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ORIGINAL RESEARCH

Pyrrolidine dithiocarbamate inhibits nuclear factor-κB pathway activation, and regulates adhesion, migration, invasion and apoptosis of endometriotic stromal cells

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ABSTRACT: The activation of nuclear factor-κB (NF-κB) has been implicated in the development and progression of endometriosis. The aim of this study is to investigate the potential application of pyrrolidine dithiocarbamate (PDTC), a potent NF-κB inhibitor, in the treatment of endometriosis. NF-κB-DNA-binding activity, IκB phosphorylation and expression of nuclear p65 protein in endometriotic ectopic stromal cells (EcSCs), endometriotic eutopic stromal cells (EuSCs) and normal endometrial stromal cells (NESCs) were detected by electrophoretic mobility shift assay and western blot analysis. Adhesion, migration, invasion and apoptosis of EcSCs were observed by means of adhesion, migration, invasion and terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling assay, respectively. Gene and protein expressions of CD44s, matrix metalloproteinase (MMP)-2, MMP-9 and survivin in EcSCs were measured by RT–PCR and western blot analysis. The results showed that PDTC in the absence or presence of interleukin (IL)-1β showed stronger inhibitory effects on NF-κB-DNA-binding activity, IκB phosphorylation and expression of nuclear p65 protein in EcSCs than those in EuSCs or NESCs. PDTC enhanced apoptosis, and suppressed IL-1β-induced cellular adhesion, migration and invasion of EcSCs. Pretreatment of EcSCs with PDTC attenuated IL-1β-induced expressions of CD44s, MMP-2, MMP-9 and survivin at gene and protein levels. All these findings suggest that PDTC induces apoptosis and down-regulates adhesion, migration and invasion of EcSCs through the suppression of various molecules. Therefore, PDTC could be used as a therapeutic agent for the treatment of endometriosis.

Key words: pyrrolidine dithiocarbamate / nuclear factor κB / endometriosis / endometrial stromal cells

Introduction

Endometriosis, the presence of functional endometrium outside the uterine cavity, is a common, chronic and estrogen-dependent gynecological disorder associated with pelvic pain and infertility. Its etiology is unclear but it is thought to be due to the implantation and maintenance of disseminated uterine endometrium, predominantly on the ovary and pelvic peritoneum. Clinical features and *in vitro* experiment have suggested that endometriotic cells are invasive and able to metastasize. The typical characteristics of endometriotic cells include: (i) adherence and attachment; (ii) migrating and invading; (iii) resistance to apoptosis (Jiang *et al.*, 2007; Banu *et al.*, 2008). These are very similar to the mechanisms operating in the invasion and metastasis of malignant tumors.

Nuclear factor-κB (NF-κB) is a transcription factor involved in cell proliferation, angiogenesis, adhesion, invasion and metastasis. It regulates many of the gene products that are critical to the initiation and establishment of the early and late stages of endometriosis, including adhesion-molecule-like CD44s (i.e. CD44 standard), matrix metalloproteinases (MMPs) and apoptosis-suppressor proteins such as survivin. *In vitro* studies show that NF-κB can be activated in endometriotic ectopic stromal cells (EcSCs) by interleukin (IL)-1β, an important cytokine that plays a critical regulatory role in endometriosis (Xiu-li *et al.*, 2009). Constitutive activation of NF-κB has also been demonstrated in endometriotic lesions and peritoneal macrophages of endometriosis patients. Agents blocking NF-κB are effective inhibitors of endometriosis development, and some drugs with known NF-κB inhibitory

properties have proved efficient at reducing endometriosis-associated symptoms in women (González-Ramos et al., 2010). So inhibiting NF-κB appears to be a promising strategy for future therapies.

Pyrrolidine dithiocarbamate (PDTC) is an antioxidant and potent NF-κB inhibitor. It has been used to suppress the activity of NF-κB in some kinds of cells (Cuzzocrea et al., 2002; Németh et al., 2003) and has been demonstrated to have an inhibitive effect on the development of experimental endometriotic implants in rats by suppressing cell proliferation (Celik et al., 2008). In the current study, we evaluated the effects of PDTC on NF-κB activation, adhesion, migration, invasion and apoptosis of EcSCs, as well as expressions of CD44s, MMP-2, MMP-9 and survivin in EcSCs to clearly demonstrate the mechanisms of PDTC for treatment of endometriosis.

Materials and Methods

Collection of tissues

Eutopic endometria and ectopic tissues were obtained from 31 patients with endometriosis (ovarian cysts: $n = 19$; ectopic peritoneal lesions: $n = 12$) by laparoscopy. Normal endometria were acquired by biopsy from 35 healthy women. The tissues were acquired during the proliferative phase of the menstrual cycle. None of the patients received any hormonal therapy during the 6 months before their operation. Peritoneum from the anterior abdominal wall was obtained from reproductive age women without endometriosis ($n = 10$) who were undergoing surgery for benign conditions. Informed consent was obtained from all patients and the study was approved by the Ethical Committee of our Institution.

Preparation of primary EcSCs, endometriotic eutopic stromal cells, normal endometrial stromal cells and human peritoneal mesothelial cells

Tissues were washed in Hank's Balanced Salt Solution (HBSS) and minced. Digestion of the tissue was then performed for 1 h at 37°C, with shaking every 15 min in 0.125% type IA collagenase (Sigma Chemical, St. Louis, MO, USA). Subsequently, supernatant was filtered through a 40-μm nylon mesh. Isolated cells were washed twice in HBSS and seeded in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS), Penicillin G (100 U/ml) and streptomycin (100 mg/ml). Stromal cultures were dissociated with 0.05% trypsin and 0.02% versene in saline, harvested by centrifugation, replanted and allowed to grow to confluence. The cells after the third passage were >99% pure as determined by immunocytochemical staining with antibodies to vimentin, cytokeratin, CD45 or von Willebrand factor (Zymed Laboratories, San Francisco, CA, USA) and were used for the following experiments.

Human peritoneal mesothelial cells (HPMCs) were enzymatically dispersed from sections of peritoneum using 0.1% type I collagenase (Worthington Biomedical, Freehold, NJ, USA) and 0.05% DNAse (Sigma). Cells were plated in 25-cm^2 tissue flasks and grown in DMEM supplemented with 10% FBS, penicillin G (100 U/ml) and streptomycin (100 mg/ml). HPMCs were confirmed by immunohistochemistry with antibodies against cytokeratin, vimentin, CD45 antigen and factor VIII (Signet, Dedham, MA, USA). Passage 2 cultures were used for experiments.

To analyze the effects of PDTC, EcSCs, endometriotic eutopic stromal cells (EuSCs) or normal endometrial stromal cells (NESCs) were pre-incubated with 5, 10, 25, 50 or 100 μM PDTC (Sigma) for 1 h before adding 10 ng/ml IL-1β (Sigma Chemical) for a further 24 h period. All experiments were repeated at least three times.

Extraction of nuclear proteins and electrophoretic mobility shift assay

Nuclear extracts were prepared using nuclear and cytoplasmic extraction reagent kit nuclear extraction reagent (Pierce, Rockford, IL, USA). The protein content was quantified by a Bradford assay (Pierce). Electrophoretic mobility shift assay (EMSA) was performed using a commercial Kit (Gel Shift Assay System, Promega, Madison, WI, USA). NF-κB consensus oligonucleotide probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was end-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase. Retardation of proteins bound to oligonucleotides was determined by electrophoresis on 4% polyacrylamide gels in the cold (25 mA). Gels were dried and exposed to films. Quantification of NF-κB signals was done by densitometrical scanning using alphaEaserFC software (Alpha Innotech, San Leandro, CA, USA).

Western blot analysis

Cells were lysed with 80 μl of lysis buffer (50 mM Tris–HCl, 125 mM NaCl, 0.1% Nonidet P-40, 5 mM ethylenediamine tetra-acetic acid, 50 mM NaF, 1 mM DTT and protease inhibitors). The protein content was quantified by a Bradford assay. Twenty micrograms protein were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electro-transferred to immobilon-P membrane (Millipore) for 2 h at 4°C. Non-specific binding was blocked with 10% dry milk in tris-buffered saline/0.1% Tween-20 for 1 h at room temperature. With constant shaking, the membranes were incubated with primary antibodies against CD44s, MMP-2, MMP-9, survivin, p65 and p-IκB (Santa Cruz) overnight at 4°C and then in secondary antibodies at room temperature for 1 h with constant shaking. The expression of target proteins was detected by an enhanced chemiluminescence Kit (Amersham) and visualized by autoradiography with hyper film. Quantification of the proteins was performed using the ratio of the target protein bands relative to glyceraldehyde-3-phosphatedehydrogenase (GAPDH).

Semi-quantitative reverse transcription-polymerase chain reaction

RNA extraction and RT–PCR were performed using a commercial Kit (Promega). Oligonucleotide primers and fragment lengths were seen in Table I. A total of 30 cycles were performed, with each cycle comprising 1 min at 94°C, 1 min at 50–60°C depending on each gene and 1.5 min at 72°C with a final extension of 8 min at 72°C. Human GAPDH reactions were amplified for 25 cycles. The reaction products were separated on 2% agarose gel, stained with 1 μg/ml of ethidium bromide and visualized using a digital imaging system. Semi-quantitative analysis was performed using the ratio of the genes tested versus GAPDH.

Adhesion assay

EcSCs were labeled with calcein-AM dye solution first. Fifty micrograms of calcein (Molecular Probes, Leiden, The Netherlands) were dissolved in 5 μl of anhydrous dimethyl sulfoxide (Sigma) and adding this solution to 5 ml of DMEM supplemented with 0.5% bovine serum albumin (BSA). Trypsinized EcSCs (1×10^6 cells/ml) were incubated in DMEM/0.5% BSA at 37°C for 45 min, with occasional mixing. The labeled EcSCs were washed to remove free dye and incubated with indicated concentrations of PDTC. When HPMCs reached confluence in 96-well plates coated with collagen IV, the calcein-labeled, pretreated EcSCs (1×10^4 cells per well) were seeded additionally. After the plates were incubated at 37°C for 60 min, the medium of each well was removed and washed. The remaining fluorescence per well was measured on a Perkin Elmer plate reader, using 485 excitation and 530 emission filters. On each plate, a standard was prepared by adding different numbers of labeled EcSCs to the wells. The amount of adherent cells was determined

Table I Sequences of PCR primers and length of PCR product.

Genes	PCR primers	Length of PCR product (bp)
CD44s	5′-CCA ACT CCA TCT GTG CAG-3′ 5′-AAC CTC CTG AAG TGC TGC-3′	300 bp
MMP-2	5′-CCACGTGACAAGCCCATGGGGCCCC-3′ 5′-GCAGCCTAGCCAGTCGGATTTGATG-3′	480 bp
MMP-9	5′-GAG GAA TAC CAG TAC CGC TAT G-3′ 5′-CAA ACC GAG TTG GAA CCA CG-3′	518 bp
Survivin	5′-GTGAATTTTTGAAACTGGACAG-3′ 5′-CCTTTCCTAAGACATTGCTAAG-3′	243 bp
GAPDH	5′-TGA TGA CAT CAA GAA GGT GGT GAA G-3′ 5′-TCC TTG GAG GCC ATG TGG GCC AT-3′	240 bp

by calibrating the measured fluorescence of the experimental wells in relation to the standard.

Cell migration and invasion assays

In vitro migration and invasion assays were performed using uncoated or Matrigel (Sigma)-coated 24-well chambers/microfilters (8 μm pore-sized polycarbonate filters), respectively (BD Bioscience, Bedford, MA, USA). Briefly, after rehydration of the chambers, the pretreated EcSCs (5×10^4 cells per chamber) in 500 μl of DMEM plus 10% FBS were seeded onto the upper chamber. In the lower chamber, 500 μl of DMEM plus 10% FBS was placed. Migration/invasion of EcSCs was measured as number of cells invaded from a defined area of the uncoated or Matrigel-coated microfilter through micropores in 24 h. The chambers were immersed in 100% methanol for 1 min for fixation, and all cells were then stained by hematoxylin. The cells remaining on the top surface of the filter were completely removed with a cotton swab, and the filter was removed from the chamber and mounted on a glass slide. These preparations were examined under a microscope at ×200 magnification. The number of infiltrating cells was counted in five regions selected at random, and the extent of infiltrating cells was determined by the mean count.

Terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling assay

The method examines DNA strand breaks during apoptosis, in which we used the Roche *in situ* cell death detection reagent (Roche Applied Science, Indianapolis, IN, USA). Briefly, pretreated cells were washed, air-dried, fixed with 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After being washed, cells were incubated with reaction mixture for 60 min at 37°C. Stained cells were mounted with mounting medium purchased from Sigma Chemical and analyzed under a fluorescence microscope (Labophot-2; Nikon, Tokyo, Japan). Pictures were captured using a Photometrics Coolsnap CF color camera (Nikon, Lewisville, TX, USA) and MetaMorph version 4.6.5 software (Universal Imaging, Downingtown, PA, USA).

Statistical analysis

For statistical analysis, the comparison of two groups was performed using *t*-test, whereas one-way analysis of variance and the Bonferroni's test *post hoc* were used for multiple comparisons. The level of significance was set at $P < 0.05$.

Results

Effects of PDTC on NF-κB activation

PDTC (10 μM) alone inhibited NF-κB-DNA-binding activity, IκB phosphorylation and expression of nuclear p65 protein in untreated EcSCs ($P < 0.01$, 0.05, 0.05), EuSCs ($P < 0.01$, 0.01, 0.05) and NESCs ($P < 0.05$, 0.05, 0.05). PDTC pre-incubation inhibited IL-1β-induced NF-κB-binding activity, IκB phosphorylation and expression of nuclear p65 protein dose-dependently in EcSCs ($P < 0.005$, 0.01, 0.005), EuSCs ($P < 0.01$, 0.005, 0.05) and NESCs ($P < 0.01$, 0.05, 0.01). PDTC showed stronger inhibitory effects on NF-κB activation in EcSCs than those in EuSCs ($P < 0.05$) or NESCs ($P < 0.05$). The inhibitory effects in NESCs were also weaker than those in EuSCs ($P < 0.05$) (Fig. 1). In addition, similar patterns of response were obtained whether cells were derived from ovarian cysts or from ectopic peritoneal lesions.

Effects of PDTC on cell adhesion

Adhesion of EcSCs to HPMCs was calculated by the ratio of the number of adhesive EcSCs to the total amount of EcSCs added. As shown in Fig. 2, pre-incubation of EcSCs with PDTC inhibited adhesion of EcSCs to HPMCs induced by IL-1β ($P < 0.01$). These effects were dose-dependent ($P < 0.01$).

Effects of PDTC on migration and invasion of EcSCs

After seeding PDTC-pretreated EcSCs in the top chamber of uncoated or Matrigel-coated chamber, we found that PDTC suppressed IL-1β-induced cell motility and invasion ($P < 0.05$, 0.01). The inhibitive effects were both dose-dependent ($P < 0.05$, 0.05) (Fig. 3). The invasive potential was determined on the basis of the ability of cells to invade a matrix barrier containing mainly laminin and type IV collagen, the major components of the basement membrane.

Effects of PDTC on cell apoptosis

We use terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling (TUNEL) assay to measure apoptosis of EcSCs to determine whether PDTC enhances the apoptosis. The results showed that IL-1β reduced apoptosis of EcSCs ($P < 0.05$). PDTC

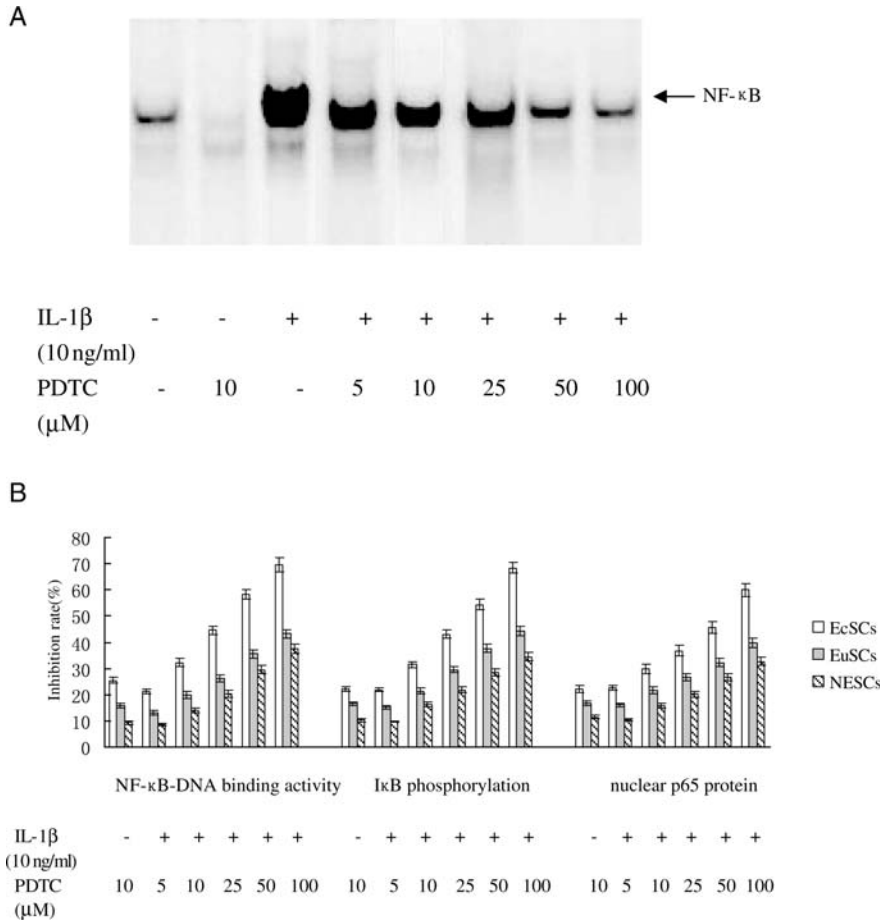

Figure 1 Effects of PDTC on NF-κB-DNA-binding activity, IκB phosphorylation and nuclear p65 protein expression in EcSCs. Cell preparations, stimulated with IL-1β, nuclear extraction and EMSA, protein extraction and western blot analysis were performed as described above. (**A**) EMSA after pretreatment of EcSCs ($n = 31$) with PDTC in the absence or presence of 10 ng/ml IL-1β. (**B**) Comparison of inhibition rate of NF-κB-binding activity, IκB phosphorylation and expression of nuclear p65 protein in EcSCs ($n = 31$), EuSCs ($n = 31$) and NESCs ($n = 35$). Values were expressed as the mean ± SE of three independent experiments.

pretreatment at a concentration of 10, 25 and 50 μM up-regulated apoptosis of EcSCs dose-dependently in the presence of IL-1β ($P < 0.01$). PDTC at a concentration of 5 μM had no influence on apoptosis compared with the control (pretreated with IL-1β only; $P > 0.05$) and there was also no difference between the apoptosis under the condition of 50 and 100 μM PDTC ($P > 0.05$; Fig. 4).

Effects of PDTC on IL-1β-induced expressions of adhesive, invasive and antiapoptotic gene products

PDTC at concentration from 5 to 100 μM dose-dependently inhibited IL-1β-induced gene and protein expressions of CD44s, MMP-2 and MMP-9 ($P < 0.05$). PDTC pretreatment at a concentration of 10, 25 and 50 μM suppressed IL-1β-induced survivin expression at gene and protein levels dose-dependently ($P < 0.05$). PDTC at a concentration of 5 μM had no influence on survivin expressions compared with the control (pretreated with IL-1β only) ($P > 0.05$), and there was also no difference between survivin expressions under the condition of 50 and 100 μM PDTC ($P > 0.05$) (Fig. 5).

Discussion

Evidence showing that NF-κB may be one of the major culprits in the pathogenesis of endometriosis is extensively discussed in the literature (Yamauchi *et al.*, 2004; Guo, 2007). Activation of NF-κB may be responsible for angiogenesis, invasiveness and inhibition of apoptosis, and for increased production of pro-inflammatory cytokines and estrogens (Lebovic *et al.*, 2001; Yamauchi *et al.*, 2004; Guo, 2007). In women with endometriosis, NF-κB was found to be highly expressed in both eutopic and ectopic tissues compared with the normal endometrium (Wang *et al.*, 2005). As PDTC has been shown to diminish NF-κB activity at multiple points of the IκB kinase (IKK)- IκB- NF-κB cascade in several cell lines (Demirbilek *et al.*, 2006; Morais *et al.*, 2006), we investigated whether this preparation could affect NF-κB activity and NF-κB-regulated cellular responses of EcSCs, as well as its mechanisms. We demonstrated here that PDTC- inhibited NF-κB activation in the presence or absence of IL-1β, as shown by EMSA. And the inhibitory effects were mainly dependent on IKK cascade, which were verified by the suppression of IκB phosphorylation and expression of nuclear p65

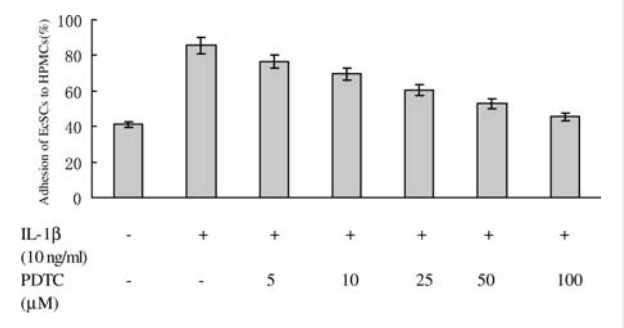

Figure 2 Effects of PDTC on adhesion of EcSCs to human peritoneal mesothelial cells (HPMCs). Labeled EcSCs ($n = 31$) incubated with indicated concentrations of PDTC in the presence of IL-1β were seeded onto the 96-well plates coated with HPMCs. After 60 min, the remaining fluorescence per well was measured. The adhesion of EcSCs to HPMCs were calculated by the ratio of the number of adhesive EcSCs to the total amount of EcSCs added. Values were expressed as the mean ± SE of three independent experiments.

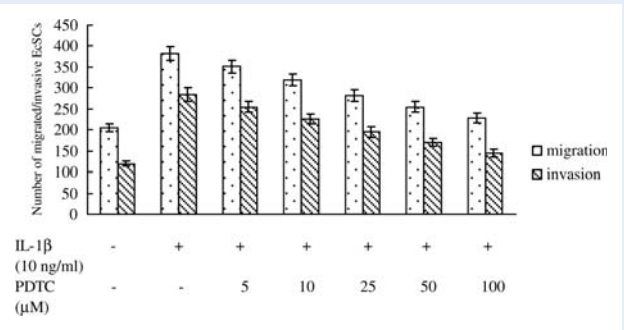

Figure 3 Effects of PDTC on migration/invasion of EcSCs. Cells ($n = 31$) were incubated with indicated concentrations of PDTC in the presence of IL-1β. Cell migration/invasion ($n = 31$) through uncoated or Matrigel-coated microfilter through micropores were determined by a Boyden chamber method. Number values were expressed as the mean ± SE of three independent experiments.

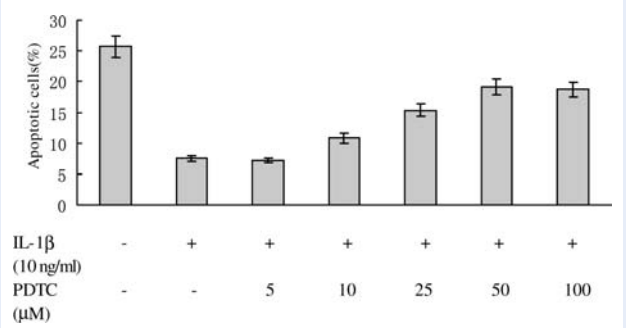

Figure 4 Effects of PDTC on apoptosis of EcSCs. Cells ($n = 31$) were incubated with indicated concentrations of PDTC in the presence of IL-1β. Cell apoptosis ($n = 31$) was determined using TUNEL assay. Numerical data were expressed as mean ± SE of three independent experiments.

protein. Further investigation is required to determine the mechanism by which PDTC inhibits IKK in EcSCs. In addition, PDTC showed stronger inhibitory effects on NF-κB activation in EcSCs compared with those in EuSCs or NESCs, and the inhibitory effects in NESCs were also weaker than those in EuSCs, suggesting that PDTC has cell-specific effects on EcSCs and that its effects could be weaker on the eutopic endometrium, especially on normal endometrium.

In order for the endometriotic cells to disperse, they need to have the capacity to adhere and attach (Jiang *et al.*, 2007). Authors have demonstrated that fragments of endometrium attach to HPMCs that cover the surface of peritoneum (Dechaud *et al.*, 2001). Our present study showed that PDTC inhibited IL-1β-induced binding of EcSCs to HPMCs, suggesting that PDTC may block the adherence of endometriotic tissue to peritoneal locations. It has been reported that the CD44 molecule, which is a major receptor for hyaluronic acid (HA), is frequently expressed by endometriotic cells (Kim *et al.*, 2007) and is partly responsible for mediating mesothelial binding through recognition of mesothelial-associated HA (Dechaud *et al.*, 2001). IL-1β has been found to stimulate increase in CD44-dependent cell binding to HA (Leir *et al.*, 2003). CD44 is one of the NF-κB regulatory target genes and can be up-regulated in human mesenchymal stem cells via the NF-κB pathway (Böcker *et al.*, 2008). In our experience, PDTC suppressed the IL-1β-induced CD44s expressions at both mRNA and protein levels, which suggested that NF-κB might be involved in the stimulation of CD44s in EcSCs by IL-1β. Thus, we assume that PDTC may impair the binding and adhesion of EcSCs to HPMCs by inhibiting CD44s expression in EcSCs.

Cell motility is required for the cells to invade and metastasize. Cellular movement requires reorganization of components of the cytoskeleton, such as microtubules, actin and microfilaments. The factors that can affect the reorganization of cytoskeleton may influence cell motility. For instance, ovarian steroids affect the migration of endometrial stromal cells derived from women with endometriosis, and this effect is likely to involve cytoskeletal reorganization (Gentilini *et al.*, 2010). It has been found that some chemokines including IL-1β act as motility-stimulating factors that induce migration and changes in the actin organization (Abd-El-Basset *et al.*, 2004). Gliotoxin, an inhibitor of NF-κB, has been proved to inhibit cell migration, which was associated with a rearrangement of the F-actin and vimentin cytoskeleton (Pozo *et al.*, 2009). Our results showed that PDTC inhibited IL-1β-induced migration of EcSCs. It may be the results of the action on EcSCs cytoskeleton, such as F-actin. This requires further research.

Establishment of ectopic sites of endometrial growth is an invasive event requiring degradation of the extracellular matrix (ECM). Our results found that when EcSCs were pretreated with PDTC, the number of filter-infiltrating cells was decreased, demonstrating that PDTC inhibited IL-1β-induced invasion ability of EcSCs. MMPs that are essential in remodeling and degradation of ECM have been improved to play an important role in the destruction of the surrounding connective tissue by endometriotic tissue and be involved in the invasive establishment and progression of endometriosis. An increased expression of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) have

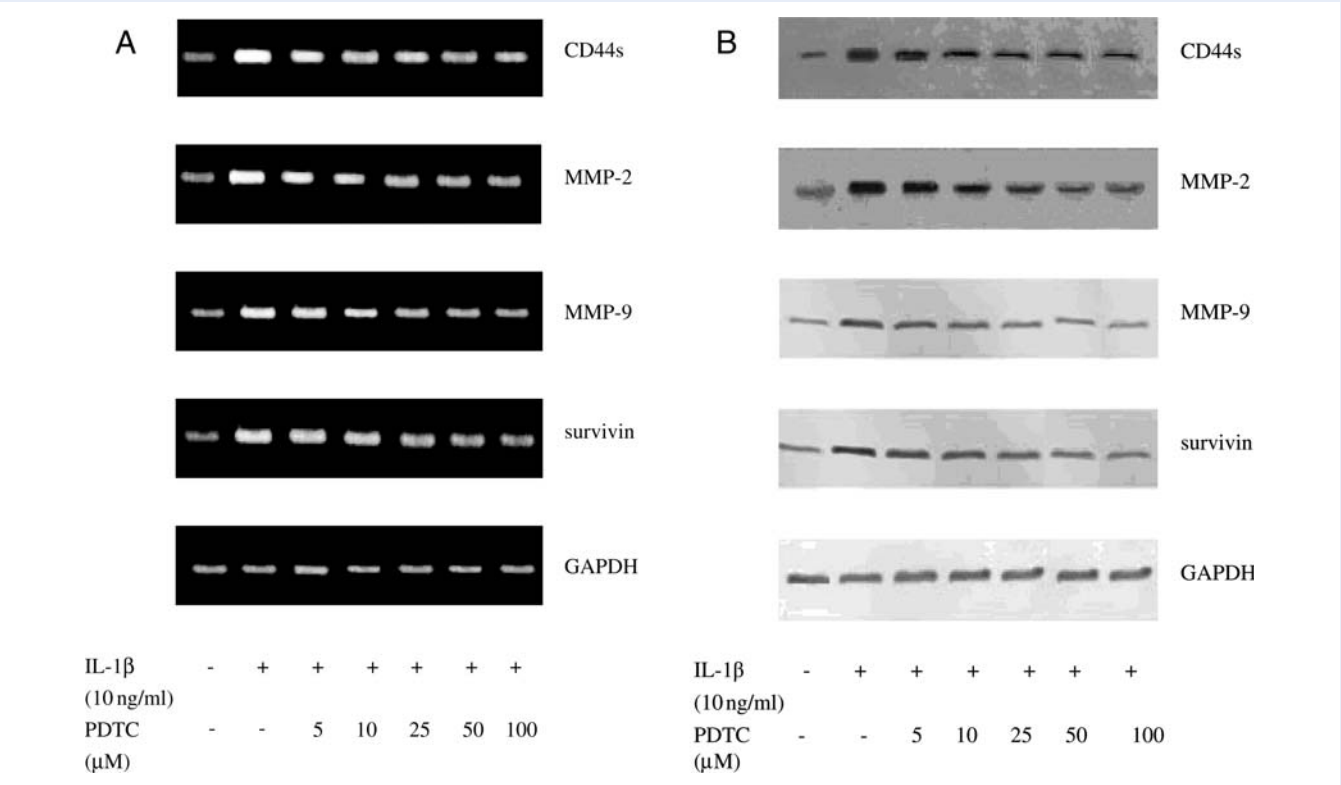

Figure 5 Effects of PTDC on gene and protein expressions of CD44s, metalloproteinases (MMP-2, MMP-9) and survivin in IL-1β-treated EcSCs ($n = 31$). Cultures were pre-incubated with indicated concentrations of PDTC for 1 h before stimulation with IL-1β. (**A**) Total RNA of the cells was extracted and analyzed by RT–PCR. Quantification was performed using the ratio of the target bands relative to GAPDH. (**B**) Cell extracts were analyzed by western blot analysis with the indicated antibodies. Quantification of the target protein bands using the ratio of the proteins tested versus GAPDH. Values were expressed as the mean ± SE of three independent experiments.

been found in ectopic tissues (Shaco-Levy *et al.*, 2008; Di Carlo *et al.*, 2009). Analysis of the MMP-9 promoter has identified an upstream NF-κB site, and NF-κB is a crucial transactivator for the expression of MMP-9 induced by IL-1β (Lin *et al.*, 2009). Although MMP-2 promoter itself does not contain a NF-κB binding site, several reports show that NF-κB can augment MMP-2 activation (Yoon *et al.*, 2002; Xie *et al.*, 2004). SN50, NF-κB inhibitor peptide, inhibited IL-1β-stimulate increases in MMP-2 expression and activity (Xie *et al.*, 2004). Our data showed that expressions of MMP-2 and MMP-9 induced by IL-1β were inhibited by PDTC, suggesting that PDTC reduced MMP-2 and MMP-9 expressions in EcSCs by inhibiting the NF-κB pathway. The inhibitory effects of PDTC on IL-1β-induced invasion are mediated through suppression of NF-κB-mediated MMP-2 and MMP-9 expression.

The underlying mechanisms by which endometrial tissues survive and grow in ectopic sites are an enigma. Normal cells undergo apoptosis when they separate from their primary tissue. However, spontaneous apoptosis of ectopic endometrial tissue is impaired in women with endometriosis (Béliard *et al.*, 2004). NF-κB plays an important role in protecting EcSCs from apoptosis by activating anti-apoptotic genes (Nasu *et al.*, 2007). It has been found that IL-1β reduces apoptosis in endometrial epithelial cells from patients with endometriosis (Bilotas *et al.*, 2010). Survivin, a member of inhibitor of apoptosis proteins, is one of the regulators of cell death (Ambrosini *et al.*, 1997) and can inhibit caspases activity and apoptosis in cells exposed to various apoptotic stimuli (Altieri and Marchisio, 1999). Tissue studies by Ueda *et al.* (2002) showed that survivin gene was aberrantly expressed in ectopic endometrium and that survivin gene expression might facilitate the apoptosis reduction and invasive phenotype in endometriosis. Survivin is one of the NF-κB target genes. Selective inhibition of NF-κB, which abolished expression of survivin, accentuated endothelial apoptosis (Liu *et al.*, 2010). In this study, we presented evidence that PDTC at a concentration of 10, 25 and 50 μM induced apoptosis of EcSCs in the presence of IL-1β and suppressed IL-1β-induced survivin expressions. Interestingly, though PDTC at a concentration of 5 μM inhibited NF-κB-activation, adhesion and migration/invasion as well as expressions of various molecules (CD44s, MMP-2 and MMP-9) of EcSCs, it had no influence on apoptosis or survivin expression. The mechanisms require further research. The results suggested that PDTC, at certain concentrations, can protect EcSCs from persistent growth by suppressing survivin expression and the inhibition of adhesion, migration and invasion and that this may not necessarily be an effect of apoptosis.

Overall, our results suggest that PDTC induces apoptosis, decreases adhesion, migration and invasion of EcSCs through the suppression of NF-κB and NF-κB-regulated genes. Given that PDTC does not induce toxicity in normal kidney cells and is well tolerated clinically (Hellmuth *et al.*, 2002), the potential of the compound in treatment of endometriosis warrants further research. Further *in vivo* studies are needed to validate these findings.

Authors' roles

J.-j.Z. performed the study design and drafted the article; Z.-m.X. performed the statistical analysis; C.-m.Z. and H.-y.D. collected the data; X.-q.J. revised the article; X.-f.W. and C. L. drew the figures.

References

Abd-El-Basset EM, Prashanth J, Ananth Lakshmi KV. Up-regulation of cytoskeletal proteins in activated microglia. *Med Princ Pract* 2004; **13**:325–333.

Altieri DC, Marchisio PC. Survivin apoptosis: an interloper between cell death and cell proliferation in cancer. *Lab Invest* 1999;**79**:1327–1333.

Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, surviving, expressed in cancer and lymphoma. *Nat Med* 1997;**3**:917–921.

Banu SK, Lee J, Speights VO Jr, Starzinski-Powitz A, Arosh JA. Cyclooxygenase-2 regulates survival, migration, and invasion of human endometriotic cells through multiple mechanisms. *Endocrinology* 2008; **149**:1180–1189.

Béliard A, Noël A, Foidart JM. Reduction of apoptosis and proliferation in endometriosis. *Fertil Steril* 2004;**82**:80–85.

Bilotas M, Meresman G, Buquet R, Sueldo C, Barañao RI. Effect of vascular endothelial growth factor and interleukin-1beta on apoptosis in endometrial cell cultures from patients with endometriosis and controls. *J Reprod Immunol* 2010;**84**:193–198.

Böcker W, Docheva D, Prall WC, Egea V, Pappou E, Rossmann O, Popov C, Mutschler W, Ries C, Schieker M. IKK-2 is required for TNF-alpha-induced invasion and proliferation of human mesenchymal stem cells. *J Mol Med* 2008;**86**:1183–1192.

Celik O, Hascalik S, Elter K, Tagluk ME, Gurates B, Aydin NE. Combating endometriosis by blocking proteasome and nuclear factor-kappaB pathways. *Hum Reprod* 2008;**23**:2458–2465.

Cuzzocrea S, Chatterjee PK, Mazzon E, Dugo L, Serraino I, Britti D, Mazzullo G, Caputi AP, Thiemermann C. Pyrrolidine dithiocarbamate attenuates the development of acute and chronic inflammation. *Br J Pharmacol* 2002;**135**:496–510.

Dechaud H, Witz CA, Montoya-Rodriguez IA, Degraffenreid LA, Schenken RS. Mesothelial cell-associated hyaluronic acid promotes adhesion of endometrial cells to mesothelium. *Fertil Steril* 2001; **76**:1012–1018.

Demirbilek S, Akin M, Gürünlüoğlu K, Aydin NE, Emre MH, Taş E, Aksoy RT, Ay S. The NF-kappaB inhibitors attenuate hepatic injury in bile duct ligated rats. *Pediatr Surg Int* 2006;**22**:655–663.

Di Carlo C, Bonifacio M, Tommaselli GA, Bifulco G, Guerra G, Nappi C. Metalloproteinases, vascular endothelial growth factor, and angiopoietin 1 and 2 in eutopic and ectopic endometrium. *Fertil Steril* 2009; **91**:2315–2323.

Gentilini D, Vigano P, Somigliana E, Vicentini LM, Vignali M, Busacca M, Di Blasio AM. Endometrial stromal cells from women with endometriosis reveal peculiar migratory behavior in response to ovarian steroids. *Fertil Steril* 2010;**93**:706–715.

González-Ramos R, Van Langendonckt A, Defrère S, Lousse JC, Colette S, Devoto L, Donnez J. Involvement of the nuclear factor-kappaB pathway in the pathogenesis of endometriosis. *Fertil Steril* 2010;**94**:1985–1994.

Guo SW. Nuclear factor-kappab (NF-kappaB): an unsuspected major culprit in the pathogenesis of endometriosis that is still at large? *Gynecol Obstet Invest* 2007;**63**:71–97.

Hellmuth M, Wetzler C, Nold M, Chang JH, Frank S, Pfeilschifter J, Mühl H. Expression of interleukin-8, heme oxygenase-1 and vascular endothelial growth factor in DLD-1 colon carcinoma cells exposed to pyrrolidine dithiocarbamate. *Carcinogenesis* 2002;**23**:1273–1279.

Jiang HQ, Li YL, Zou J. Effect of recombinant human endostatin on endometriosis in mice. *Chin Med J (Engl)* 2007;**120**:1241–1246.

Kim HO, Yang KM, Kang IS, Koong MK, Kim HS, Zhang X, Kim I. Expression of CD44s, vascular endothelial growth factor, matrix metalloproteinase-2 and Ki-67 in peritoneal, rectovaginal and ovarian endometriosis. *J Reprod Med* 2007;**52**:207–213.

Lebovic DI, Chao VA, Martini JF, Taylor RN. IL-1beta induction of RANTES (regulated upon activation, normal T cell expressed and secreted) chemokine gene expression in endometriotic stromal cells depends on a nuclear factor-kappaB site in the proximal promoter. *J Clin Endocrinol Metab* 2001;**86**:4759–4764.

Leir SH, Holgate ST, Lackie PM. Inflammatory cytokines can enhance CD44-mediated airway epithelial cell adhesion independently of CD44 expression. *Am J Physiol Lung Cell Mol Physiol* 2003;**285**:L1305–1311.

Lin CC, Kuo CT, Cheng CY, Wu CY, Lee CW, Hsieh HL, Lee IT, Yang CM. IL-1 beta promotes A549 cell migration via MAPKs/AP-1 and NF-kappaB-dependent matrix metalloproteinase-9 expression. *Cell Signal* 2009;**21**:1652–1662.

Liu C, Liang B, Wang Q, Wu J, Zou MH. Activation of AMP-activated protein kinase alpha1 alleviates endothelial cell apoptosis by increasing the expression of anti-apoptotic proteins Bcl-2 and surviving. *J Biol Chem* 2010;**285**:15346–15355.

Morais C, Pat B, Gobe G, Johnson DW, Healy H. Pyrrolidine dithiocarbamate exerts anti-proliferative and pro-apoptotic effects in renal cell carcinoma cell lines. *Nephrol Dial Transplant* 2006; **21**:3377–3388.

Nasu K, Nishida M, Ueda T, Yuge A, Takai N, Narahara H. Application of the nuclear factor-kappaB inhibitor BAY 11–7085 for the treatment of endometriosis: an *in vitro* study. *Am J Physiol Endocrinol Metab* 2007; **293**:E16–23.

Németh ZH, Deitch EA, Szabó C, Haskó G. Pyrrolidinedithiocarbamate inhibits NF-kappaB activation and IL-8 production in intestinal epithelial cells. *Immunol Lett* 2003;**85**:41–46.

Pozo M, Izquierdo MC, de Nicolás R, Egido J, Ortiz A, González-Cabrero J. Gliotoxin inhibits neointimal hyperplasia after vascular injury in rats. *J Vasc Res* 2009;**46**:278–289.

Shaco-Levy R, Sharabi S, Benharroch D, Piura B, Sion-Vardy N. Matrix metalloproteinases 2 and 9, E-cadherin, and beta-catenin expression in endometriosis, low-grade endometrial carcinoma and non-neoplastic eutopic endometrium. *Eur J Obstet Gynecol Reprod Biol* 2008;**139**:226–232.

Ueda M, Yamashita Y, Takehara M, Terai Y, Kumagai K, Ueki K, Kanda K, Yamaguchi H, Akise D, Hung YC *et al.* Survivin gene expression in endometriosis. *J Clin Endocrinol Metab* 2002;**87**:3452–3459.

Wang F, He YL, Peng DX, Liu MB. Expressions of nuclear factor-kappaB and intercellular adhesion molecule-1 in endometriosis. *Di Yi Jun Yi Da Xue Xue Bao* 2005;**25**:703–705.

Xie Z, Singh M, Singh K. Differential regulation of matrix metalloproteinase-2 and -9 expression and activity in adult rat cardiac fibroblasts in response to interleukin-1beta. *J Biol Chem* 2004; **279**:39513–39519.

Xiu-li W, Su-ping H, Hui-hua D, Zhi-xue Y, Shi-long F, Pin-hong L. NF-kappaB decoy oligonucleotides suppress RANTES expression and monocyte chemotactic activity via NF-kappaB inactivation in stromal cells of ectopic endometrium. *J Clin Immunol* 2009;**29**:387–395.

Yamauchi N, Harada T, Taniguchi F, Yoshida S, Iwabe T, Terakawa N. Tumor necrosis factor-alpha induced the release of interleukin-6 from endometriotic stromal cells by the nuclear factor-kappaB and mitogen-activated protein kinase pathways. *Fertil Steril* 2004;**82** Suppl 3:1023–1028.

Yoon SO, Park SJ, Yoon SY, Yun CH, Chung AS. Sustained production of H(2)O(2) activates pro-matrix metalloproteinase-2 through receptor tyrosine kinases/phosphatidylinositol 3-kinase/NF-kappa B pathway. *J Biol Chem* 2002;**277**:30271–30282.