Decreased Expression of Early Growth Response-1 and Its Role in Uterine Leiomyoma Growth

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ABSTRACT

Expression of early growth response (Egr)-1, a transcriptional factor implicated in growth regulation, is suppressed in several malignant tumors. The present study investigated the expression of Egr-1 and related genes in uterine leiomyoma and normal myometrium to determine possible contributions of Egr-1 to neoplastic growth in leiomyoma cells. Levels of Egr-1 transcripts were decreased in all leiomyomas (n = 20) to approximately 10% of levels in corresponding myometrium, where basal expression was high. Preoperative leuprorelin acetate therapy increased levels of Egr-1 mRNA in normal myometrium only. Northern blot analysis using additional sample sets (n = 5) revealed the full-length Egr-1 transcript. Western blot analysis (n = 5) confirmed decreased expression of Egr-1 protein. Southern blot analysis of the Egr-1 gene and microsatellite analysis of the chromosomal location at 5q31 (D5S414, D5S500, and D5S476) revealed neither DNA recombination nor loss of heterozygosity in leiomyomas. Moreover, Egr-1 retained identical responsiveness to phorbol 12-myristate 13-acetate in primary cultures derived from both leiomyoma and normal tissues. Electrophoretic mobility shift analysis revealed that phorbol 12-myristate 13-acetate-induced Egr-1 in leiomyoma cells retained DNA binding ability. Egr-1 thus appears functionally intact in leiomyoma cells. Finally, consistent with the role of Egr-1 in growth inhibition, transfection of Egr-1 expression vector into a myometrial cell line (KW) that expresses low levels of Egr-1 and displays rapid growth inhibited thymidine uptake in these cells. Egr-1 may display tumor-suppressing activity and offers a potential target for leiomyoma management.

INTRODUCTION

Early growth response (Egr)-1, a DNA-binding transcription factor structurally related to the tumor suppressor gene Wilms’ tumor gene (WT1), is expressed ubiquitously in human tissues (1). In normal cells, Egr-1 is immediately induced in response to extrinsic signals, including serum, phorbol esters, and some growth factors. Egr-1 binds to the GC-rich consensus DNA sequences of promoters, regulating the transcription of numerous genes associated with biological responses such as macrophage differentiation (2), immune responses of myeloid cell precursors (2), luteinizing hormone secretion of pituitary cells (3), memory formation in neuronal cells (4), and ovulatory response in granulosa cells (5). Expression of Egr-1 also underlies diverse pathophysiological responses such as survival responses to damaging irradiation (6), development of vascular occlusion in arteriosclerosis (7), thrombosis formation after lung transplantation (8), and formation of severe pulmonary emphysema (9).

In addition to these diverse actions in nonneoplastic cells, expression of Egr-1 is commonly down-regulated in tumor cells, including breast cancer, lung cancer, lymphoma, and leukemia (10–14), and in fresh surgical specimens of glioblastoma and breast, hepatocellular, and esophageal carcinoma, contrasting sharply with their normal tissue counterparts (15). Reexpression of Egr-1 leads to inhibited cell proliferation, decreased tumor growth, inhibited colony formation, and restoration of normal phenotypes such as increased attachment and contact inhibition (16–19). Given these tumor-suppressing functions, Egr-1 represents a therapeutic target in these tumors (1, 13, 16, 20).

Several different mechanisms have been proposed to explain the tumor-suppressive action of Egr-1 (21). Egr-1 directly up-regulates transforming growth factor (TGF)-β1 in a fibrosarcoma cell line (HT1080), PTEN (phosphatase tensin homolog) in 293T human fetal kidney cells (22), p53 in numerous cells such as human prostate cancer (PC-3) and melanoma cells (22, 23), p73 (a member of the p53 family) in neuroblastoma cells (24), and tumor necrosis factor α in monocytic cell lines (25). Both TGF-β1 and PTEN induce cell cycle arrest by modulating the cyclin-dependent pathway. PTEN, p53, and p73, in addition to tumor necrosis factor α, lead to apoptosis (18, 22, 24). Egr-1 also directly transactivates expression of fibronectin and plasminogen activator, enhancing cell attachment and a more normal phenotype (16). Tumor-suppressive action would be achieved as a sum of these various actions, but the major mechanisms mediating tumor-suppressive activity by Egr-1 probably depend predominantly on cell type and on the strength and duration of the inductive stimuli for Egr-1.

In contrast to tumor-suppressive activity, Egr-1 reportedly plays an oncogenic role in prostate cancer. Egr-1 is expressed at a higher level and promotes cell growth in prostate cancer compared with normal tissues (26, 27). Experimental inhibition of Egr-1 expression using oligonucleotides reverses transformation of prostate cancer cells both in vitro and in vivo (28). Egr-1 deficiency delays progression of prostate carcinoma in Egr-1−/− mice (29). The molecular basis of this paradoxical action of Egr-1 in prostate tumor remains undetermined, and numerous genes that are up-regulated by Egr-1 in prostate cells have been implicated as mediating oncogenic actions that stimulate cell growth, such as insulin-like growth factor II, platelet-derived growth factor-A, platelet-derived growth factor-B, and vascular endothelial growth factor (21). In prostate tumors, TGF-β1 induced by Egr-1 is thought to accelerate tumor progression by inducing matrix formation and activation of other growth factors that attach to the matrix for optimal effects on the secreting cells and tumor-supporting endothelial cells, unlike other tumors, in which TGF-β1 predominantly inhibits epithelial cell growth (21).

The present study investigated Egr-1 expression in uterine leiomyoma with benign monoclonal proliferation of transformed myocytes. Egr-1 expression was consistently down-regulated in leiomyomas compared with surrounding myometrium, and restored expression of Egr-1 inhibited myometrium-derived KW cell growth in vitro. These findings suggest that Egr-1 may display tumor-suppressing activity, offering a potential target for leiomyoma management.

MATERIALS AND METHODS

Tissue Sources. Uterine tissues were obtained from women undergoing hysterectomy for uterine leiomyoma after approval from the institutional review board. Patients were randomly selected and enrolled in the study, and

Received 3/9/03; revised 3/24/04; accepted 4/26/04.


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written informed consent was obtained in all cases. Women with evidence of adenomyosis and/or endometriosis at the time of laparotomy were excluded. Subserial and intramural leiomyoma specimens were obtained from leiomyoma tissues just beneath the capsule of the nodule, with corresponding myometrial samples taken ≥2 cm away from the nodule. All specimens were histologically confirmed as ordinary leiomyoma. Tissue samples were dissected immediately after surgery, snap-frozen in liquid nitrogen, and stored at −74°C for up to 3 months for Western blotting and for up to 1 year for reverse transcription-PCR assays. The mean age of patients was 43 years (range, 32–48 years). Menstrual cycle was determined according to histology of the endometrium obtained intraoperatively in preoperatively untreated women. For women who had undergone preoperative treatment, leuprolreacin [LA (1.88 mg s.c. every 4 weeks)] was administered for 8–21 weeks before the operation.

**Cell Culture.** Isolation and culture of smooth muscle cells from leiomyoma tissues and myometrium and morphological validation of these primary cells as smooth muscle cells were performed as described previously (30–32). Briefly, leiomyoma tissue was minced using scissors and digested with collagenase type B (1 mg/ml; Roche) and DNase I (0.15 mg/ml; Sigma, St. Louis, MO) for 3–4 h with vigorous shaking in a water bath at 37°C. Digests were filtered through three layers of sterile gauze and then cultured in DMEM/Ham’s F-12 (1:1) supplemented with 10% fetal bovine serum (Sigma), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 100 µg/ml kanamycin (Invitrogen, Carlsbad, CA). About 1 week was usually required for freshly isolated cells to reach confluence. Cultures were maintained for no more than 4 weeks. Primary cells were confirmed to display features of uterine muscle cells as smooth muscle cells were performed as described previously (30–32). The phenotype of KW cells resembles that of normal myometrium in terms of gene expression (31, 33, 34). KW cells were originally established from myometrial smooth muscle cells and were provided by Dr. Osamu Matsuo (Kinki University, Osaka, Japan) (33). The phenotype of KW cells resembles that of normal myometrium in terms of gene expression (31, 33, 34).

For Egfr-1 induction, primary and KW cells were serum starved for 12 and 6 h, respectively, and then stimulated with 8 ng phosphol 12-phyryate (PMA) or 2 µM deoxynucleotide triphosphates, 1 unit of RNase inhibitors, and 0.5 µl of reverse transcriptase (Invitrogen) for 40 min at 42°C. The reaction mixture contained 50 pmol of random hexamers, 200 µM deoxynucleotide triphosphates, 1 unit of RNAse inhibitors, and 1 unit of reverse transcriptase (ReverseAce; Toyobo, Tokyo, Japan). Of the resulting cDNA, 1% was used as a template for subsequent real-time PCR reactions.

**Construction of DNA Templates for Control PCRs.** Templates for PCR standards were first amplified from cDNA and then subcloned into a PCR2.1 vector using TA cloning kits (Invitrogen). The fidelity of each template was confirmed using an ABI Prism 310 automatic DNA sequencer (PE Applied Biosystems, Foster City, CA). Primer sequences are listed in Table 1. DNA concentrations were determined spectrophotometrically.

**Real-Time PCR for Quantification of Transcripts.** Products were PCR amplified and detected in real time using a LightCycler (Roche). Primer pairs used for quantitative PCR reactions were the same as those used to construct DNA standards (Table 1). Each PCR reaction mixture (5 µl) comprised 1 µl of serially diluted DNA template or sample cDNA, 0.5 µm each of gene-specific primers, 2 mm magnesium sulfate, and 0.5 µl of Master Mix (LightCycler Faststart DNA master SYBR Green I kit; Roche) PCR products were detected as double-stranded DNA according to the fluorescence of SYBR Green I, which specifically binds to double-stranded DNA at the end of the elongation phase in each PCR cycle. The theoretical basis of quantification using real-time PCR has been described elsewhere (35). Briefly, on plots of log-arithmically transformed fluorescence intensity against cycle number, the noise band was defined as the beginning of the log linear phase of amplification for each reaction beyond the background. Crossing points (number of cycles required to reach noise band level) were calculated and plotted against the common logarithm of the standard copy number to generate a standard curve.

Level of noise band was corrected when the mean squared error of the standard curve exceeded 0.1. Quantitative values of unknown samples were calculated from plots based on standard curves for the same assay.

**Northern Blotting.** Total RNA or Poly(A)^+ -rich RNA prepared from total RNA using mRNA purification kits (Amersham Biosciences, Piscataway, NJ) was analyzed by Northern blotting. An Egfr-1 DNA probe was excised from pSVegfr-1 plasmids containing full-length cDNA of human Egfr-1 (donated by Dr. Tetsuo Hiran; Division of Behavioral Sciences, Faculty of Integrated Arts and Sciences, Hiroshima University, Hiroshima, Japan) and radiolabeled with [32 P]dCTP by random priming using Megaprime II kits (Amersham, Arlington Heights, IL). For quantitative comparisons, signal intensity was measured densitometrically, and values were normalized to that of glyceraldehyde-3-phosphate dehydrogenase.

**Western Blotting.** Samples were Western blotted, and signals were detected as described previously (30). Anti-Egr-1 antibodies (sc-189 and sc-588) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

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**Table 1 Oligonucleotide sequences used for PCR and EMSA**

<table>
<thead>
<tr>
<th>Target gene name</th>
<th>Objectives</th>
<th>Forward primer*</th>
<th>Reverse primer*</th>
<th>Annealing temperature (°C)*</th>
<th>Size of amplicon (bp)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Egr-1</strong></td>
<td>Real-time PCR</td>
<td>Egfr1479F: AAGTTTGTCGACGGACGATG</td>
<td>Egfr1678R: CAGGGGATGTTAGTGAGTTG</td>
<td>63</td>
<td>200</td>
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<tr>
<td></td>
<td>EMSA</td>
<td>Egfr1conF: AGAGACTGCAGGCAGAAGC</td>
<td>Egfr1conR: CCCCGGCGCGCGCGAG</td>
<td>60</td>
<td>190</td>
</tr>
<tr>
<td><strong>WT1</strong></td>
<td>Real-time PCR</td>
<td>WT13159F: ACAGATGCAAGACGAGAAGC</td>
<td>WT1548R: CCTGGTGTTGGTCTTCAGG</td>
<td>62</td>
<td>266</td>
</tr>
<tr>
<td><strong>NAB2</strong></td>
<td>Real-time PCR</td>
<td>NB21018F: TGACAGCGCAGAAAGAGGA</td>
<td>NB21223R: AGGTGTCCTCGGTGTCATT</td>
<td>60</td>
<td>211</td>
</tr>
<tr>
<td><strong>CSRIP1</strong></td>
<td>Real-time PCR</td>
<td>CSRIP1/47F: CTCGCAAAGATGCCCCAAGT</td>
<td>CSRIP1/257R: GGCCCATACTTTCGCG</td>
<td>60</td>
<td>195</td>
</tr>
<tr>
<td><strong>TGFB1</strong></td>
<td>Real-time PCR</td>
<td>TGFB1/341F: TTGTGATTCGCCACGGGTG</td>
<td>TGFB1/625R: GGCACCTGAAGAGACGAGAG</td>
<td>60</td>
<td>195</td>
</tr>
<tr>
<td><strong>Aromatase</strong></td>
<td>Real-time PCR</td>
<td>Aromatase/207F: TTGTGTTCTTCTTCTTCTC</td>
<td>Aromatase/208: AAAAGAGCCAGGATTTCAC</td>
<td>72, 60, and 395</td>
<td>398</td>
</tr>
<tr>
<td><strong>c-fos</strong></td>
<td>Real-time PCR</td>
<td>c-fos/249F: TACCAACGAGACTCTCTTC</td>
<td>c-fos/510R: GTGCTTGCGAGTCTTC</td>
<td>69 and 65</td>
<td>282</td>
</tr>
<tr>
<td><strong>HMGIC</strong></td>
<td>Real-time PCR</td>
<td>HMGIC/464F: AAGAGAGACGAGAGGAGGAC</td>
<td>HMGIC/636R: CTCTTGTTGCCACGACTTCG</td>
<td>70 and 65</td>
<td>191</td>
</tr>
<tr>
<td><strong>GAPDH</strong></td>
<td>Real-time PCR</td>
<td>GAPDH/246F: CACCATCTCCTCGGAAAAGT</td>
<td>GAPDH/1456R: GGTCTTCTCCTGCTTGCTT</td>
<td>70 and 65</td>
<td>239</td>
</tr>
<tr>
<td><strong>Sp1</strong></td>
<td>Real-time PCR</td>
<td>Sp1/106F: AATCCAGTGGGCGGGGC</td>
<td>Sp1/109: GCCGTGGTCTGGGACCTGGC</td>
<td>55</td>
<td>279</td>
</tr>
</tbody>
</table>

* EMSA, electrophoretic mobility shift analysis; Egr, early growth response; TGFB, transforming growth factor; HMGIC, high mobility group protein I; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LOH, loss of heterozygosity.

* Multiple temperatures refer to molecules annealed using two or more sequential steps.

* 6-Carboxy-fluorescein (6-FAM)-labeled oligonucleotides.

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Preparation of Nuclear Protein. Mined tissue samples were disrupted using a Dounce homogenizer, and myometrial smooth muscle cells were suspended in hypotonic buffer (10 mM HEPES, 10 mM KCl, 1 mM DTT, 0.5 mM EGTA, and 0.5 mM EDTA (pH 7.4)) containing protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Cells were then resuspended in the same hypotonic buffer containing 0.3% NP40 and quickly vortex mixed. After centrifugation at 100 × g for 5 min, nuclear pellets were washed twice by resuspension and pelleting in hypotonic buffer containing 250 mM sucrose. Pellets were resuspended in 0.5 ml of 0.45 mM NaCl in hypotonic buffer and continuously vortex mixed at 4°C for 30 min. After salt extraction, nuclear pellets were centrifuged at 10,000 × g for 30 min, and supernatants were mixed with stock buffer [20 mM HEPES (pH 7.9), 1 mM EDTA, 100 mM KCl, 1 mM DTT, and 20% glycerol] and stored at −80°C. Protein concentrations were determined in 50-fold dilutions of nuclear extracts using BCA kits (Fierce Chemical Co., Rockford, IL).

Electrophoretic Mobility Shift Analysis. Two synthetic oligonucleotides (Egr-1F and Egr-1R; Table 1) were annealed and purified by PCR (Bio-Rad, Hercules, CA) chromatography to construct a double-stranded oligonucleotide probe corresponding to the Egr-1 consensus sequence (CCGCCC) with cohesive ends. An oligonucleotide probe for Sp1 was similarly produced and purified from Sp1/F and Sp1/R oligonucleotides. The resulting double-stranded probe for Egr-1 was labeled according to the Klenow procedure using [32P]dCTP. Nuclear protein (10 μg) was incubated with 15,000 cpm of probe in 1× binding buffer [20 mM HEPES (pH 7.6), 0.2 mM EDTA, 20% glycerol, 75 mM KCl, 2 μg/poly(dI-dC)/poly(dI-dC) copolymer] for 15 min at room temperature. For competition assays, nonradio-labeled Egr-1 or Sp1 double-stranded oligonucleotides (100-fold excess of radiolabeled probe) were added and incubated for 30 min on ice before addition of the radiolabeled probe. Nuclear extracts were incubated for supershift assays with 1 μl of antibody (for either Egr-1 or Sp1) for 30 min on ice before addition of the radiolabeled probe. The resulting DNA-protein complexes were analyzed using 6% polyacrylamide gels with 0.5× Tris-borate EDTA electrophoresis buffer. Gels were dried and exposed to X-ray film. Antibodies were purchased from Santa Cruz Biotechnology.

Analysis of Loss of Heterozygosity. Three loci of sequence-tagged site markers mapped close to the Egr-1 gene were amplified using multiplex PCR. Reaction mixtures (10 μl) comprised 50 ng of genomic DNA, 150 mM deoxyribonucleotide triphosphates, 0.8 μM each of D5S500 primers, and 0.2 μM each of D5S176 and D5S550 primers. Forward primers of each locus were labeled with 6-carboxy-fluorescein at the 5′ ends. Resulting PCR products were denatured and analyzed by capillary electrophoresis on the ABI Prism 310 automated DNA sequencer. Sizes of DNA fragments were analyzed using GeneScan version 3.1 software (PE Applied Biosystems).

Southern Blotting. Genomic DNA was extracted from frozen tissues using a DNA tissue kit (Qiagen KK, Tokyo, Japan). Genomic DNA (10 μg) was digested using 50 units of EcoRI, BamHI, or HindIII and Southern blotted with a human Egr-1 cDNA probe covalently labeled with alkaline phosphatase using an AlkPhos direct nucleic acid labeling kit (Amersham). Blots were hybridized at 55°C in gold hybridization buffer (Amersham) containing 8 μM urea and stringently washed at 60°C according to the instructions of the manufacturer. Signals were detected on X-ray film (Hyperfilm; Amersham) using a CDP-star detection kit (Amersham).

Transient Transfection Assay. Expression vector for Egr-1 (pcDNA3.1mEgr-1) was provided by Dr. Hajime Karabayashi (Gunma University, Gunma, Japan). An expression vector for secretory-type alkaline phosphatase (pSEAP control vector) was purchased (Clontech, Palo Alto, CA) and cotransfected to monitor transfection efficiency. A pCDNA3.1 vector was also purchased (Clontech, Tokyo, Japan). Genomic DNA (10 μg) was digested using 50 units of EcoRI, BamHI, or HindIII and Southern blotted with a human Egr-1 cDNA probe covalently labeled with alkaline phosphatase using an AlkPhos direct nucleic acid labeling kit (Amersham). Blots were hybridized at 55°C in gold hybridization buffer (Amersham) containing 8 μM urea and stringently washed at 60°C according to the instructions of the manufacturer. Signals were detected on X-ray film (Hyperfilm; Amersham) using a CDP-star detection kit (Amersham).

RESULTS

Expression of Egr-1 and Related Genes

Real-Time PCR Analysis. We quantified mRNA levels for Egr-1 and related genes using real-time RCR. Levels of each transcript did not correlate with clinical parameters of age, menstrual cycle, size of leiomyoma nodules, number of leiomyomas in the same uterus, or relative location of leiomyoma nodules within the uterus. Transcript levels were only affected by preoperative administration of LA, and only use of LA was included in the following analysis.

Levels of Egr-1 transcripts were decreased in all leiomyomas examined (n = 20), compared with corresponding myometrium (Fig. 1). Preoperative exposure to LA increased mean levels of Egr-1 transcript in myometrium, but not in leiomyoma (P < 0.05). No correlation was found between size of leiomyoma nodules and levels of Egr-1 transcripts (data not shown).

We next examined expression of WT1, a modulator of Egr-1 function (18). Levels of WT1 transcripts did not differ between leiomyomas and corresponding myometrium, whereas significantly increased levels of these transcripts were observed after preoperative LA treatment (P < 0.05, ANOVA). Levels of WT1 transcripts in individual samples displayed weak but positive correlations with levels of Egr-1 transcripts in both myometrium (r = 0.58; P = 0.03, Spearman’s rank order correlation test) and leiomyoma (r = 0.63, P = 0.003) [data not shown]. Another important modulator of Egr-1 function, nerve growth factor-induced gene A-binding protein-2 (NAB2), was also examined. NAB2 acts as a transcriptional corepressor specific for Egr-1, but levels did not differ significantly between leiomyoma and corresponding myometrium. Preoperative LA administration significantly increased the levels of NAB2 transcripts (r = 0.57, P < 0.05, ANOVA), whereas transcripts of NAB2 were not observed in leiomyomas examined (n = 0). Levels of Nb2 transcripts in individual samples displayed positive correlations with Egr-1 levels in myometrium (r = 0.50, P < 0.05) and leiomyoma (r = 0.46, P = 0.02).

Expression of another immediate early gene, c-fos, was examined. Levels of c-fos transcript in 18 of 20 leiomyomas were lower than the levels in corresponding myometrium. The remaining two leiomyomas expressed similar or slightly higher levels of c-fos than the corresponding myometrium. Mean levels of transcripts differed significantly between leiomyoma and corresponding myometrium, irrespective of preoperative LA treatment (P < 0). Preoperative LA therapy only increased the mean level of c-fos transcript in myometrium, as seen with Egr-1. Levels of c-fos transcripts in individual samples correlated with levels of Egr-1, irrespective of preoperative LA exposure (r = 0.77, P < 0.001), whereas amounts of transcript were 2 orders of magnitude lower than those of Egr-1. Expression of aromatase was also elevated in leiomyomas from the non-LA-treated group, and this increase was abolished by preoperative LA therapy, consistent with our previous findings (30, 32).

To validate quantitative assays, we also compared expressions of other genes expressed in leiomyoma tissues at levels different from those in myometrium. Levels of cytokine-rich protein 1 transcripts, originally cloned as a transcription factor expression by leiomyoma at high levels, were increased in leiomyomas, as described elsewhere (36). This increase was nullified by preoperative LA therapy. Levels of TGF-β1 transcript were then measured because expression of 4679
TGF-β1 may be higher in leiomyomas (37, 38). No significant difference in levels of TGF-β1 was identified between leiomyoma and myometrium, and no changes between LA-treated and non-LA-treated groups were found. Levels of TGF-β1 expression in individual samples correlated positively with levels of Egr-1 expression (0.63, \( P < 0.001 \)).

**Northern Blotting.** To demonstrate that Egr-1 transcripts detected by real-time PCR were full length, samples were Northern blotted using full-length cDNA for Egr-1 as a probe. Fig. 2 shows that both leiomyomas and surrounding myometrial samples expressed a single band of 3.8 \( \times 10^3 \) nucleotides. Sizes of signals corresponded to those of Egr-1 transcripts detected from other cell types (12). This result negated the possibility of gross abnormality in Egr-1 transcripts, although the presence of small mutations could not be excluded.

**Western Blotting.** Western blotting confirmed decreased levels of Egr-1 protein in leiomyoma tissues. In five sets of tissue samples, the Egr-1 protein level in myometrium was higher than that in corresponding leiomyomas (Fig. 3).

**DNA Binding Activity of Egr-1 in Myometrial Cells**

DNA binding of Egr-1 in uterine smooth muscle cells was confirmed by electrophoretic mobility shift analysis. Nuclear extracts were prepared from leiomyoma cells treated with or without PMA, which is a potent inducer of Egr-1 (2). Use of PMA enhanced detected binding activity without changing binding conduct. Fig. 4 shows that nuclear extracts from PMA-treated cells displayed a bandshift in the presence of Egr-1 probe, and this was competitively abolished on the addition of 100-fold molar excess of unlabeled probe. The band detected by Egr-1 probe underwent a supershift to a slower migrating position on addition of antibody to Egr-1. Notwithstanding the similarity in binding sequences between Egr-1 and Sp1, neither treatment with oligonucleotides containing the consensus sequence for Sp1 binding nor treatment with anti-Sp1 antibody displayed any effect on mobility or intensity of the observed bands. This suggests the fidelity of the Egr-1 probe used for this experiment. Faint but significant binding was detected even for non-PMA-treated leiomyoma cells on overexposed film, although this can barely be seen in Fig. 4. Binding activity of Egr-1 transcripts was also detected in PMA-treated smooth muscle cells (14).
Analysis of Egr-1 Gene Structure in Leiomyoma

To identify genomic alterations capable of explaining the decreased expression of Egr-1, five pairs of genomic DNA samples obtained from leiomyoma nodules and corresponding myometrium were hybridized using labeled Egr-1 cDNA. None of the DNA fragments digested by EcoRI, HindIII, or BamHI differed between leiomyomas and the corresponding myometrium (data not shown).

Short tandem repeat polymorphisms near the Egr-1 gene were analyzed to exclude heterozygous deletion of Egr-1. D5S414, Egr-1, D5S500, and D5S476 were located on 5q31 in this order toward the telomere, within a 3-Mb interval. Among leiomyoma nodules (n = 36) and corresponding myometrium (n = 26) obtained from the 26 patients analyzed, 7, 12, and 14 nodules were informative for the D5S414, D5S500, and D5S476 loci, respectively. No nodules displayed loss of heterozygosity for any locus.

Leiomyoma Cells Retain the Ability to Express Egr-1 and c-fos Genes

Inducibility of Egr-1 and c-fos genes in leiomyoma cells was examined. PMA increased Egr-1 expression in both leiomyoma and control myometrial cells (Fig. 5, A and B). No differences were identified in baseline levels of transcripts, time intervals required to reach maximum induction, or magnitude of induction from baseline expression. Western blotting confirmed induction of Egr-1 protein in leiomyoma cells maintained in serum-free media (Fig. 5C).

Despite the presence of PMA in media, Egr-1 and c-fos transcripts displayed profound and rapid decreases after 4 and 2 h of PMA treatment, respectively (Fig. 5A).
independent experiments, each of which was conducted in triplicate. Thymidine uptake factors are possible inhibitors of cells proliferation (Fig. 6 also demonstrated by Western blot analysis because both growth factor B1, and transforming growth factor β3 expression. Cells were lysed at 12 and 24 h of transfection. Anti-Egr-1 antibody (sc-189) was used at a concentration of 1:5000. Arrowheads indicate the expected size for each protein.

**Fig. 6.** Growth of KW cells transiently transfected with expression vector for early growth response (Egr)-1. A, incorporation of [3H]thymidine is expressed as a percentage of incorporation of each control. Each column and bar represent the mean ± SE of eight independent experiments, each of which was conducted in triplicate. Thymidine uptake was significantly decreased in the group transfected with 1.0 μg of Egr-1 vector compared with controls (Wilcoxon’s signed rank correlation test). B, total cell lysate obtained from collateral transfection experiments was Western blotted to detect Egr-1, transforming growth factor β1, and transforming growth factor β3 expression. Cells were lysed at 12 and 24 h of transfection. Anti-Egr-1 antibody (sc-189) was used at a concentration of 1:5000. Arrowheads indicate the expected size for each protein.

**Restored Expression of Egr-1 Inhibits Growth of Myometrial Cells**

To examine the effect of reexpression of Egr-1 on cell growth, myometrium-derived KW cells, in which endogenous expression of Egr-1 protein is barely detectable, were transiently transfected with different doses of Egr-1 expression vectors. At 24 h after transfection, DNA synthesis was measured according to thymidine uptake during an additional 1-h incubation with [3H]thymidine. Cells transfected with 1.0 μg of Egr-1 vector exhibited lower uptakes than control cells transfected with empty vectors in all eight independent experiments conducted in triplicate (P < 0.05, Wilcoxon’s signed rank test). Similarity, cells transfected with 0.5 μg of Egr-1 tended to display lower uptake than control cells in six of the eight experiments, although differences were not significant (P = 0.09). Fig. 6A shows the means ± SE of thymidine uptake for eight independent experiments, normalized to each control level. Protein levels of Egr-1 expressed in cells reached detectable levels at 12 and 24 h of transfection (Fig. 6B). Concomitant induction of TGF-β1 and TGF-β3 was also demonstrated by Western blot analysis because both growth factors are possible inhibitors of cells proliferation (Fig. 6B).

**DISCUSSION**

We demonstrated that Egr-1 expression is consistently decreased in leiomyoma compared with myometrium and that this is probably attributable to transcriptional down-regulation. Given the tumor suppressor function of Egr-1 shown in other tumor cells, high expression in normal myometrium and consistent decreases in leiomyoma suggest a protective role against the neoplastic phenotype. Egr-1 inhibition of cell proliferation in myometrium-derived KW cells was empirically confirmed.

Our results indicate that decreased expression of Egr-1 is not attributable to genomic alteration but rather to certain transcriptional down-regulators. Contrasting with impaired expression of both basal and gonadotropin-releasing hormone (GnRH) agonist-induced levels seen *in vivo*, leiomyoma cells *in vitro* expressed Egr-1 to the same extent as normal myometrial cells, both at basal and PMA-induced levels. Egr-1 expression thus seems to be inhibited by factors that would be present in leiomyoma tissues *in vivo* and absent in primary cells maintained *in vitro*. Although the mechanisms of Egr-1 down-regulation remain undetermined, concomitant down-regulation of c-fos might offer a clue toward understanding these mechanisms. Expression of c-fos is also consistently down-regulated in leiomyoma, and a significant positive correlation was identified between Egr-1 and c-fos levels in individual samples. Other researchers have noted similar down-regulation of c-fos in leiomyoma (42, 43).

The simplest explanation for this concomitant down-regulation of transcripts is alternation of certain transcriptional factors that bind to *cis*-elements shared by both genes. Both c-fos and Egr-1 promoters contain serum-responsive elements as major *cis*-regulatory elements of transcription (44). These *cis*-responsive element regions comprise a dyad symmetry element for binding serum-responsive factors, and a contiguous Ets motif for binding ternary complex factors such as Elk-1. These promoters can be activated by mitogen-activated protein kinase (MAPK) through phosphorylation of Elk-1 as a constituent of the ternary complex (44, 45). Accordingly, decreased expression of both genes might be attributable to decreased MAPK activity. A preliminary experiment showed, however, that MAPK activity in leiomyoma was somewhat increased compared with surrounding myometrium.² This was an unexpected result, but it agrees with a recent study reporting that phosphorylated extracellular signal-regulated kinase 1/2 is increased in leiomyoma compared with normal myometrium (46). Paradoxical association of activated MAPK and down-regulated c-fos has been noted previously in other transformed cells (47). This paradox can be explained as follows: besides MAPK signal cascades, activation of c-fos transcription depends on transient relaxation of chromatin structure through hyperacetylation of H3/H4 nucleosomal histones (48, 49). This process is mediated by histone acetyltransferase activity and antagonized by histone deacetylase activity. Activated Elk-1 binding to serum-responsive elements of c-fos promoter initially interacts with cAMP response element binding protein (CREB)-binding protein (CBP)/p300, a coactivator possessing histone acetyltransferase activity, enhancing transcription. In contrast, constitutively phosphorylated Elk-1, under sustained activation of the MAPK cascade, changes the interaction partner and permanently recruits histone deacetylase 1 to the promoter, in turn repressing c-fos transcription through hypoacetylation of histones (50). This is a mechanism for transcriptional shutoff of the c-fos gene after transient activation induced by growth factor stimulation. The Egr-1 promoter shares similar serum-responsive elements and Ets binding sites with the c-fos promoter, and transcriptional regulation of Egr-1 closely parallels that of c-fos in numerous cells under various conditions, as seen from our results. Sustained activity of MAPK in leiomyoma may thus lead to down-regulation of Egr-1 expression through mechanisms similar to those found in c-fos.

Reexpression of Egr-1 retarded cell growth in myometrium-derived cells. This is suggestive of a growth-inhibitory function of Egr-1, as observed in tumors of many organs except the prostate. Among the several mechanisms proposed for the tumor-suppressive actions of Egr-1, we examined expression of TGF-β1 and TGF-β3 in cells transfected with Egr-1 vectors. Up-regulation of both isoforms after transfection was confirmed. Actually, levels of Egr-1 mRNA in individual samples correlated positively with

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¹ Unpublished data.
those of TGF-β1 mRNA, even for leiomyoma samples in vivo. However, contrary to expectations for low Egr-1 expression, no decrease in the mean level of TGF-β1 transcript was detected in leiomyoma samples compared with myometrium. We could not exclude the possibility that the real-time PCR method used in this study was not sensitive enough to detect any difference, but another possibility is the existence of factors more potent than Egr-1 that elevate basal expression of TGF-β1 in leiomyoma. If no difference exists in TGF-β1 level between leiomyoma and myometrium, TGF-β1 would not represent the critical factor mediating the growth-inhibitory function of Egr-1. Moreover, TGF-β1 and TGF-β3 have reportedly displayed both accelerative and repressive effects on myometrial cell proliferation, depending on dose, cell origin (normal myometrium or leiomyoma), and culture method (38, 51, 52). Additional experiments using primary cells are needed to determine the significance of impaired expression of Egr-1 in leiomyoma and the role of TGF-βs as a mediator of Egr-1 action.

If Egr-1 down-regulation is important in the etiology or pathogenesis of leiomyoma, one might expect LA to increase Egr-1 levels in leiomyoma because LA therapy ameliorates the disease. However, leiomyoma tissues displayed no increase in Egr-1 mRNA expression after preoperative LA therapy. The most probable explanation is that, in addition to Egr-1, leiomyoma growth is simultaneously regulated by numerous other factors, such as growth factors and cell cycle- and apoptosis-related factors. LA and, more probably, LA-induced estrogen deficiency alter expression of these growth-related genes in addition to Egr-1 (46), with the net effect of retarding neoplastic growth. This complex action of LA would explain shrinkage of leiomyoma cells without detectable up-regulation of Egr-1 in leiomyoma. A second possibility is that the growth-inhibitory action of Egr-1 is mediated by other factors, which would represent the target of GnRH agonist action. GnRH agonists would thus act downstream of Egr-1 and so decrease leiomyoma growth without increasing Egr-1 itself. Another possibility is dissociation between transcript and protein levels, and studies are needed to determine whether protein levels for Egr-1 are increased in leiomyoma after LA therapy.

Our results revealed that preoperative GnRH agonist treatment increased Egr-1 expression in myometrium. In pituitary gonadotropes, GnRH binds to a GnRH receptor and triggers protein kinase C activation, leading to MAPK activation and subsequent enhanced transcription of the Egr-1 gene (53). Because myometrium expresses the GnRH receptor (54), a similar signaling pathway from GnRH to the Egr-1 gene might exist in myometrial cells. Our preliminary experiments confirmed GnRH agonist-induced activation of MAPK and Egr-1 transcription in myometrial cells (data not shown). Considering the inhibitory action of Egr-1 on cell growth, GnRH agonist-induced activation of Egr-1 may mediate part of the direct growth-inhibitory actions of GnRH agonists on myometrial cells (31). Contrary to myometrium, leiomyomas, albeit positive for a GnRH receptor, did not increase their Egr-1 mRNA level in response to preoperative GnRH agonist treatment. This may mean that down-regulatory mechanism (factor) of Egr-1 expression overcomes GnRH receptor-dependent stimulation or function downstream of it.

In summary, Egr-1 expression is significantly decreased in leiomyoma, and this is probably due to down-regulated transcription rather than alteration of the Egr-1 gene. Reactivation of the Egr-1 gene inhibited the growth of myometrium-derived cells. Egr-1 represents a potential target for leiomyoma therapies, and further study is required to define the pathophysiological roles of Egr-1 expression in normal myometrial and leiomyoma cells.

ACKNOWLEDGMENTS

We thank Drs. Osamu Matsuo, Hajime Kurabayashi, and Tetsuo Hirano for providing KW cells, mouse Egr-1 vector, and human Egr-1 vectors, respectively. We are also indebted to Noriko Minami for excellent technical assistance.

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