Concentration-dependent effects of a selective estrogen receptor modulator raloxifene on proliferation and apoptosis in human uterine leiomyoma cells cultured in vitro

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BACKGROUND: This study was conducted to elucidate the effects of raloxifene on proliferation and apoptosis in cultured human uterine leiomyoma cells. METHODS: The monolayer cultures were treated with graded concentrations (10⁻⁹, 10⁻⁸ and 10⁻⁷ M) of raloxifene and 10⁻⁷ M 17β-estradiol (E₂). Cell viability, percentage of proliferating cell nuclear antigen (PCNA)-positive cells, percentage of terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick-end labelling (TUNEL)-positive cells and the expression of PCNA and Bcl-2 proteins were assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazoliol assay, immunocytochemistry, TUNEL assay and western blot analysis, respectively. RESULTS: Compared with untreated cultures, the number of viable cultured cells, percentage of PCNA-positive cells and PCNA protein expression were significantly decreased by treatment with 10⁻⁹ M raloxifene, but increased by treatment with either 10⁻⁸ M or 10⁻⁷ M raloxifene. In contrast, the percentage of TUNEL-positive cells was significantly increased and Bcl-2 protein expression was significantly decreased by treatment with 10⁻⁹ M raloxifene, whereas they were not affected by treatment with either 10⁻⁸ or 10⁻⁷ M raloxifene. CONCLUSIONS: In cultured leiomyoma cells, low concentration (10⁻⁹ M) of raloxifene may inhibit the growth of leiomyoma cells, whereas high concentrations (10⁻⁸ M, 10⁻⁷ M) of raloxifene may promote their growth.

Key words: apoptosis/leiomyoma/proliferation/raloxifene/selective estrogen receptor modulator

Introduction

Uterine leiomyomas (fibroids) are the most common type of solid tumour in adult women, clinically apparent in at least 25% of those of reproductive age (Buttram et al., 1981; Cramer et al., 1990; Steward, 2001). Because leiomyomas do not occur before the menarche and markedly shrink in hypoestrogenic conditions, such as late menopause, ovariectomy or gonadotropin-releasing hormone agonist therapy, it is presumed that growth of these tumours depends on estrogens (Robboy et al., 2000; Steward, 2001). The physiological effects of estrogens are mediated by the estrogen receptor (ER), of which both subtypes, ERα and ERβ, were found in fibroids (Benassayag et al., 1999; Wang et al., 2001).

Selective estrogen receptor modulators (SERMs) are ER ligands that display an unusual tissue-selective pharmacology: they are agonists in some tissues (bone, liver and the cardiovascular system), antagonists in the breast and mixed agonists/antagonists in the uterus (Lewis and Jordan, 2005). SERMs act on their target receptors by diffusing into the cell and binding to ERα or ERβ. The binding causes a conformational change and the dimerization of the receptors, which facilitates binding of co-regulatory proteins (co-activators and co-repressors) that activate or repress transcriptional activation of estrogen target genes (Osborne et al., 2000). Raloxifene, a benzothiophene derivative, is the most widely studied of the newer SERMs. It has been approved for the treatment and prevention of post-menopausal osteoporosis. Preclinical data have revealed that raloxifene may have a beneficial effect on uterine leiomyomas (Black et al., 1994; Brant et al., 1996; Fuchs-Young et al., 1996; Porter et al., 1998; Walker et al., 2000). The results of clinical studies also confirmed the efficacy of raloxifene on leiomyomas (Jirecek et al., 2004; Palomba et al., 2001, 2005). Moreover, Palomba et al. reported that raloxifene showed antiproliferative and proapoptotic
actions on leiomyoma cells from post-menopausal women (Palomba et al., 2005). However, there are no experimental data demonstrating the direct effects of raloxifene on cultured human leiomyoma cells.

Therefore, in order to evaluate the effects of raloxifene on the growth of leiomyoma, we examined the effects of raloxifene on proliferation and apoptosis in human uterine leiomyoma cells cultured in vitro.

Materials and methods

Tissue collection
Twelve uterine leiomyoma tissues were obtained from premenopausal women with regular menstrual cycles who underwent abdominal hysterectomy or myomectomy for medically indicated reasons at Kobe University Hospital. Informed consent was obtained from each patient before surgery for the use of uterine tissues for the present study. The institutional review board approved the use of uterine tissues for culture experiments. The patients ranged in age from 42 to 51 years, with a mean age of 48.4 years, and had received no hormonal therapy for at least six menstrual cycles before surgery. The histological diagnosis of each uterine specimen was examined. Samples were excluded from the study if accurate date of menstrual cycle could not be assigned or if unexpected pathology was found (e.g. adenomyosis or leiomyosarcoma).

Cell culture
Uterine leiomyoma tissues, dissected from endometrial cell layers, were cut into small pieces and digested in 0.2% collagenase (wt/vol) at 37°C for 3–5 h, as previously described (Matsuo et al., 1997). Leiomyoma cells were collected by centrifugation 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 were plated at densities of approximately 1 × 10^5 cells/dish in 10 cm² culture dishes, 4 × 10^4 cells/well in two-well chamber glass slides and 1 × 10^4/well in 96-well tissue culture plates. The isolated leiomyoma cells in culture dishes and two-well chamber slides were subcultured at 37°C for 120 h in a humidified atmosphere of 5% CO₂–95% air in Phenol Red-free Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (vol/vol; Invitrogen Life Technologies, Inc., Grand Island, NY, USA). The isolated leiomyoma cells in 96-well tissue culture plates were subcultured for 72 h under the conditions described above. The monolayer cultures at approximately 70% confluence were treated with graded concentrations (10⁻⁹, 10⁻⁸ and 10⁻⁷ M) of raloxifene hydrochloride (Eli Lilly and Co., Indianapolis, IN, USA) according to manufacturer’s protocol for proliferation assay. Experiments were performed in triplicate. Results were expressed as the OD at 490 nm.

Cell proliferation assay
The cell viabilities of cultured leiomyoma cells were colorimetrically determined using a cell proliferation assay kit (Cell Titer 96 AQueous One Solution Cell Proliferation Assay, Promega Corp., Madison, WI, USA). After being treated in the absence or presence of graded concentrations (10⁻⁹, 10⁻⁸ and 10⁻⁷ M) of raloxifene and 10⁻⁷ M E₂ in serum-free DMEM for 24, 48 and 72 h in a 96-well tissue culture plate, 20 µl Cell Titer 96 AQueous One Solution Reagent containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) and phenazine ethosulphate was added to each well, and cultured cells were incubated at 37°C in a humidified, 5% CO₂ atmosphere for 4 h. The absorbance of soluble formazan produced by cellular reduction of the MTS was measured at 490 nm using an MTP-120 enzyme-linked immunosorbent assay plate reader (Corona Electric Co., Osaka, Japan). The MTS tetrazolium compound is bioreduced by cells into formazan product by reduced nicotinamide adenine dinucleotide phosphate or reduced nicotinamide adenine dinucleotide produced by dehydrogenase enzymes in metabolically active cells (Berridge and Tan, 1993). The quantity of formazan product, as measured by the amount of 490 nm absorbance, is directly proportional to the number of living cells in culture. Experiments were performed in triplicate. Results were expressed as the OD at 490 nm.

Immunocytochemical staining for proliferating cell nuclear antigen
After being treated in the absence or presence of graded concentrations (10⁻⁹, 10⁻⁸ and 10⁻⁷ M) of raloxifene and 10⁻⁷ M E₂ in serum-free DMEM for 48 h, the leiomyoma cells cultured in two-well chamber slides were washed three times with PBS, fixed in methanol at 4°C for 20 min and again washed with PBS three times. The fixed cells were subjected to immunocytochemical staining by the avidin/biotin immunoperoxidase method using a polyvalent immunoperoxidase kit (Omnitags, Lipshow, MI, USA) according to manufacturer’s instructions. A mouse monoclonal antibody to human proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used as the primary antibody at a dilution of 1:80. To assure the specificity of the immunological reaction, cultured cells were subjected to the same immunoperoxidase method, except that the primary antibody was replaced by nonimmune murine IgG (Miles, Elkhart, IN, USA) at the same dilution as the specific antibody. The replacement of the specific primary antibody with non-immune murine IgG resulted in a lack of positive immunostaining for PCNA.

Immunocytochemical staining was analysed by two investigators in a blinded fashion without knowledge of the experimental group. The PCNA-positive rate was determined by observing more than 1000 nuclei for each experimental sample and was used for evaluating the proliferating activity of leiomyoma cells.

In situ terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick-end labelling assay
In situ labelling of fragmented DNA in cultured leiomyoma cells was performed with the terminal deoxynucleotidyltransferase-mediated 2'-deoxyuridine 5'-triphosphate nick-end labelling (TUNEL) assay, using the ApopTag in situ apoptosis detection kit (Intergen Co., Purchase, NY, USA) according to manufacturer’s protocol for monolayer cultures. Leiomyoma cells were subcultured in two-well glass chamber slides for 120 h and then cultured under serum deprivation conditions for 48 h in the absence or presence of graded concentrations (10⁻⁹, 10⁻⁸ and 10⁻⁷ M) of raloxifene and 10⁻⁷ M E₂. At the termination of cultures, nucleotide-sized DNA fragments were tailed with digoxigenin-deoxy-UTP and then bound with peroxidase-conjugated antidigoxigenin antibodies. The nuclei were counterstained with haematoxylin (Zymed Laboratories, Inc., San Francisco, CA, USA) for determining the TUNEL-positive rate of cultured leiomyoma cells.

Apoptosis of cultured leiomyoma cells was analysed by two investigators in a blinded fashion without knowledge of the experimental group. All stained nuclei were scored as positive for apoptosis. The TUNEL-positive rate was determined by observing more than 1000 nuclei for each experimental sample.
Western blot analysis for PCNA and Bcl-2
Proteins were extracted from cultured leiomyoma cells as described previously (Shimomura et al., 1998). After being treated in the absence or presence of graded concentrations (10⁻⁹, 10⁻⁸ and 10⁻⁷ M) of raloxifene and 10⁻⁷ M E₂ in serum-free DMEM for 48 h, cells were lysed at 4°C for 20 min in the presence of a lysis buffer consisting of 150 mM NaCl, 2 mM phenylmethylsulphonylfluoride, 1% Nonidet P-40, 0.5% deoxycholate, 1 mg/liter aprotinin, 0.1% sodium dodecyl sulphate and 50 mM Tris–HCl, pH 7.5. The lysates were subsequently centrifuged at 13,000 for 30 min at 4°C, and the supernatants were collected. Protein content in the supernatants was determined by the Bradford assay (Bradford, 1976). Each 100 µg aliquot of the protein extract from cultured leiomyoma cells were separated by NuPAGE Novex 4–12% Bis–Tris Gel (Invitrogen Life Technologies, Carlsbad, CA, USA) under a reducing condition using 200 V for 50 min. The proteins were then electrophoretically transferred from gels to PVDF transfer membranes (Amersham Biosciences). The blots were exposed overnight to a mouse monoclonal antibody to PCNA (Santa Cruz Biotechnology, Inc.) and a mouse monoclonal antibody to Bcl-2 (Santa Cruz Biotechnology, Inc.) at dilutions of 1:200 and 1:200. The membranes were visualized by exposure to X-OMAT film (Eastman Kodak Co., Rochester, NY, USA). The radioautograms were then scanned and quantified with ES Epson 8500 (Seiko Epson Co., Japan). The experiments were repeated with at least six different cultured specimens with similar results, and the reported results are representative.

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

Results

Effects of graded concentrations of raloxifene on the number of viable cultured leiomyoma cells
Comparative effects of treatment with graded concentrations of raloxifene and 10⁻⁷ M E₂ for 24, 48 and 72 h on the number of viable cultured leiomyoma cells were determined by MTS assay (Figure 1). Compared with untreated control cultures, treatment with 10⁻⁹ M raloxifene for 48 and 72 h significantly decreased the number of viable cultured leiomyoma cells, whereas treatment with either 10⁻⁷ M raloxifene or 10⁻⁸ M E₂ for 24, 48 and 72 h or 10⁻⁸ M raloxifene for 48 and 72 h significantly increased the number of viable cultured leiomyoma cells (P < 0.05). 10⁻⁷ M raloxifene and 10⁻⁷ M E₂ at 24 h, 10⁻⁸ M raloxifene at 48 and 72 h; P < 0.01, 10⁻⁸ and 10⁻⁷ M raloxifene, 10⁻⁷ M E₂ at 48 and 72 h).

Effects of graded concentrations of raloxifene on PCNA expression
Figure 2A represents immunocytochemical staining for PCNA in leiomyoma cells cultured for 48 h (a–e) in the absence or presence of graded concentrations of raloxifene and 10⁻⁷ M E₂. PCNA-positive nuclei in cultured leiomyoma cells treated with 10⁻⁹ M raloxifene (Figure 2Ac) for 48 h were apparently less than those in untreated control cultures for 48 h (Figure 2Aa). The number of PCNA-positive nuclei in cultured leiomyoma cells treated with either 10⁻⁸ M (Figure 2Ad), 10⁻⁷ M raloxifene (Figure 2Ae) or 10⁻⁷ M E₂ (Figure 2Ab) was more abundant than that in untreated control cultures (Figure 2Aa). Replacement of the primary antibody with non-immune murine IgG resulted in a lack of positive immunostaining for PCNA in cultured leiomyoma cell nuclei (data not shown).

Figure 2B shows the percentage of PCNA-positive nuclei of leiomyoma cells cultured in the absence or presence of either graded concentrations of raloxifene or 10⁻⁷ M E₂ for 48 h. Compared with untreated control cultures, treatment with 10⁻⁹ M raloxifene decreased the percentage PCNA-positive nuclei of cultured leiomyoma cells (P < 0.05). A significant increase in the PCNA-positive percentage was obtained by 48 h treatment with either raloxifene at concentrations higher than 10⁻⁸ M or 10⁻⁷ M E₂ (P < 0.01).

Western blot analysis of proteins extracted from leiomyoma cells cultured in the absence or presence of either graded concentrations of raloxifene or 10⁻⁷ M E₂ for 48 h revealed that cultured leiomyoma cells contained immunoreactive PCNA with a molecular mass of approximately 36 kDa. Compared with untreated control cultures, treatment with 10⁻⁹ M raloxifene significantly (P < 0.05) decreased 36 kDa PCNA expression in cultured leiomyoma cells (Figure 3). In contrast, treatment with either raloxifene at concentrations of 10⁻⁸ and 10⁻⁷ M or 10⁻⁷ M E₂ significantly (P < 0.01) increased 36 kDa PCNA expression compared with untreated control cultures (Figure 3).

Effects of graded concentrations of raloxifene on the TUNEL-positive rate
Figure 4A represents the distribution of TUNEL-positive nuclei in leiomyoma cells cultured for 48 h (a–e) in the absence or presence of either graded concentrations of raloxifene or 10⁻⁷ M E₂. Compared with untreated control cultures...
at 48 h (Figure 4Aa), TUNEL-positive nuclei were more abundant in cultured leiomyoma cells treated with $10^{-9}$ M raloxifene (Figure 4Ac) for 48 h, although TUNEL-positive nuclei were not affected by 48 h treatment with either $10^{-8}$ M (Figure 4Ad) and $10^{-7}$ M raloxifene (Figure 4 Ae) or $10^{-7}$ M E2 (Figure 4Ab) for 48 h. The number of PCNA-positive nuclei in cultured leiomyoma cells treated with either $10^{-9}$ M raloxifene (d) and $10^{-7}$ M raloxifene (e) or $10^{-7}$ M E2 (b) for 48 h was remarkably more abundant than that in untreated control cultures (a) for 48 h. Bars, 5 µm. Original magnification, ×400. (B) The mean percentage of PCNA-positive nuclei of leiomyoma cells cultured in the absence or presence of graded concentrations of raloxifene and $10^{-7}$ M E2 for 48 h. Results represent the mean ± SD of six independent experiments performed in triplicate. *$P < 0.05$; **$P < 0.01$ (versus percentage of PCNA-positive nuclei in untreated leiomyoma cells in control cultures).

Effects of graded concentrations of raloxifene on Bcl-2 protein expression

Effects of treatment with graded concentrations of raloxifene and $10^{-7}$ M E2 for 48 h on Bcl-2 protein expression in cultured leiomyoma cells were assessed by western blot analysis (Figure 5). Compared with untreated control cultures, treatment with either $10^{-9}$ M raloxifene or $10^{-7}$ M E2 significantly ($P < 0.05$) decreased 26 kDa Bcl-2 protein expression in cultured leiomyoma cell, whereas treatment with raloxifene at concentrations of $10^{-8}$ and $10^{-7}$ M did not affect 26 kDa Bcl-2 protein expression compared with untreated control cultures.

Discussion

The present study demonstrates for the first time the direct effects of raloxifene on proliferation and apoptosis in human...
uterine leiomyoma cell cultured in vitro. We found in this study that $10^{-9}$ M raloxifene might inhibit the growth of uterine leiomyoma cells by down-regulating proliferation and by up-regulating apoptosis in those cells, whereas $10^{-8}$ and $10^{-7}$ M of raloxifene might promote cell growth by up-regulating proliferation without affecting apoptosis in those cells.

As for the effects of raloxifene on uterine leiomyomas, it has been demonstrated in the clinical trials that raloxifene induces the decrease in the size of uterine leiomyomas in premenopausal women (Jirecek et al., 2004). Furthermore, studies on animal models have also demonstrated that raloxifene inhibits the proliferation of rat leiomyoma cells in vitro (Fuchs-Young et al., 1996), the administration of raloxifene induces a fast regression of abdominal wall estrogen-induced leiomyomas in guinea pigs (Porter et al., 1998) and the treatment with tamoxifen or with a raloxifene analogue reduces the size of leiomyomas and the incidence (Walker et al., 2000). These studies have indicated the anti-estrogenic effects of raloxifene on leiomyoma.

However, in premenopausal women, Palomba et al. (2002) have shown no significant effect on uterine and leiomyoma size after raloxifene administration at the dose of 60 or 180 mg/day. Jirecek et al. (2004) have indicated that the different efficacy of raloxifene between different doses and patient groups within premenopausal women may be explained by the different ratio of circulating raloxifene and estrogen, and because the circulating serum estrogen decreases with age within premenopausal women, raloxifene seems to be more effective in the treatment of leiomyomas in elderly premenopausal women than in younger ones. Palomba et al. (2004, 2005) have described that in perimenopausal women with low sex hormone level, high dose (180 mg/day) of raloxifene probably only inhibits leiomyoma growth but does not have any clinical effect on uterine and leiomyoma dimensions.

Clinical trials with raloxifene have demonstrated that the serum concentration of raloxifene is in the $10^{-9}$ M range when given the currently recommended dose of 30–150 mg/day (Kim et al., 2002). Moreover, raloxifene is rapidly absorbed from the gastrointestinal tract and undergoes extensive first-pass glucuronidation, approximately 60% of a dose is absorbed, but absolute bioavailability is only 2%
showed that 10^{-9} and 10^{-8} M and close to 10^{-9} M. Our findings showed that 10^{-9} M raloxifene exerted growth inhibitory effects on cultured uterine leiomyoma cells by decreasing proliferation and increasing apoptosis in those cells with decreasing PCNA and decreasing Bcl-2 expressions. These findings are basically consistent with the preclinical and clinical findings previously mentioned.

Besides, it has been shown in other tissues that raloxifene acts on the metabolism, the central nervous system and the cardiovascular system as an estrogenic agonist, whereas it shows an estrogenic antagonist effect on the breast (Riggs and Hartmann, 2003). The tissue-specific actions of SERMs can be explained by three interactive mechanisms: (i) differential ER conformation upon ligand binding, (ii) differential expression and binding to the ER of coregulatory protein and (iii) differential ER expression and gene activation via non-estrogen response element (ERE) interaction (tethered pathway) (Lewis and Jordan, 2005). That is to say, individual SERM may induce specific and unique changes in receptor conformations. Crystallographic data of ERα and ERβ complexed with a variety of SERMs have revealed that different ligands produce distinct ligand-binding domain conformations of ER, and Helix 12, the most C-terminal of these, has been identified as the critical core of the receptor’s AF-2 domain by virtue of its role in regulating co-activator binding to the ligand-bound ERα (Danielian et al., 1992; Henttu et al., 1997; Shiau et al., 1998). The relative concentration of co-activators (SRC-1, SRC-2, SRC-3/AIB1) or co-repressors and their selective interaction with ERα is another possibility, which may explain the target site specificity for SERM action (Schiff et al., 2003; Shao and Brown, 2004). Furthermore, SERM–receptor complex may interact with the AP-1 proteins, c-Jun and c-Fos through tethered pathway, instead of binding to the ERE activate transcription (Paech et al., 1997). Another idea for the mechanism of SERM action is that the ratio of ERα to ERβ is important for expressing the estrogen-like actions of anti-estrogens because on most instances, ERα acts as an activator, whereas ERβ can inhibit the action of ERα by forming heterodimers with it (Peterson et al., 2000).

In contrast, our findings showed that high concentrations (10^{-8} M, 10^{-7} M) of raloxifene promoted the growth of cultured leiomyoma cells by increasing proliferation without decreasing the apoptosis of those cells. That is to say, low concentration of raloxifene inhibited the growth of leiomyoma cells, whereas high concentrations of raloxifene promoted it. Raloxifene at different concentrations exerted different actions on cultured leiomyoma cells. Interestingly, other authors (Hibner et al., 2004) have demonstrated that high concentrations (10 ng/ml, 100 ng/ml) of raloxifene promote the growth of endometrial cancer cells 	extit{in vitro}. The above concentrations are properly in the 10^{-8} and 10^{-7} M ranges. In that study, the growth of those cells did not differ from that of the control cells when cells were cultured with a low concentration of raloxifene (1 ng/ml, it is just in the 10^{-9} M range). Thus, we hypothesize whether raloxifene has the concentration-specific effects in the same tissue. A potential explanation is that raloxifene at different concentrations may change the expression of ER or the ratio of ERα/ERβ or the expression of cofactors. In addition, it was reported that raloxifene-resistant MCF-7 cell model could be established by culturing MCF-7 cells for 12 months in medium containing 10^{-6} M raloxifene. When cultured with 10^{-6} M raloxifene, raloxifene-resistant cells grew significantly faster than MCF-7 cells. The studies about the phenomenon suggested that the nuclear factor-κB (NF-κB) signal transduction pathway could be involved in the survival of raloxifene-resistant MCF-7 cells (Liu et al., 2003). NF-κB may be possible to explain the effect of high concentration of raloxifene on leiomyoma cells. However, the precise mechanism underlying the effects of different concentrations of raloxifene in uterine leiomyoma cells remains to be determined. Further study will be necessary to conform the hypothesis and to investigate the effects of raloxifene at different concentrations on ER and cofactors expression in cultured leiomyoma cells.

In conclusion, the present study suggests that low concentration of raloxifene may inhibit cultured leiomyoma cells growth by down-regulating proliferation and by up-regulating apoptosis, although high concentrations may promote cell growth by up-regulating proliferation without affecting apoptosis in those cells. Since such high concentrations of raloxifene are almost never reached according to the present clinical dose of raloxifene, raloxifene may be a relatively safe compound for the women with uterine leiomyoma.

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References


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