive-age women. Progesterone stimulates their growth, whereas treatment with progesterone receptor (PR) antagonists or selective progesterone receptor modulators shrinks these tumors. Molecular mechanisms underlying these observations are unknown.

Objective: Bcl-2 is a key protein that inhibits apoptosis. It was proposed that growth enhancement of leiomyoma cells by progesterone was mediated via bcl-2 induction. Here we test the hypothesis that PR regulates the bcl-2 gene by directly binding to its promoter.

Results: The pure progesterone agonist R5020 increased the total number of viable primary human leiomyoma smooth muscle (LSM) cells in culture. Progesterone or R5020 (10^{-6} M) significantly increased bcl-2 mRNA levels after 2 and 4 h by 9.2- and 3.4-fold, respectively, in LSM cells. Transient transfection with deletion mutants of bcl-2 promoter showed that the −1281/−258-bp region conferred responsiveness to progesterone induction in the presence of PR-A. We identified a palindromic progesterone response element (PRE) at −553/−539 bp. EMSA showed that PR in nuclear extracts from LSM cells bound specifically to this PRE. Chromatin immunoprecipitation-PCR confirmed *in situ* recruitment of PR to the −629/−388-bp region bearing the PRE. *In vivo*, bcl-2 mRNA levels correlated significantly with total PR mRNA levels in leiomyoma tissues.

Conclusion: Taken together, progesterone via PR interacts with the bcl-2 promoter to induce its expression in leiomyoma tissue. This may explain, in part, the progesterone-dependent enhancement of growth in uterine leiomyoma. **(*J Clin Endocrinol Metab* 92: 4459–4466, 2007)**

UTERINE LEIOMYOMAS ARE the most common gynecological tumors in women of reproductive age. Growing evidence from biochemical, histological, clinical, and pharmacological studies indicates that progesterone and progesterone receptor (PR) play key roles in uterine leiomyoma growth and development (1). Several investigators have shown an increased concentration of both PR isoforms (PR-A and PR-B) in leiomyoma tissue, compared with adjacent myometrium (2, 3). Furthermore, increases in mitotic activity occur in leiomyoma tissue relative to the adjacent myometrial tissue during the luteal phase and after treatment with medroxyprogesterone acetate (4, 5). Additionally, *in vitro* studies showed that progesterone suppresses apoptosis and stimulates proliferation of leiomyoma smooth muscle (LSM) cells in culture, whereas PR modulators inhibit proliferation and induce apoptosis in these cells (6–11).

Clinical studies with both progestins and mifepristone also indicate that progesterone may be a key hormone that regulates leiomyoma growth. The synthetic progestins, medroxyprogesterone acetate and norethindrone, when used as add-back therapy in combination with GnRH agonists, attenuate or reverse the inhibitory effects of GnRH agonists on leiomyoma size, whereas mifepristone and asoprisnil were shown to reduce leiomyoma volume (12–14). All these findings suggested that progesterone plays an important role in leiomyoma growth; however, we know little about the underlying mechanisms.

The Bcl-2 protooncogene was discovered in lymphomas composed of B cells (15). The product of the bcl-2 gene, when elevated in cells either *in vivo* or *in vitro*, prevents the normal course of apoptotic cell death in a variety of cells induced by tropic factor deprivation or other stimuli without altering proliferation (16). In addition to extending the life span of certain cells, bcl-2 can promote cell replication by reducing the requirements for growth factors (17). It seems therefore that bcl-2 protein may play an important role in the growth of tumors.

Previous studies on steroid hormone responsive tissues, such as breast and endometrium, suggested that bcl-2 mRNA and protein levels might be regulated by progesterone or synthetic progestins in these tissues (18–20). In leiomyoma relative to the adjacent myometrium, bcl-2 expression was increased, and its expression was most abundant during the

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Abbreviations: cFBS, Charcoal-stripped fetal bovine serum; ChIP, chromatin immunoprecipitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; LSM, leiomyoma smooth muscle; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; PR, progesterone receptor; PRE, progesterone response element.

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secretory phase of the menstrual cycle (2, 6). In cultured LSM cells, progesterone also markedly increased bcl-2 protein levels, whereas selective PR modulators significantly suppressed them (6, 9), suggesting that up-regulation of bcl-2 protein by progesterone may be important for the growth of leiomyoma. However, no information is currently available on the mechanism by which progesterone regulates bcl-2 expression.

Here we demonstrate the transcriptional mechanism responsible for progesterone and PR-dependent induction of bcl-2 in LSM cells using primary culture as a model system. We describe the binding of PR to a specific region on the bcl-2 promoter that confers responsiveness to progesterone.

Materials and Methods

Tissue collection and cell culture

Human uterine leiomyoma and matched myometrium were collected from 21 premenopausal women (mean age 40 yr, range 27–50) undergoing hysterectomy. The size of the tumors ranged from 3.5 to 12 cm in diameter. The subjects had not received any hormonal treatment during the 6 months before surgery. The tissues were collected from the Prentice Women's Hospital at Northwestern University with the approval of its institutional review board. Each uterine specimen was evaluated histologically by a pathologist. Nine samples were obtained during the luteal phase, seven during the follicular phase, and five during menstruation. We isolated LSM cells from the peripheral portions (~1 cm to the capsule) of the leiomyoma and cultured them as previously described (21). Cells used in the experiments were passaged two or three times.

Cell viability assay

The effects of the progesterone agonist R5020 on the viability of LSM cells were evaluated by the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay (American Type Culture Collection, Manassas, VA), as previously described (9). The cells were cultured to 70% confluence and starved in serum-free medium for 24 h. Then cells were treated in the presence or absence of various concentrations of R5020 (10^{-8}, 10^{-7}, 10^{-6} M) in phenol red-free DMEM/F12 with 2% charcoal-stripped fetal bovine serum (cFBS) for 96 h and the MTT assay was done.

RNA preparation and real-time quantitative PCR

Total RNA from LSM cells and tissues of leiomyoma and matched myometrium was extracted using Tri-reagent (Sigma-Aldrich, St. Louis, MO). cDNAs were made from 2 μg total RNA with the Superscript III first-strand synthesis system (Invitrogen, Carlsbad, CA). Bcl-2, PR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were amplified by real-time PCR using the ABI TaqMan gene expression system (Applied Biosystems, Foster City, CA) and the ABI 7900 sequence detection system. All gene expression was normalized to GAPDH. Data reported are the mean fold change ± SD for triplicate measurements.

Immunoblotting

Cell lysates were prepared in modified radioimmunoprecipitation assay buffer and analyzed by immunoblotting as described previously (22) using mouse monoclonal anti-PR (kindly provided by Dr. Dean Edwards, Baylor College of Medicine, Houston, TX), monoclonal anti-bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA), and monoclonal anti-β-actin (Sigma-Aldrich) antibodies.

Transient transfections and luciferase assays

Progressive deletion mutants of the bcl-2 promoter fused to the luciferase reporter gene in pGL3 plasmids were constructed as previously described (23, 24). The −1281/−1- and −258/−1-bp promoter fragments in the pGL3 plasmids were sequenced for fidelity. Transient transfection of LSM cells was carried out with the Fugene HD reagent (Roche Applied Science, Indianapolis, IN) with the following plasmids: 1) 1 μg of pGL3–basic luciferase reporter plasmid containing −1281/−1 bp or −258/−1 bp fragments of bcl-2 promoter region; 2) 300 ng of pCMV-β-gal plasmid as an internal control (Promega, Madison, WI); 3) 1 μg of pSG5 expression vector containing a full-length human PR-A or PR-B cDNA [PR-A and PR-B vectors were graciously provided by Dr. Pierre Chambon (25)]; and 4) 1 μg of the established progesterone-responsive reporter plasmid pPRE/GRE.E1b.Luc (kindly provided by Dr. Ming-Jer Tsai, Baylor College of Medicine, Houston, TX) as a positive control (26). After transfection for 5 h, the cells were incubated in phenol red-free DMEM/F12 with 10% cFBS overnight. Then the cells were starved for 5 h and treated for 40 h with 10^{-6} M R5020 in 5% cFBS. Luciferase assays were performed using luciferase reporter assay kit (Promega). Luciferase activity was normalized to β-gal values. PR-A and PR-B were selectively overexpressed to enhance progestogenic effect and also to assess their individual roles. To examine the transfection efficiency in primarily cultured LSM cells, cells were transfected with pmaxGFP (Amaxa Biosystems, Gaithersburg, MD) and counted for green fluorescent protein (GFP)-positive cells by flow cytometry.

Chromatin immunoprecipitation (ChIP) assays

LSM cells (untransfected) were incubated with 10^{-6} M progesterone or vehicle (ethanol) for 1 h. ChIP assays were performed as described previously using a kit from Upstate Biotechnology (Lake Placid, NY) (22). The sonicated chromatin fraction was immunoprecipitated with an equal amount of either rabbit IgG or anti-PR antibody (Abcam, Cambridge, MA), which recognizes both PR-A and PR-B. Chromosomal DNA was purified and analyzed by PCR for the presence of the bcl-2 promoter (−629/−388 bp). The primer sequences used for PCR were: forward: 5′-CTGGAGAGTGCTGAAGATTG-3′; reverse: 5′-CACACTACAAGTAACACGGC-3′.

EMSA

LSM cells (untransfected) were treated for 2 h with 10^{-6} M progesterone. Nuclear protein was extracted from whole cells using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL). EMSA was performed using a LightShift chemiluminescent EMSA kit (Pierce). The sequence of the double-stranded oligonucleotide used for EMSA was 5′-GACAGAGGATCATGCTGTACTTAAA-3′, which is identical with a 25-bp-long sequence (−559/−535 bp) within the promoter region of the bcl-2 gene and contains an imperfect 15-bp palindromic progesterone response element (PRE; −553/−539). The mutant probe is an unlabeled 25-bp oligonucleotide, in which 3 bp of the 15-bp PRE at −549, − 543, and −540 positions were replaced by As.

Biotin end-labeled oligonucleotide probes were purchased from Sigma-Aldrich and annealed to the complementary oligonucleotides. Twenty femtomoles of labeled probe and 3 μg of nuclear extract were incubated for 20 min at room temperature in a reaction mix containing 5% (vol/vol) glycerol, 10 mM Tris-HCl, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, and 2 μg of poly(dI-dC). For DNA competition EMSA, the unlabeled wild-type or mutant probe (4 pmol) was added simultaneously with the labeled probe. For supershift experiments, 5 μg of PR antibody were added, and incubation was continued for an additional 40 min on ice. Nondenaturing polyacrylamide gels (5%) were used to resolve protein-DNA complexes. Protein-DNA samples were transferred to the Biodyne B precut modified nylon membrane (Pierce). Protein-DNA complexes were detected using a LightShift chemiluminescent EMSA kit (Pierce) and exposed to the CL-XPosure film (Pierce).

Statistical analysis

Differences among groups were analyzed by one-way ANOVA followed by Fisher's protected least-significant difference test, using Statview (Abacus Concepts, Inc., Berkeley, CA). Significance was accepted at $P < 0.05$.

Results

Effects of the progesterone agonist R5020 on leiomyoma cell growth

The PR levels in cultured LSM cells were examined by immunoblotting using whole-cell lysates from leiomyoma tissue, LSM cells at various passages (P0, P1, P2, or P3). Consistent with previously published data, cultured cells contained lower levels of PR, compared with leiomyoma tissue (Fig. 1A, please note that the exposure time for tissue is 10 sec, but it takes about 1 min to detect the bands in cultured cells) (27). It has previously been shown that LSM cells cultured in serum-free media become quiescent and are then fully stimulated to reenter the cell cycle after incubation with 10% fetal bovine serum or show half-maximal stimulation to reenter the cell cycle when incubated with 2% fetal bovine serum (21, 28). Using LSM cells grown in charcoal-stripped serum and phenol red-free media and subsequently made quiescent under serum-free conditions, we evaluated the effects of treatment with various concentrations of R5020 on cell viability using the MTT assay. Exposure of LSM cells to R5020 increased the number of viable cells in a dose-dependent manner (Fig. 1B). The number of viable cells increased by 1.4-fold when treated with 10^{-6} M R5020, which was significantly higher than untreated control cells ($P = 0.008$). These data suggest that the progesterone agonist R5020 promotes LSM cell growth.

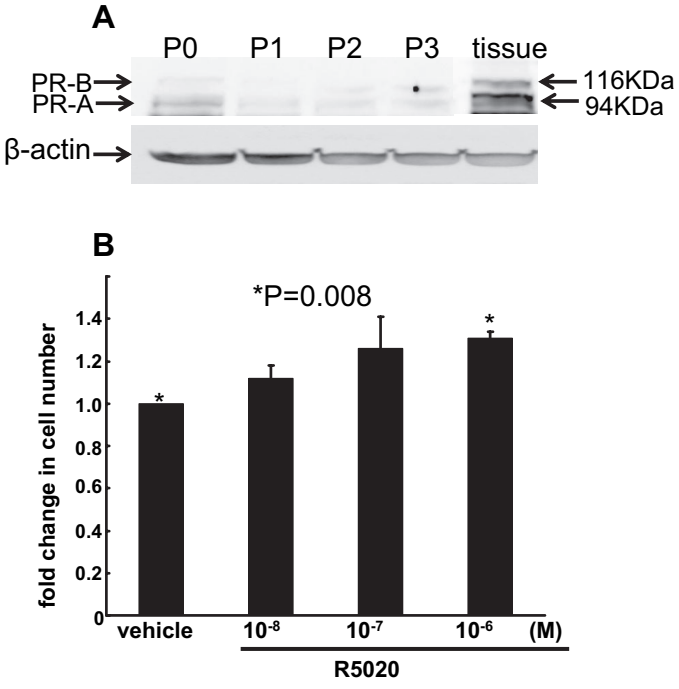

FIG. 1. A, Comparison of PR protein levels in tissue and cultured LSM cells. Cells were passaged and cultured to 80% confluency. Cell lysates were prepared for immunoblotting with anti-PR and β-actin (loading control) antibodies. B, Effects of various concentrations of R5020 on the number of viable cultured human LSM cells in the presence of 2% cFBS, as assessed by MTT assay. Compared with the ethanol (vehicle)-treated control, treatment with 10^{-6} M R5020 for 96 h significantly increased the number of viable LSM cells. The data are expressed as mean ± SEM fold change from the control value in triplicate experiments repeated three times on primary LSM cells isolated from three subjects. P0, Passage 0; P1, passage 1; P2, passage 2; P3, passage 3.

Regulation of bcl-2 mRNA and protein levels in leiomyoma cells

We investigated the effects of progesterone and its synthetic agonist R5020 on the expression of genes that regulate apoptosis and proliferation, namely bcl-2, bcl-x, and c-*myc*, in cultured LSM cells. Progesterone (10^{-6} M) increased bcl-2 mRNA by 9.3-fold at 2 h ($P = 0.009$) (Fig. 2A). Treatment with R5020 (10^{-6} M) resulted in a 1.6-fold induction of bcl-2 mRNA at 2 h, which increased to 3.4-fold induction at 4 h ($P = 0.04$), followed by a decline toward control levels at 8 h (Fig. 2A). Both progesterone and R5020 resulted in significant but modest inductions of c-*myc* mRNA levels at 1 h but not at 2, 4, or 8 h (Fig. 2B). Bcl-x mRNA levels were not affected by progestins at any of the time points (Fig. 2C). Treatment with R5020 (10^{-6} M) for 72 h increased bcl-2 protein levels (Fig. 2D). These results demonstrate that progesterone or its agonist R5020 has a limited regulatory effect on bcl-x and c-*myc* expression in LSM cells, but it significantly enhances bcl-2 expression, suggesting that progesterone supports cell survival through up-regulation of the antiapoptotic gene bcl-2 in these cells.

Regulation of bcl-2 promoter activity by PR and R5020

The transfection efficiency was examined by transfecting cells with pmaxGFP for 48 h and counting GFP-positive cells by flow cytometry; the positive rate is approximately 20% (Fig. 3A). Luciferase reporter constructs containing −1281/−1 or −258/−1 bp (relative to the translation start site) of the 5′-flanking region of the human bcl-2 promoter were transfected into LMS cells along with a human PR expression vector expressing one of the isoforms of PR: PR-A or PR-B. The pGL3-basic and pSG5 plasmids were transfected as controls. After transfection and incubation in the presence or absence of 10^{-6} M R5020 for 40 h, the cells were collected and assayed for luciferase activity. In the absence of PR expression vectors, R5020 had a modest stimulatory effect on the −1281-bp but not the −258-bp luciferase construct (Fig. 3B). Cotransfection of the PR-A expression vector in the presence of R5020 significantly induced the −1281-bp promoter, demonstrating that progestin-responsive regulatory region exists between −1281 and −258 bp of the bcl-2 promoter. Unliganded PR-A also up-regulated the −1281 promoter, although not to the magnitude of liganded PR-A. Interestingly, the −1281 promoter did not respond to PR-B. The −258 promoter did not respond to progestins for either PR-A or PR-B. These data show a specificity of the bcl-2 promoter to PR-A. To demonstrate that the PR-B expression vector encodes functional PR-B, the cells were cotransfected with an established progesterone responsive plasmid pPRE/GRE.E1b.Luc and treated with R5020 for 40 h. As shown in Fig. 3C, PR-B in the presence of R5020 significantly stimulated pPRE/GRE.E1b.Luc, but PR-A had a significantly weaker effect.

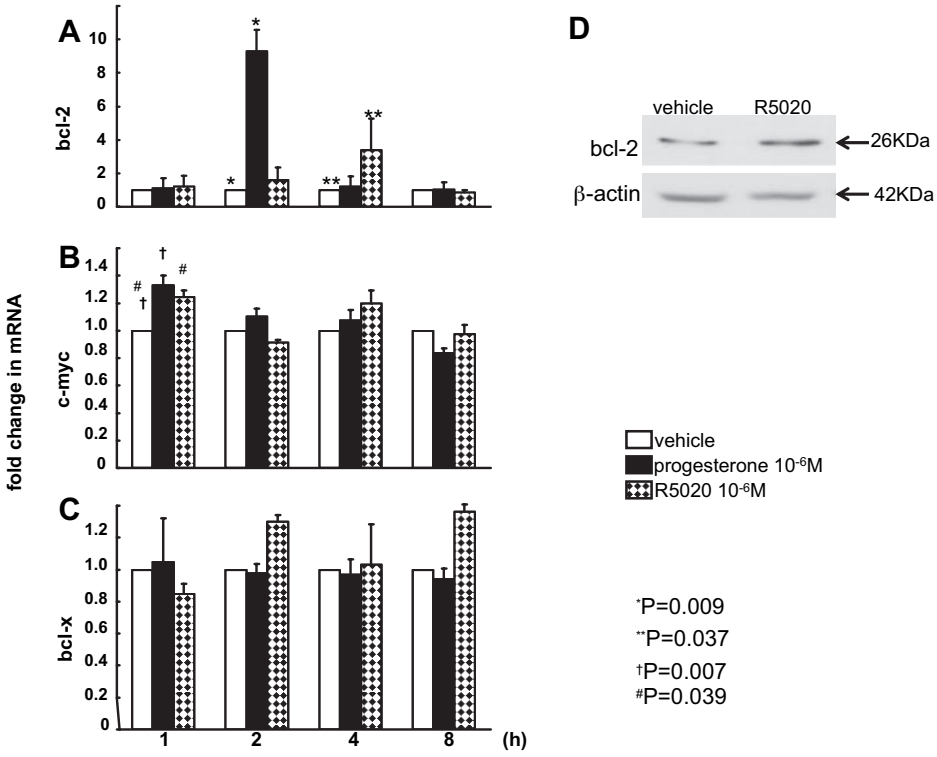

FIG. 2. Time-dependent induction of mRNA and protein levels of bcl-2, mRNA levels of c-*myc*, and bcl-x by progesterone or R5020 in primary LSM cells. LSM cells were treated with progesterone (10^{-6} M), R5020 (10^{-6} M) or ethanol (vehicle) as control, for 1, 2, 4, and 8 h. Total RNA was extracted and analyzed for the expression of bcl-2 (A), c-*myc* (B), and bcl-xl (C) via real-time quantitative RT-PCR. D, LSM cells were treated with R5020 (10^{-6} M) or ethanol (vehicle) as control for 72 h. Cell lysates were prepared for immunoblotting with anti-bcl-2 and β-actin (loading control) antibodies. The data are expressed as fold increase, compared with vehicle-treated cells and represent mean ± SD of triplicate experiments.

Binding of PR to a PRE in the bcl-2 promoter

Because progesterone or R5020 regulated bcl-2 gene expression and promoter activity, we looked for a PRE in the promoter region. Using the transcription element search system (http://www.cbil.upenn.edu/tess/), a 15-bp PRE-like sequence GGATCAtgcTGTACT at −553/−539 from the ATG start site was identified in the bcl-2 promoter, which is 93% homologous to the consensus PRE sequence described by Lieberman *et al.* (29).

To elucidate whether nuclear proteins bind specifically to this regulatory region, an EMSA was performed using nuclear extracts from LSM cells and a 25-bp probe identical with the sequence that flanked the PRE. These cells had been incubated with progesterone for 2 h before harvesting to ensure PR was occupied by ligand. Figure 4 shows that a specific DNA-protein complex was formed when a biotin-labeled oligonucleotide probe was incubated with the nuclear extract (lane 2). Addition of a 200-fold excess of unlabeled wild-type oligonucleotide decreased binding of extracts to the labeled oligonucleotide demonstrating specificity. In addition, competition with an unlabeled and mutated oligonucleotide in which three bases within the putative PRE were mutated did not disrupt the protein-DNA binding. Lane 5 shows that inclusion of anti-PR antibody resulted in total immunodepletion of the protein-DNA complex, whereas normal IgG had no effect (lane 6). These data suggest that PR binds to the PRE in the bcl-2 gene promoter in a specific manner.

ChIP assays were performed to confirm that the bcl-2 promoter region containing the PRE was occupied by endogenous PR *in situ*. Sheared chromatin isolated from cultured LSM cells, treated with progesterone or vehicle for 1 h, was immunoprecipitated with anti-PR antibody. The presence of bcl-2 promoter DNA in the immunoprecipitate was determined by PCR amplification. As shown in Fig. 5B, bcl-2 DNA was specifically immunoprecipitated with the anti-PR antibody but not with a nonspecific IgG. Treatment with progesterone significantly enhanced binding of PR to this site. These results demonstrate that endogenous PR binds *in situ* to the bcl-2 promoter region.

The findings from ChIP, EMSA, and reporter gene assays strongly support the notion that progesterone and PR highly regulate the promoter activity of the bcl-2 gene through a PRE located at −553 to −539. In addition, PR-A appeared to play a predominant role in this regulation.

An in vivo correlation between bcl-2 and PR mRNA in leiomyoma tissue

To determine whether there is an *in vivo* interaction between bcl-2 and PR expression in leiomyoma tissue, we analyzed mRNA levels of bcl-2 and PR in human leiomyoma and matched adjacent myometrial tissues from 18 subjects using real-time PCR. To allow comparisons of data obtained from samples of different subjects, the mRNA levels of bcl-2 and PR were normalized to GAPDH, and then the mRNA level in leiomyoma tissue was expressed as a multiple of that in the matched myometrium, allowing the detection of relative mRNA differences between the two tissues. Among the specimens from 18 subjects, eight leiomyoma samples had distinctly higher levels of bcl-2 than those in myometrial tissues, five leiomyoma samples showed lower bcl-2 mRNA levels, and bcl-2 levels in leiomyoma and myometrial tissues were similar in five subjects. The mean mRNA level of bcl-2 in leiomyoma samples was approximately 1.4-fold higher than that in myometrial samples. This observed trend did not reach statistical significance ($P = 0.12$). Importantly, subjects

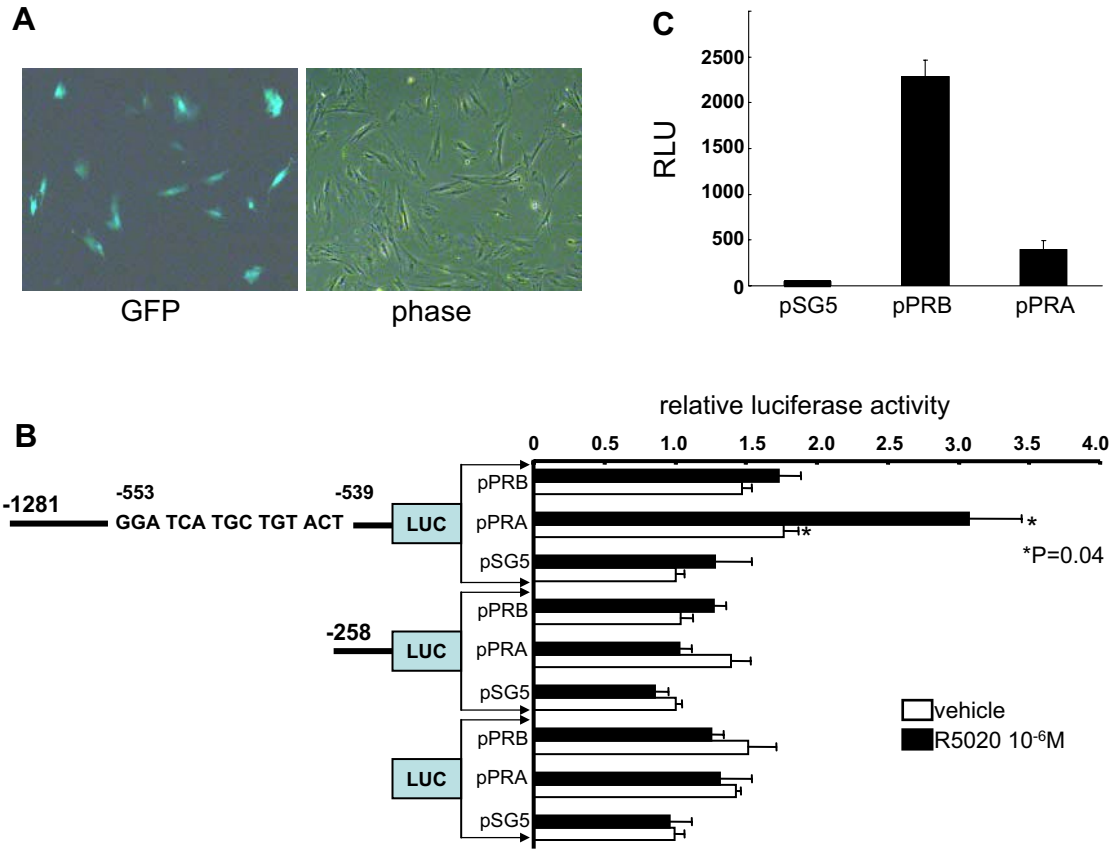

FIG. 3. Transcriptional activation of bcl-2 promoter reporter constructs by R5020. A, Photomicrographs showing representative images of GFP-positive cells. B, LSM cells were transiently transfected with luciferase fusion vectors containing −1281- or −258-bp of 5-flanking region relative to the bcl-2 translation start site. The cells were also transiently cotransfected with expression vectors encoding either PR-A or PR-B. The cells were treated with ethanol as control or 10^{-6} M R5020 for 40 h. Luciferase activity of each construct was normalized to β-gal and then to that of the cells cotransfected with pGL3-basic and pSG5 empty vector incubated in the absence of R5020, which was set to 1. C, LSM cells were cotransfected with an established progesterone responsive reporter plasmid pPRE/GRE.E1b.Luc and with expression vectors PR-A and PR-B, and then the cells were treated and extracted for luciferase assay as in B. The experiments were done independently three times. *Error bars* show SD values among triplicates from a single representative transfection. pSG5, Empty control vector for PR-A and PR-B.

with higher levels of bcl-2 mRNA in leiomyoma also had comparably higher levels of PR mRNA, and vice versa. Regression analysis indicated a highly significant correlation between bcl-2 mRNA level and PR mRNA level ($R = 0.82033$, $P = 0.000031$) in leiomyoma tissue (Fig. 6). This suggested that expression of bcl-2 is strongly and positively associated with the PR status *in vivo* in leiomyoma tissue.

Interestingly, mean bcl-2 and PR mRNA levels were higher in luteal *vs.* follicular phase leiomyoma samples, whereas opposite trends were observed in matched myometrial samples. None of these trends, however, reached statistical significance.

Discussion

Bcl-2 is an important protein involved in the control of apoptosis. To our knowledge, the present study is the first to report a progesterone regulatory region in the promoter of this gene. Previous studies have shown that progesterone down-regulated bcl-2 levels in endometrium and breast cancer cells (18, 19). Microarray analysis of mouse uterine tissue showed that bcl-2 mRNA levels decreased after progesterone treatment (20). Progesterone and its agonist R5020 treatment stimulated bcl-2 mRNA and promoter activity via PR in primary LSM cells, which is consistent with a previously published study of Matsu *et al.* (6). However, Matsu *et al.* reported that bcl-2 protein was uniformly increased in leiomyoma tissues and cells relative to normal myometrium, which is different from our data showing that leiomyoma (*vs.* myometrial) bcl-2 mRNA levels are higher in some subjects but not in others, suggesting that some posttranslational regulation may control bcl-2 protein levels. The contrasting effects of progesterone on bcl-2 expression in leiomyoma *vs.* endometrium is intriguing but somewhat predictable and is consistent with the opposite functions of progesterone in endometrium and myometrium. For example, progesterone prevents estrogen-dependent growth of endometrial tissue, whereas progesterone enhances growth in uterine leiomyoma and breast tissue (30, 31). The mechanism underlying the contrasting effects of progesterone on bcl-2 expression in endometrium and leiomyoma is unclear. One possibility is that in endometrium, progesterone functions indirectly through antagonizing the effect of estradiol on bcl-2 regulation (19), whereas in leiomyoma, as we found in this study, it up-regulates bcl-2 gene expression.

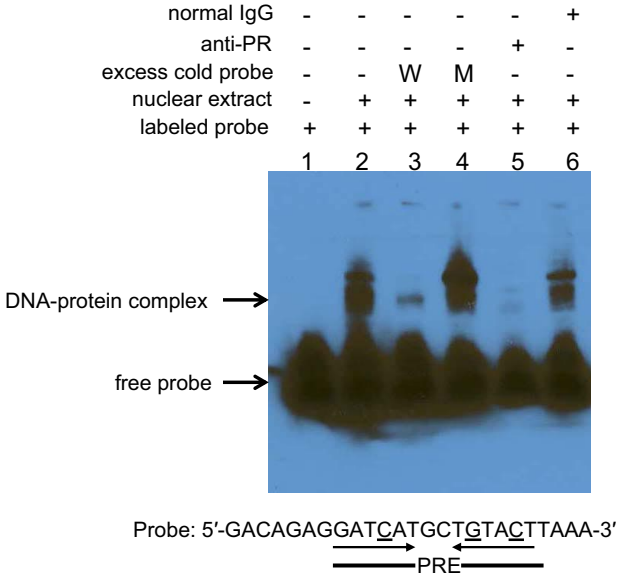

FIG. 4. EMSA analysis of PR binding region of bcl-2 promoter. EMSAs were performed using samples of nuclear extracts (3 μg) from LSM cells treated with progesterone for 2 h and biotin-labeled oligonucleotide probes containing putative PR binding sites and its flanking sequence. Competition studies were performed using 200-fold excess of cold unlabeled wild-type (W; lane 3) or mutant (M; lane 4) probes. Immunodepletion of complexes was performed using 5 μg of anti-PR (lane 5) antibody. Normal IgG was added as a negative control (lane 6). The indirect repeats in the palindromic PRE in the probe are indicated by *arrows*. The bases changed to As in the mutated probe are *underlined*. The figure represents one of the three independently performed experiments.

Here we demonstrated that R5020 increased total viable primary LSM cells. Others have also reported that progestins stimulate proliferation and inhibit apoptosis of primary LSM cells, whereas the selective PR modulator asoprisnil inhibits proliferation and induces apoptosis in the same cells (5–11). Our report further explored the molecular mechanisms by which progestin stimulation of leiomyoma growth occurs. Our working hypothesis is that progestin-dependent growth

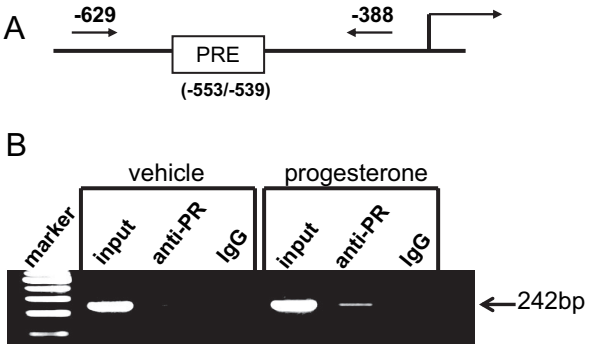

FIG. 5. *In vivo* binding of human PR to the bcl-2 promoter in LSM cells. Chromatin prepared from LSM cells treated for 1 h with 10^{-6} M progesterone or vehicle (ethanol) was subjected to the ChIP procedure using an anti-PR antibody or a nonspecific anti-rabbit IgG. The isolated DNA fragments were amplified by 40 cycles of PCR using primers covering −629/−388 (relative to bcl-2 translation start site) of 5′-flanking region of bcl-2. Input, Nonprecipitated chromatin; anti-PR, chromatin precipitated with a specific antibody against PR; IgG, chromatin precipitated with nonspecific rabbit IgG.

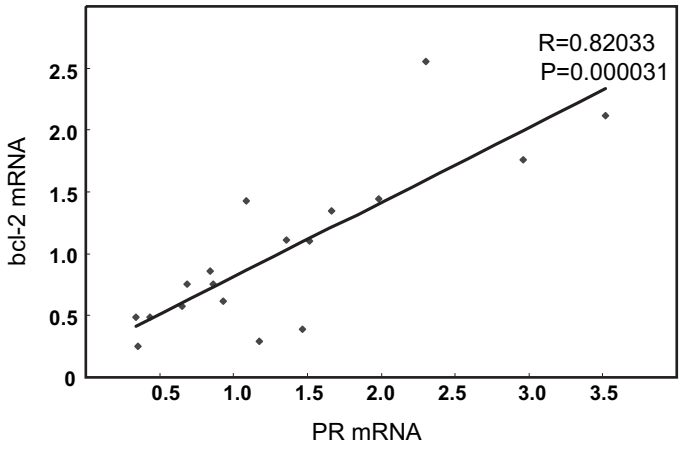

FIG. 6. Correlation between mRNA levels of bcl-2 and PR in human leiomyoma and myometrial tissues. Overall, 36 samples from 18 subjects were analyzed; 18 samples were obtained from leiomyomas and 18 from adjacent myometrial tissues. To allow comparisons of data obtained from samples from different subjects, mRNA levels in the myometrial tissues were normalized to 1. Regression analysis indicated bcl-2 mRNA levels correlated significantly and positively with PR mRNA levels (R = 0.82033, P = 0.000031).

stimulation involves enhancement of bcl-2 expression, predicting a progesterone-PR-mediated regulatory region upstream of the bcl-2 promoter. The above data are consistent with this hypothesis.

Transfection of either PR-A or PR-B expression vector together with the bcl-2 promoter region to the LSM cells provided an opportunity to study selective effects of each PR isoform. Progestin-dependent stimulation of bcl-2 promoter activity could clearly be demonstrated in the presence of PR-A but not with the full-length isoform PR-B. This is consistent with previously published work on distinct biological roles of PR-A and PR-B in various tissues (32). For example, selective knockout of PR-A *vs.* PR-B in mice demonstrated an important role of PR-A in uterine physiology, whereas PR-B knockout mice did not exhibit a uterine phenotype (33, 34). Thus, it is conceivable that PR-A, but not PR-B, selectively mediates progesterone action on the bcl-2 gene in LSM cells in culture.

PR binds to the palindromic consensus sequence located in the promoter or enhancer regions of target genes (29, 35). After analysis of proximal promoter region of bcl-2 gene, we identified a 15-bp PRE at −553/−539 relative to the ATG start site, which contains two palindromic repeats and closely resembles the consensus sequence described by Beato (36) and Lieberman *et al.* (29). Although the identified sequence does not perfectly match the consensus sequence (93% homology), it contains the most important nucleotides for receptor binding, including the four major guanine/cytosine contact points (37, 38). Additionally, the PRE found in the bcl-2 gene does not have complete dyad symmetry with respect to the two 6-bp elements, which is consistent with studies indicating that steroid hormone receptors are not binding to the two half-sites in an equivalent manner (39). EMSAs using wild-type and mutant competitor DNA fragments and anti-PR antibody clearly demonstrated that the 15-bp PRE binds to the nuclear proteins present in proges-

terone-stimulated LSM cells in a specific manner. *In situ* recruitment of PR to the promoter region bearing this PRE was further confirmed by ChIP. Thus, our results are suggestive that binding of ligand-activated PR to this PRE is important for regulation of bcl-2 by progesterone. It was also shown that PR interacts rapidly with SH3 domains of various cytoplasmic signaling molecules, including c-Src tyrosine kinases (40). A potential role of this nongenomic mechanism should be explored regarding progesterone action in leiomyoma.

In summary, we demonstrated that the PRE-like sequence in the human bcl-2 promoter region specifically binds PR and confers progesterone responsiveness to the human bcl-2 gene, thus providing one explanation of the ability of progesterone to regulate bcl-2 expression in leiomyoma cells. We plan to analyze this PRE-like sequence further using site-directed mutagenesis, investigate the interaction of PR with other transcription factors and *cis*-acting regulatory elements, and evaluate whether this sequence also responds to other members of the PR subfamily of nuclear receptors, *i.e.* glucocorticoid, androgen, and mineralcorticoid receptors.

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