ICI182,780 is used in adjuvant therapies of breast cancer. As a complete estrogen receptor (ER) blocker, ICI182,780 may antagonize the effects of estrogen on the cardiovascular system. Estrogen inhibits the proliferation of vascular smooth muscle cells (VSMCs), which is one of the mechanisms that estrogen can exert cardioprotective effects. In the present study, to assess the effects of ICI182,780 on the proliferation of VSMCs, we cultured VSMCs isolated from rat aorta with or without the ER antagonist ICI182,780. The results of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, bromodeoxyuridine incorporation assay, viable cell count, immunochemical staining for proliferating cell nuclear antigen (PCNA), and S-phase ratio determined by flow cytometry revealed a remarkable proliferation of VSMCs after ICI182,780 treatment. ICI182,780 significantly enhanced cell growth in a dose-dependent manner (10^{-8}–10^{-5} M). Furthermore, the number of PCNA-positive cells and the S-phase progression of VSMCs increased after treatment with ICI182,780. Reverse transcriptase-polymerase chain reaction (RT–PCR) and western blot analysis showed that the mRNA and protein level of cyclin D1 in VSMCs increased under the treatment of ICI182,780. These data suggested that ICI182,780 can promote the growth of VSMCs, which might produce some adverse effects on the cardiovascular system.

Keywords  ICI182,780; antiestrogen; vascular smooth muscle cells; proliferation

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Introduction

The antiestrogen agent ICI182,780 has been approved by the US Food and Drug Administration for the treatment of hormone-receptor-positive metastatic breast cancer in post-menopausal women. ICI182,780 completely blocks the estrogen receptor (ER), inhibits the transcriptional activities of the ER, and induces a rapid proteasome-dependent degradation of the receptor. These effects are associated with its anti-proliferative activities in the breast glands. However, due to its anti-estrogenic activities, ICI182,780 may produce some adverse effects on the cardiovascular system.

Women generally experience the initial manifestations of coronary artery disease 10 years later than men, suggesting that estrogen may play a cardioprotective role. The proliferation of vascular smooth muscle cells (VSMCs) is the key event in the development of atherosclerosis. Several studies have shown that estrogen inhibits the proliferation of VSMCs [1–3]. Two subtypes of the ER, namely ERα and ERβ, are expressed in VSMCs, and they appear to mediate the inhibitive effects of estrogen on VSMCs proliferation [4–6]. The absence of functional ERα is associated with premature coronary artery disease [7]. ERα is expressed in the majority of artery samples obtained from normal pre- and post-menopausal women, but in very few arteries obtained from women with coronary artery disease [8]. The methylation of the gene encoding ERα in human coronary atherosclerotic plaques was increased compared with the gene in the normal aortic tissue, which corroborated the above-mentioned results [9]. On the basis of these potential effects of estrogen and ER, ICI182,780, a complete ER blocker, might promote the proliferation of VSMCs. However, previous studies on ICI182,780 treatment have not given enough consideration to this possibility. Thus, in the present study, we performed a series of experiments to assess the effects of ICI182,780 on the proliferation of VSMCs.
Materials and Methods

Cell culture and identification

Rat aortic smooth muscle cells (RASMCs) were isolated from the aorta of 8-week-old female Wistar rats (Animal center, Shandong University). Aortic slices were stripped off the endothelium and adventitia, rinsed, and cut into small pieces. The primary culture of RASMCs was maintained in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, Logan, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a humidified atmosphere of 95% air and 5% CO2. The smooth muscle cell (SMC) was confirmed by standard immunocytochemistry staining with rabbit anti-smooth muscle α-actin polyclonal antibody (1:100 dilution; Boster, Wuhan, China). The cells from passages four to seven were used for subsequent experiments. The study was approved by the Animal Care Committee of Shandong University and was performed according to the Guidelines for the Use of Experimental Animals by the Ministry of Health, China.

Cell proliferation assay by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method

A total of 5000 cells was seeded per well in 96-well culture plates and incubated in DMEM with 10% FBS at 37°C and 5% CO2 for 24 h. Then, the cells were starved in phenol-red-free DMEM (Hyclone) with 0.4% dextran-coated charcoal-stripped FBS (DCC-FBS; Hyclone) for 48 h, and subsequently, the medium was replaced with new phenol-red-free DMEM containing 5% DCC-FBS. The cells were subject to different treatments as follows: vehicle (0.1% alcohol), 10⁻⁸ M 17β-estradiol (Sigma, St. Louis, USA), or ICI182,780 (Tocris Bioscience, Ellisville, USA) of the different final concentrations of 10⁻⁸, 10⁻⁷, 10⁻⁶, and 10⁻⁵ M. After incubation for 48 h, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well. After further incubation at 37°C and 5% CO2 for 4 h, the culture medium was aspirated and replaced with 150 µl/well of DMSO. The plates were then agitated for 10 min, and the optical density (OD) was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Rad, Hercules, USA).

Analysis of DNA synthesis by 5-bromo-2'-deoxyuridine incorporation assay

RASMCs proliferation was examined using a 5-bromo-2'-deoxyuridine (BrdU) cell proliferation assay kit (Millipore Chemicon, Temecula, USA) according to the manufacturer’s protocol. The cells were treated as described in the MTT experiment. The quiescent cells were incubated for another 24 h with or without ICI182,780 at various final concentrations (10⁻⁸, 10⁻⁷, 10⁻⁶, and 10⁻⁵ M). Then, BrdU was added to the medium and the cells were incubated for 4 h. After denaturation of DNA, anti-BrdU monoclonal antibody was added, and the samples were allowed to stand at room temperature for 1 h. After washing with wash buffer, peroxidase-labeled goat anti-mouse IgG was added and the samples were incubated at 37°C for 30 min. The immune complexes were detected using 3,3′,5,5′-tetramethyl-benzidine as the substrate, and the absorbance at 450 nm was determined. The absorbance values correlated directly to the amount of DNA synthesized. The relative proliferation rates were presented as the percentage of vehicle control. On the basis of the acquired values above, we determined the effective concentration of ICI182,780 (10⁻⁶ M) for the subsequent experiments.

Viable cell number

For cell number experiments, the RASMCs were plated at a density of 5 × 10³ cells per well in a 24-well plate, allowed to attach overnight, and then starved as described above for 48 h. Subsequently, the medium was replaced every 24 h for 3 days with phenol-red-free DMEM containing 5% DCC-FBS with either ICI182,780 (10⁻⁶ M) or the vehicle. The medium was changed every time after counting the cells. The number of cells was determined using a Coulter counter, and a trypan-blue exclusion test was performed to determine the cell viability.

Immunocytochemistry for detecting proliferating cell nuclear antigen expression

For immunocytochemical analysis, 5 × 10⁵ cells were seeded per well in 6-well plates with a slide in each well. After synchronization as described above for 24 h, the cells were maintained in 5% DCC-FBS phenol-red-free DMEM with either ICI182,780 (10⁻⁶ M) or the vehicle. After incubation for 48 h, the cells were fixed with 4% paraformaldehyde, and then stained by standard immunocytochemical techniques with Histostain-Plus kit (Zymed, San Diego, USA). A mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA) protein (Santa Cruz Biotechnology, Santa Cruz, USA) was used at a dilution of 1:100 as the primary antibody. Phosphate-buffered saline (PBS) solution was used as the negative control. Biotinylated goat anti-mouse IgG secondary antibody was used to react with the primary antibody, and all steps were performed according to the protocol of the kit. Five high-power fields in each slide were randomly selected and 500 cells were randomly counted. Accordingly, the percent of cells with positively stained nuclei was calculated.
Cell cycle distribution
RASMCs were plated at a density of $5 \times 10^5$ cells per well in 6-well plates and incubated overnight. After synchroniza-
tion for 24 h, the cells were maintained in 5% DCC-FBS phenol-red-free DMEM containing either ICI182,780 ($10^{-6}$ M) or the vehicle for 48 h. The cells were trypsi-
nized and centrifuged at $400 \text{ g}$ for 5 min. The pellets were washed twice in PBS solution, and the cells were fixed with 70% cold alcohol overnight at 4°C. Then, the alcohol was removed and cells were washed with PBS. Finally, the cells were incubated in a mixture of 50 mg/l propidium iodide (PI) and 3000 U/l RNase for 30 min, and the cell cycle was analyzed by flow cytometry (Cytomics 500; Beckman Coulter, Brea, USA).

Evaluation of cyclin D1 expression by RT–PCR
and western blot
RASMCs were seeded onto 6-well plates and cultured until 70–80% confluence, then starved in phenol-red-free DMEM containing 0.4% DCC-FBS for 24 h. The cells were further incubated in 5% DCC-FBS phenol-red-free DMEM containing either ICI182,780 ($10^{-6}$ M) or the vehicle for 24 h. Then the cells were washed with PBS twice and immediately homogenized in Trizol reagent (Invitrogen, Carlsbad, USA). The total RNA was extracted according to the manufacturer’s instructions. Reverse transcriptase-polymerase chain reaction (RT–PCR) was performed using the primers based on the rat cyclin D1 cDNA sequences. The sequences of the PCR primers were as follows: 5’-CCAGAGCGCGAGGAGAACAA-3’ (sense) and 5’-GAGGCCGTAGTAGGACAGGA-3’ (antisense). β-actin was amplified in parallel to ascertain the integrity of the cDNA. The amplification profile consisted of 31 cycles with denaturation at 95°C for 8 s, annealing at 59°C for 40 s, extension at 72°C for 60 s, with an initial denaturation at 95°C for 5 min, and a final extension of 7 min at 72°C. The PCR products were fractionated on 2% agarose gels and visualized using ethidium bromide.

The amplified PCR products were subjected to agarose gel electrophoresis and subsequently transferred to a nitrocellulose membrane for 3 h. The membrane was blocked for 6 h at room temperature in tris-buffered saline (TBS) containing 5% skimmed milk powder and for another 2 h with the appropriate primary antibodies (1:500 polyclonal rabbit anti-cyclin D1, 1:500 polyclonal rabbit anti-β-actin). Subsequently, the membranes were washed four times (15 min each) in TBS and incubated with horseradish peroxidase-labeled anti-rabbit IgG for 2 h. The signal was detected by electrochemical luminescence western blotting detection reagents (Amersham Pharmacia Biotech., Piscataway, USA). The relative expression level of cyclin D1 (cyclin D1/β-actin) was measured in terms of the absorbance and the area of each blot by densitometry analysis.

Statistical analysis
All experiments were performed in triplicate with consistent results. Statistically significant results were obtained by one-way analysis of variance. A value of $P < 0.05$ was considered significant. Data are presented as mean ± SD.

Results
VSMCs identification
Phase-contrast microscopy of the cultured cells revealed a typical ‘hill and valley’ appearance similar to that of SMC cultures. In addition, ~100% cells from passages four to seven were positively stained for smooth muscle α-actin. Thus, the cultured cells were identified as SMCs.

Effect of ICI182,780 on the proliferation of RASMCs
To evaluate the dose–response effects of ICI182,780 on RASMCs proliferation, the MTT assay was performed with 17β-estradiol as a control. Results showed ICI182,780 promoted the growth of RASMCs in a concentration-dependent manner in the range of $10^{-8}$–$10^{-5}$ M. A significant increase of the growth was observed at $10^{-6}$ M and higher concentration ($10^{-5}$ M). The growth of RASMCs was enhanced by about 2-fold compared with that of untreated cells. As compared with vehicle treatment, 17β-estradiol significantly inhibited the growth of RASMCs (Fig. 1).

Effect of ICI182,780 on DNA synthesis
Compared with the vehicle, ICI182,780 promoted a dose-dependent increase in DNA synthesis in RASMCs over a concentration range of $10^{-8}$–$10^{-5}$ M (Fig. 2). Compared with untreated cells, RASMCs treated with $10^{-6}$ M ICI182,780 exhibited markedly enhanced proliferation rate (relative proliferation rate was 166% ± 15.5%; $P < 0.05$). At a higher concentration ($10^{-5}$ M), ICI182,780 further stimulated the proliferation of RASMCs by about 200% ($P < 0.01$). Therefore, $10^{-6}$ M was chosen as the effective concentration of ICI182,780 for the subsequent experiments.
Effect of ICI182,780 on viable cell number

Cell growth was markedly enhanced at 24, 48, and 72 h after treatment with $10^{-6}$ M ICI182,780. Cell number count showed that ICI182,780 ($10^{-6}$ M) promoted the viability and proliferation of RASMCs (Fig. 3).

Effect of ICI182,780 on immunochemical staining for PCNA

PCNA is an important marker of cellular proliferative state, and its level was increased in the late G1 phase, reach the maximum in the S phase, and decrease markedly in the G2 and the M phases \cite{10,11}. The effect of ICI182,780 on PCNA expression in RASMCs was evaluated by immuno-cytochemical staining. The results revealed that the number of PCNA-positive cells was increased after treatment with ICI182,780 ($10^{-6}$ M). There was a significant difference between the ICI182,780 treatment group and the control group ($P < 0.01$; Fig. 4).

Effect of ICI182,780 on cell cycle

The effects of ICI182,780 on cell cycle were also determined by flow cytometry. The treatment with ICI182,780 ($10^{-6}$ M) significantly enhanced the percentage of RASMCs that progressed to the S phase. Compared with control group, the percentage of cells in the S phase was doubled after ICI182,780 treatment (40.2% vs. 19.8%, $P < 0.01$), suggesting that ICI182,780 significantly increased DNA synthesis in RASMCs (Fig. 5).

Effect of ICI182,780 on cyclin D1 expressions

Cyclin D1 serves as a growth factor sensor and plays a critical role in the transition and progress of the cell cycle \cite{12}. Therefore, we examined the effects of ICI182,780 on cyclin D1 expression. After 24-h treatment of cells with ICI182,780 ($10^{-6}$ M), the mRNA and protein expressions of cyclin D1 were significantly elevated in RASMCs ($P < 0.05$; Figs. 6 and 7).
Discussion

In this study, the results suggested that ICI182,780 promotes the proliferation of VSMCs, and it might overcome the cardioprotective effect of estrogen and produce some adverse effects when used for the treatment of breast cancer.

ER belongs to the nuclear receptors superfamily. In the classical mechanism of estrogen action, estrogen diffuses into the cell and binds to the nuclear ER. This nuclear estrogen–ER complex can directly bind to estrogen-response element (ERE) sequences and regulate the transcription of target genes. The nuclear estrogen–ER complex can also indirectly activate or inhibit the activities of some transcriptional factors such as activator protein 1 through protein–protein interactions [13], which induces the recruitment of co-regulatory proteins to the promoter region of estrogen-response genes, elevates or depresses the mRNA levels and associated protein production. ICI182,780, which completely blocks both ERα and ERβ [14], has been shown to down-regulate the expression of ERα and ERβ mRNA and promote the degradation of ER [15]. In the present study, we found that ICI182,780, as a complete ER blocker, up-regulated the expression of cyclin D1 mRNA, elevated the level of cyclin D1 protein, and significantly promoted the growth of VSMCs.

Recent evidences support the existence of membrane ER, and signaling via membrane ER (responsible for membrane-initiated steroid signaling) can also have an indirect effect on gene transcription [16–18]. Furthermore, the membrane ER might be involved in the regulation of the growth of VSMCs. ICI182,780 may bind to the membrane ER and promote the proliferation of VSMCs. Previous studies revealed that 17β-estradiol could inhibit the proliferation of VSMCs by stimulating the synthesis of caveolin 1 [19,20] that located in the cellular membrane. Caveolin 1 as a critical upstream factor could modulate cell cycle by inhibiting the expression of cyclin D1 [21,22]. Caveolin 1 may also play a role in the action of ICI182,780. Thus, further studies are needed to clarify the precise mechanism of ICI182,780 on the growth of VSMCs.

In conclusion, the present study showed that ICI182,780 promotes the proliferation of VSMCs, indicating that ICI182,780 might produce some adverse effects on cardiovascular system when used for the treatment of breast cancer, especially in post-menopausal women with or at high risk of coronary artery disease.

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