Progesterone receptor modulator CDB-2914 down-regulates vascular endothelial growth factor, adrenomedullin and their receptors and modulates progesterone receptor content in cultured human uterine leiomyoma cells

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BACKGROUND: This study was conducted to evaluate the effects of graded concentrations (10–8, 10–7 and 10–6 M) of progesterone receptor (PR) modulator CDB-2914 on the protein contents of PR, of vascular endothelial growth factor (VEGF), adrenomedullin (ADM) and their receptors in cultured human uterine leiomyoma and matching myometrial cells. METHODS: PR-A, PR-B, VEGF-A, VEGF-B, VEGF receptor (VEGFR)-1, VEGFR-2, ADM and ADM receptor (ADMR) contents were assessed by Western blot analysis. RESULTS: Treatment with 100 ng/ml progesterone increased VEGF-A, VEGF-B and ADM contents in cultured leiomyoma cells and normal myometrial cells. The concomitant treatment with 10–6 M CDB-2914 significantly decreased the progesterone-induced VEGF-A, VEGF-B and ADM contents in cultured leiomyoma cells but not in normal myometrial cells. CDB-2914 treatment alone decreased VEGFR-1, VEGFR-2 and ADMR contents in cultured leiomyoma cells but not in normal myometrical cells. CDB-2914 treatment increased PR-A and decreased PR-B contents in cultured leiomyoma cells in a dose-dependent manner compared with untreated cultures, whereas no significant changes in PR isoform contents were observed in normal myometrial cells. CONCLUSIONS: These results suggest that CDB-2914 down-regulates VEGF, ADM and their receptor contents and modulates PR isoform contents in cultured leiomyoma cells in a cell-type-specific manner.

Key words: adrenomedullin/CDB-2914/leiomyoma/progesterone receptor modulator/vascular endothelial growth factor

Introduction

Uterine leiomyomas are associated with irregular vascular networks (Walocha et al., 2003), and angiogenic growth factors including vascular endothelial growth factor (VEGF) and adrenomedullin (ADM) have been involved in the angiogenesis of uterine leiomyomas (Hague et al., 2000; Gentry et al., 2001).

VEGF belongs to a gene family that includes VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor. VEGF acts as a key regulator in both physiological and tumour-associated angiogenesis via VEGF receptor (VEGFR) tyrosine kinases, VEGFR-1 (also known as fms-like tyrosine kinase-1) and VEGFR-2 (also known as fetal liver kinase-1 or kinase insert domain region) (Cross et al., 2003; Ferrara et al., 2003). VEGFR-2 is considered to be the major mediator of the physiological and pathological effects of VEGF-A on endothelial cells, including cell proliferation, survival, migration and permeability (Cross et al., 2003). In addition, VEGF-A-induced increase in vascular permeability is thought to contribute to angiogenesis and tumour growth (Ferrara, 2002). Previous studies have demonstrated that uterine myometrial cells express VEGF, VEGFR-1 and VEGFR-2 mRNAs and proliferate in response to VEGF (Brown et al., 1997) and that uterine leiomyomas exhibit a higher expression of VEGF-A than in adjacent normal myometrium (Gentry et al., 2001).

ADM also acts as an angiogenic growth factor (Zhao et al., 1998; Ribatti et al., 2003), as an autocrine growth factor in several cancer cell lines (Miller et al., 1996) and as an apoptosis survival factor in rat endothelial cells (Kato et al., 1997). ADM belongs to the calcitonin superfamily of peptides that includes calcitonin, calcitonin gene-related peptide and amylin (Kuwasako et al., 2004). ADM expression has been reported to be associated with vascular density and endothelial cell proliferation in uterine leiomyomas and myometrium (Hague et al., 2000). Thus, VEGF and ADM are thought to play an important role in
regulating angiogenesis and/or proliferation in leiomyomas and normal myometrium.

Accumulating data support the concept that progesterone plays a vital role in uterine leiomyoma growth (Rein et al., 1995; Matsuo et al., 1997; Shimomura et al., 1998; Kurachi et al., 2001; Maruo et al., 2004). The action of progesterone on target tissues is known to be mediated through interaction with progesterone receptor (PR), which belongs to the nuclear receptor family. PR exists as two isoforms, PR-A and PR-B (Kastner et al., 1990). PR-A is a truncated form of PR-B, lacking 164 amino acids at the N-terminus (Vegeto et al., 1993). Several studies have demonstrated that the expression of PR mRNA and proteins is up-regulated in uterine leiomyomas compared with myometrium (Brandon et al., 1993; Viville et al., 1997; Nisolle et al., 1999) with a consistent dominance of PR-A over PR-B (Viville et al., 1997; Nisolle et al., 1999), suggesting a pivotal role for PR in leiomyoma growth. In this context, recent clinical trials using progesterone antagonist RU486 (Eisinger et al., 2003) and selective PR modulator asoprisnil (J867) (Chwalisz et al., 1993), have recently demonstrated that CDB-2914 inhibits the growth of cultured human uterine leiomyoma cells and induces apoptosis of these cells in a dose- and time-dependent manner (Xu et al., 2005). The effect of CDB-2914 on the expression of these angiogenic growth factors in leiomyoma cells and normal myometrial cells remains to be elucidated. However, the possibility cannot be excluded that CDB-2914 may affect VEGF and ADM expressions in those cells by modulating the function and expression of PR. In female genital organs, progesterone has been reported not only to induce VEGF mRNA in the endometria of cynomolgus monkeys (Greb et al., 1997) and human decidual stromal cells (Ancelin et al., 2002) but also to increase uterine ADM receptor (ADMR) mRNA in adult ovariectomized rats and Eker rat uterine smooth muscle cell line (Thota et al., 2003; Thota and Yallampalli, 2005), suggesting that both VEGF and ADM may be progesterone-regulated genes. In this study, we examined the effects of progesterone on VEGF-A, VEGF-B and ADM protein contents in cultured human uterine leiomyoma cells and normal myometrial cells in the absence or presence of CDB-2914 by Western blot analysis. In addition, the dose-dependent effects of CDB-2914 on VEGF-A, VEGF-B, VEGFR-1, VEGFR-2, ADM, ADMR, PR-A and PR-B protein contents in those cells were examined in comparison with the effects on cultured normal myometrial cells.

Materials and methods

Tissue collection

Twenty-six uterine leiomyoma tissues and adjacent normal myometrium were obtained from Japanese women with regular menstrual cycles, who underwent hysterectomy for uterine leiomyomas at Kobe University Hospital. Informed consent was obtained from each patient before surgery for the use of uterine leiomyoma and myometrial tissues for this study. The Institutional Review Board approved the use of uterine leiomyoma and myometrial tissues for culture experiments. The patients’ age ranged from 31 to 44 years, with a mean age of 36.8 years, and the patients had received no hormonal therapy for at least 6 months before surgery. The histological diagnosis of each uterine specimen was examined. Samples were excluded from the study if accurate menstrual cycle dates could not be assigned or if unexpected pathology was found (e.g. adenomyosis). Each uterine specimen was examined by a pathologist for histological evaluation. Endometrial tissues were obtained from the extirpated uterus, and the day of the menstrual cycle was determined by endometrial histological dating according to the method of Noyes et al. (1950). Fifteen samples were collected from the proliferative phase of the menstrual cycle, and 11 samples were from the secretory phase of the menstrual cycle.

Cell culture

Uterine leiomyoma tissues and adjacent normal myometrium were obtained from the same individual. The central parts of leiomyoma tissues were collected by the careful removal of pseudo-capsules and fibrous septa materials. Tissues obtained were dissected from endometrial layers, cut into small pieces and digested in 0.2% collagenase (wt/vol) at 37°C for 3–5 h (Matsuo et al., 1997). The collagenase treatment has been shown to provide a pure population with smooth muscle cell characteristics without stromal or glandular epithelial cell contamination (Matsuo et al., 1997), and cultured leiomyoma cells were confirmed by immunocytochemistry to be positive for the muscle-specific protein, desmin, and negative for cytokeratin and cytoskeletal protein specific to epithelial cells, cytokeratin 19 (Matsuo et al., 1997). The leiomyoma cells and normal myometrial cells were collected by centripugation at 460 × g for 5 min and washed three times with phosphate-buffered saline (PBS) containing 1% antibiotic solution. Cell viability was determined by Trypan Blue exclusion test. The isolated leiomyoma cells and normal myometrial cells were plated at densities of approximately 1 × 10⁶ cells/dish in 10-cm² culture dishes for 5–7 days and then subcultured at 37°C for 120 h in a humidified atmosphere of 5% CO₂–95% air in Phenol Red-free Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (v/v; Life Technologies Inc., Grand Island, NY, USA). Monolayer cultures approaching 70% confluence were treated with progesterone in the absence or presence of graded concentrations (10⁻⁸, 10⁻⁷ and 10⁻⁶ M) of CDB-2914 (HRA Pharma, Paris, France) in serum-free, Phenol Red-free DMEM for 48 h. CDB-2914 was dissolved in absolute ethanol before use. Final concentration of ethanol in culture media was <0.01%, and the same concentration of ethanol was used as a vehicle in control cultures. After subculture of isolated cells for 120 h, we confirmed that there were no significant differences in VEGF-A, VEGF-B, VEGFR-1, VEGFR-2, ADM and ADMR protein contents in untreated leiomyoma cells and untreated normal myometrial cells obtained from the different menstrual phases.

Western blot analysis for VEGF, VEGFR, ADM, ADMR, PR-A and PR-B

Proteins were extracted from cultured leiomyoma cells and normal myometrial cells as described previously (Shimomura et al., 1998). At the end of the culture period, cells were lysed at 4°C for 20 min in the presence of a lysis buffer consisting of 150 mM NaCl, 2 mM phenylmethylsulphonyl fluoride (PMSF), 1% Nonidet P-40, 0.5% deoxycholate, 1 mg/l aprotinin, 0.1% sodium dodecyl sulphate (SDS) and 50 mM Tris–HCL (pH 7.5). The lysates were subsequently centrifuged at 13 000 × g for 30 min at 4°C, and the supernatants were collected. Protein content in the supernatants was determined by the Bradford assay (Bradford, 1976), and 100 μg aliquots were resolved on a 10% SDS–polyacrylamide gel under reducing conditions. The proteins were then electrophoretically transferred onto nitrocellulose membranes.
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The blots were exposed overnight to goat polyclonal antibodies to VEGF-A and VEGF-B (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal antibodies to VEGFR-1 and VEGFR-2 (Cell Signaling Technology Inc., Livermore, CA, USA), a goat polyclonal antibody to ADM (Santa Cruz Biotechnology), a goat polyclonal antibody to ADMR (Santa Cruz Biotechnology) or a mouse monoclonal antibody to PR (Santa Cruz Biotechnology) at a dilution of 1:500, 1:400, 1:500, 1:400 and 1:500, respectively.

The membranes were incubated for 1 h with horse-radish peroxidase-conjugated anti-mouse, anti-rabbit or anti-goat secondary antibody (Amersham Biosciences, Arlington Heights, IL, USA) that was diluted at 1:1000 with blocking buffer. The antigen–antibody complexes were detected with the enhanced chemiluminescence detection system (Amersham Biosciences). Hybridization signals were visualized by exposure to X-OMAT film (Eastman Kodak Co., Rochester, NY, USA). The radioautograms were then scanned and quantified with ChemiImager 4400 (Astec Co., Ltd, Osaka, Japan). The experiments were repeated with at least three independent cultured specimens, and the reported results are representative.

**Statistical analysis**
The data were expressed as the mean ± SD from at least three independent experiments. Statistical significance was determined using Student’s t-test and one-way analysis of variance. A difference with a P < 0.05 was considered statistically significant.

**Results**

**Effects of progesterone on VEGF-A, VEGF-B and ADM contents in cultured leiomyoma cells and normal myometrial cells in the absence or presence of CDB-2914**

Western blot analysis showed that cultured leiomyoma cells treated for 48 h with 100 ng/ml progesterone alone significantly (P < 0.05) augmented VEGF-A, VEGF-B and ADM contents compared with untreated control cultures (Figure 1, upper panel). The concomitant treatment with 10–6 M CDB-2914 significantly (P < 0.05) antagonized the progesterone-induced stimulatory effects on VEGF-A, VEGF-B and ADM contents (Figure 1, upper panel). Treatment with 10–6 M CDB-2914 alone significantly (P < 0.05) decreased VEGF-A, VEGF-B and ADM contents in leiomyoma cells compared with untreated control cultures (Figure 1, upper panel). On the contrary, in normal myometrial cells, treatment with 100 ng/ml progesterone alone significantly (P < 0.05) augmented VEGF-A, VEGF-B and ADM contents compared with untreated control cultures.

**Figure 1.** Effects of treatment with 100 ng/ml progesterone for 48 h on vascular endothelial growth factor (VEGF)-A, VEGF-B and adrenomedullin (ADM) contents in cultured leiomyoma cells and normal myometrial cells in the absence or presence of 10–6 M CDB-2914, as assessed by Western blot analysis. β-actin was used to ensure the even loading of each specimen. Results (and those in Figures 2–6) represent the mean ± SD of the fold increase over the control value of at least three independent experiments performed in triplicate. *P < 0.05 versus untreated control cultures, †P < 0.05 versus treatment with progesterone alone.
cultures (Figure 1, lower panel). However, the concomitant treatment with $10^{-6}$ M CDB-2914 tended to antagonize the progesterone-induced stimulatory effects on VEGF-A, VEGF-B and ADM contents, but this effect did not reach statistical significance (Figure 1, lower panel). Treatment with $10^{-5}$ M CDB-2914 alone had no apparent effects on VEGF-A, VEGF-B and ADM contents in normal myometrial cells compared with untreated control cultures (Figure 1, lower panel).

**Effects of graded concentrations of CDB-2914 on VEGF-A content**

Treatment with CDB-2914 at concentrations $\geq 10^{-7}$ M significantly ($P < 0.05$) decreased VEGF-A contents in leiomyoma cells compared with untreated control cultures (Figure 2, upper panel). In normal myometrial cells, however, treatment with graded concentrations of CDB-2914 had no effect on VEGF-A content (Figure 2, lower panel).

**Effects of graded concentrations of CDB-2914 on VEGF-B content**

Although treatment with either $10^{-8}$ or $10^{-7}$ M CDB-2914 did not affect VEGF-B content in leiomyoma cells cultured for 48 h, treatment with $10^{-6}$ M CDB-2914 significantly ($P < 0.05$) decreased VEGF-B content in those cells compared with untreated control cultures (Figure 3, upper panel). In normal myometrial cells, however, treatment with graded concentrations of CDB-2914 did not affect VEGF-B content (Figure 3, lower panel).

**Effects of graded concentrations of CDB-2914 on ADM and ADMR content**

Treatment with CDB-2914 at concentrations $\geq 10^{-7}$ M significantly ($P < 0.05$) decreased ADM content in leiomyoma cells cultured for 48 h compared with untreated control cultures (Figure 5, lower panel). However, treatment with graded concentrations of CDB-2914 did not affect VEGF-B content (Figure 3, lower panel).

**Effects of graded concentrations of CDB-2914 on VEGFR-1 and VEGFR-2 content**

Treatment with CDB-2914 at concentrations $\geq 10^{-8}$ M significantly ($P < 0.05$) decreased VEGFR-1 content in leiomyoma cells cultured for 48 h compared with untreated control cultures (Figure 4, upper panel). Furthermore, VEGFR-2 content in leiomyoma cells was significantly ($P < 0.05$) reduced by the treatment with $10^{-6}$ M CDB-2914 (Figure 4, lower panel). In normal myometrial cells, however, treatment with graded concentrations of CDB-2914 did not affect VEGF-2 content (Figure 4, lower panel).
upper panel). There was a significant difference ($P < 0.01$) in ADM content between $10^{-7}$ and $10^{-6}$ M CDB-2914 treatments. In normal myometrial cells, however, treatment with graded concentrations of CDB-2914 did not affect ADM content (Figure 5, upper panel). Furthermore, treatment with CDB-2914 at concentrations $\geq 10^{-8}$ M significantly ($P < 0.01$) decreased ADMR content in leiomyoma cells cultured for 48 h compared with untreated control cultures (Figure 5, lower panel). In normal myometrial cells, however, treatment with graded concentrations of CDB-2914 did not affect ADMR content (Figure 5, lower panel).

**Effects of graded concentrations of CDB-2914 on PR-A and PR-B content**

Compared with untreated control cultures, treatment with CDB-2914 at concentrations $\geq 10^{-7}$ M significantly ($P < 0.05$)
CDB-2914 effects on human leiomyoma and myometrial cells

Increased PR-A content in leiomyoma cells cultured for 48 h, whereas treatment with CDB-2914 at concentrations ≥10^{-8} M significantly (P < 0.05) decreased PR-B content (Figure 6, upper panel), resulting in a significant (P < 0.01) increase in the PR-A/PR-B ratio (Figure 6, upper panel). There was a significant difference (P < 0.05) in PR-A content between 10^{-8} and 10^{-7} M CDB-2914 treatments. In normal myometrial cells, however, treatment with graded concentrations of CDB-2914 affected neither PR-A and PR-B contents nor the PR-A/PR-B ratio (Figure 6, lower panel).

Figure 5. Effects of graded concentrations of CDB-2914 on adrenomedullin (ADM) and ADM receptor (ADM-R) contents in cultured leiomyoma cells and normal myometrial cells, assessed by Western blot analysis. *P < 0.05 versus untreated control cultures, †P < 0.01 versus untreated control cultures.
the action of CDB-2914 may be cell-type-specific and that contents in normal myometrial cells. These results suggest that treatment did not affect VEGFR-1, VEGFR-2 and ADMR dose-dependent, whereas graded concentrations of CDB-2914 VEGFR-2 and ADM contents in leiomyoma cells were furthermore, the inhibitory effects of CDB-2914 on VEGFR-1, VEGF-A, VEGF-B and ADM contents may be PR dependent in those cells. How- ever, CDB-2914 had no significant inhibitory effects on PR-A and PR-B or VEGF-A, VEGF-B, VEGFR-1, VEGFR-2, ADM and ADMR in leiomyoma cells. Moreover, the increase in the PR-A/PR-B ratio correlated with the decrease in the protein contents of VEGF-A, VEGF-B, VEGFR-1, VEGFR-2, ADM and ADMR in leiomyoma cells treated with CDB-2914. In contrast to CDB-2914-induced modulation of the PR-A/PR-B ratio in leiomyoma cells, treatment with CDB-2914 had no apparent effects on PR-A and PR-B or VEGF-A, VEGF-B, VEGFR-1, VEGFR-2, ADM and ADMR contents in normal myometrial cells.

The relative levels of PR-A and PR-B expressions in a cell are reported to be critical for appropriate cellular response to progesterone (Vegeto et al., 1993). The two PR isoforms have different transcriptional activities (Tung et al., 1993; Vegeto et al., 1993; Wen et al., 1994). PR-B functions as a transcriptional
activator of progesterone-responsive genes, whereas PR-A functions as a ligand-dependent repressor of PR-B transcriptional activity (Vegeto et al., 1993). Wu et al. (2004) have provided evidence that PR-B is the dominant inducer of VEGF mRNA in breast cancer cells and that PR-A may suppress PR-B-dependent induction of VEGF. Additionally, in a co-transfection study of PR-A or PR-B in human myometrial cells taken at the time of Caesarean section, Pieber et al. (2001) demonstrated that reporter expression was significantly induced by the presence of progesterone in myometrial cells when PR-B was overexpressed but not when there was excess of PR-A in these cells. A precise mechanism by which PR-A and PR-B modulate VEGF and ADM protein contents in cultured leiomyoma cells and normal myometrial cells remains to be elucidated. However, it could be postulated that decreased PR-B modulate VEGF and ADM protein contents in cultured leiomyoma cells in the absence of comparable effects on normal myometrial cells when PR-B was overexpressed but not when there was excess of PR-A in these cells. A precise mechanism by which PR-A and PR-B modulate VEGF and ADM protein contents in cultured leiomyoma cells and normal myometrial cells remains to be elucidated. However, it could be postulated that decreased PR-B content may lead to the decreased gene transcription of VEGF and ADM and that increased PR-A content in cultured leiomyoma cells treated with CDB-2914 may act to suppress the transcriptional activities of PR-B, resulting in the decrease in VEGF and ADM gene transcription in those cells.

On the contrary, the reason why CDB-2914 does not induce a significant induction of VEGF and ADM contents in cultured normal myometrial cells remains to be determined. Unlike in leiomyoma cells, no apparent alteration of PR isoform content and PR-A/PR-B ratio was found in normal myometrial cells in response to CDB-2914 treatment. However, whether CDB-2914-induced modulation of the PR-A/PR-B ratio in cultured leiomyoma cells and normal myometrial cells causes the differential gene transcription for VEGF and ADM remains to be elucidated. An additional study is needed to determine the effect of overexpression of PR-A and PR-B on the expression of the VEGF and ADM genes and their receptors in cultured leiomyoma cells and normal myometrial cells by using transient transfection. CDB-2914 may differently regulate the recruitment of coactivators and corepressors in cultured leiomyoma cells and normal myometrial cells. Coactivators and corepressors are nuclear proteins that modulate the transcriptional activity of nuclear receptors. Coactivators enhance the transcriptional activity of nuclear receptors, whereas corepressors elicit the inhibitory effects (Chwalisz et al., 2005). Progesterone agonists promote the interactions of the nuclear receptor with coactivators, whereas progesterone antagonist favours interactions with corepressors or inhibits interactions with coactivators (Chwalisz et al., 2005). The ability of RU486 to activate gene transcription is shown to be modulated by the ratio of coactivators to corepressors (Liu et al., 2002). The effect of CDB-2914 on the expression of coactivators and corepressors in cultured leiomyoma and normal myometrial cells remains unknown at present, and further investigation will be needed. However, it seems reasonable to speculate that CDB-2914 may exhibit the progesterone-antagonistic effects by enhancing the expression of corepressors and suppressing the expression of coactivators in leiomyoma cells without affecting the induction of these coregulators in normal myometrial cells. Collectively, CDB-2914 can selectively down-regulate the expression of the VEGF/VEGFR and ADM/ADMR systems in cultured leiomyoma cells in the absence of comparable effects on normal myometrial cells.

In conclusion, we have demonstrated for the first time that progesterone up-regulates VEGF-A, VEGF-B and ADM protein contents in both cultured human uterine leiomyoma cells and normal myometrial cells but that CDB-2914 treatment selectively down-regulates VEGF-A, VEGF-B and ADM together with VEGFR-1, VEGFR-2 and ADMR contents in leiomyoma cells without affecting the contents of these angiogenic growth factors and their receptors in normal myometrial cells. In addition, we have shown that the increase in the PR-A/PR-B ratio correlated with the decrease in VEGF, VEGFR, ADM and ADMR contents in leiomyoma cells treated with CDB-2914, but not in normal myometrial cells. These results suggest that CDB-2914 may inhibit growth and angiogenesis of uterine leiomyomas in a cell-type-specific manner through disrupting the VEGF/VEGFR and ADM/ADMR systems. Further studies will be necessary to clarify the precise molecular mechanism underlying the cell-type-specific action of CDB-2914 in leiomyoma cells.

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