Silencing of cyclooxygenase-2 inhibits the growth, invasion and migration of ovarian cancer cells

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Abstract. The present study aimed to investigate the effect of downregulating cyclooxygenase-2 (COX-2) expression on the growth of human ovarian cancer cells. The COX-2-specific small interfering RNA (siRNA) plasmid vector was constructed and then transfected into ovarian cancer cells. The expression of COX-2 mRNA and protein was detected by quantitative polymerase chain reaction and western blot analysis, respectively. Cell proliferation, apoptosis, cell cycle distribution and cell migration were assessed following knockdown of COX-2 by RNA interference (RNAi). Western blot analysis was used to identify differentially expressed angiogenesis- and cell cycle-associated proteins in cells with silenced COX-2. The expression levels of COX-2 in ovarian cancer cells transfected with siRNA were decreased, leading to a significant inhibition of ovarian cancer cell proliferation, migration and invasion. Western blot analysis revealed that silencing of COX-2 may inhibit vascular endothelial growth factor, matrix metalloproteinase (MMP)‑2 and MMP-9 protein expression. In conclusion, the present study demonstrated that RNAi can effectively silence COX-2 gene expression and inhibit the growth of ovarian cancer cells, which indicates that there is a potential of targeting COX-2 as a novel gene therapy approach for the treatment of ovarian cancer.

Introduction

Ovarian cancer is one of the most common types of fatal tumors of the female reproductive tract and also a major cause of mortality resulting from gynaecological malignancies (1). Epithelial ovarian cancer is the predominant form among ovarian cancers and accounts for 90% of incidences; the majority of mortalities are due to this malignancy (2). There is a lack of effective screening and early detection strategies; therefore, the majority of females are diagnosed with advanced-stage metastatic cancer for which surgical and pharmaceutical treatment options are significantly less effective (3,4). Standard treatment options include debulking followed by chemotherapy with platinum agents. Although there is a good response to primary surgery and chemotherapy treatments, the recurrence rates are high (>60%) and salvage therapies available are not curative (5-8). Therefore, it is important to understand the molecular mechanisms underlying this disease in order to develop novel treatment strategies to improve the clinical outcomes for these patients.

Cyclooxygenase-2 (COX-2) is inducible by inflammatory stimuli, including cytokines, growth factors and tumor promoters, and is upregulated in a variety of malignancies. COX-2 upregulation favors the growth of malignant cells by stimulating proliferation and angiogenesis (9,10). A large number of previous studies demonstrated that COX-2 is overexpressed in ovarian cancer (11-13). Furthermore, Arico et al (14) found that COX-2 can induce angiogenesis via vascular endothelial growth factor (VEGF) and prostaglandin production and can also inhibit apoptosis by inducing the anti-apoptotic factor B-cell lymphoma 2 as well as activating anti-apoptotic signaling through Akt/protein kinase B (one of the serine/threonine kinases). These results suggest that COX-2 has a significant role in the generation and progression of solid tumors and the inhibition of COX-2 may inhibit the growth of a variety of solid malignancies. Therefore, downregulation of COX-2 in cancer cells may prove useful in improving clinical outcomes in cancer patients.

RNA interference (RNAi) is a powerful method for gene inactivation (15-17) and cancer gene therapy (18). The basic mechanism of RNAi starts with a long double-stranded RNA that is processed into small interfering RNAs (siRNAs) of ~21 nt (19,20). The advantage of RNAi technology is that it can be used to target a large number of different genes, which are involved in a number of distinct cellular pathways. The technology of RNA silencing is poised to have a major impact on the treatment of human disease, particularly cancer (19).

The aim of the present study was to investigate the role of COX-2 in the growth of human ovarian cancer cells. The effect of RNAi-induced COX-2 suppression on the proliferation, invasion and migration of ovarian cancer cells was also evaluated.
Materials and methods

Cell culture. SKOV3 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Modified McCoy's 5A Medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL, Carlsbad, CA, USA). The cells were maintained in a humidified 37°C incubator with 5% CO₂.

siRNA design and conduct. In total, three different siRNAs were designed against COX-2 mRNA as suggested by the method used in a study by Elbashir et al. (21). The targeting sequences corresponding to the siRNAs against COX-2 (GeneBank accession no. NM_000963.2) were as follows: Bases 290-310 (siRNA-1, 5'-AAACTGCTCAACACCGGAATT-3'), bases 456-477 (siRNA-2, 5'-TCACATTTGATTGACAGTCCA-3') and 517-538 (siRNA-3, 5'-CCCTCTCTAACCTCTCTATT-3'). The interference vector pGenesil-COX-2 was constructed using the pGenesil-1 vector. In brief, three pairs of oligonucleotide fragments were designed, synthesized and annealed, pGenesil-1, which consisted of human U6 short hairpin RNA (shRNA) promoter, was used to generate a series of siRNA expression vectors by inserting three pairs of annealed oligonucleotides. The recombinant plasmid vectors pGenesil-1-COX-2(1), pGenesil-1-COX-2(2) and pGenesil-1-COX-2(3) were then repeatedly excised and ligated successively. Thus, the tandem recombinant vector pGenesil-1-COX-2(1+2+3) was constructed and called pGenesil-1-COX-2. All the sequences inserted were verified by DNA sequencing. Plasmid pGenesil-1-KB was used to serve as a control for the empty vector. All the siRNA sequences were checked in terms of their specificity using the Basic Local Alignment Search Tool (National Library of Medicine, Bethesda, MD, USA) database and did not exhibit any homology to other human genes.

The SKOV3 cells were randomly divided into three groups: Untransfected control group, empty vector transfection group that served as a blank control, and the pGenesil-1-COX-2 siRNA expression plasmid transfected siRNA group. In total, 5x10⁵ cells were seeded in six-well plates and grown overnight prior to transfection. All the plasmids were transiently transfected into SKOV3 cells by Lipofectamine™ 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions and incubated in serum-starved media at 37°C in 5% CO₂. At 8 h post-transfection, the medium was replaced with fresh complete medium containing 10% FBS. The cells were harvested 48 h after transfection and used for the evaluation of COX-2 expression. The transfection efficiency was monitored by measuring the percentage of fluorescent cells among a total of 1,000 cells using fluorescence microscopy.

RNA isolation and quantitative polymerase chain reaction (qPCR). To evaluate COX-2 mRNA expression, the cells were harvested following transfection. The total RNA was extracted from the cells using TRIzol reagent (Invitrogen Life Technologies) for reverse transcription. The RNA was transcribed to cDNA using the Superscript First-Strand Synthesis kit (Takara, Dalian, China) following the manufacturer's instructions. qPCR assays were performed using SYBR-Green Real-Time PCR Master Mix (Toyobo, Osaka, Japan) and RT-PCR amplification equipment (7300 Real-Time PCR System; Applied Biosystems, Foster City, CA, USA) using specific primers: COX-2 sense, 5'-CCCTTGGGTGTCAAAGGTAAA-3' and antisense, 5'-AAACTGATGGGTAAGTCTCTG-3'; and β-actin sense, 5'-GCGAGCACAGAGCCTCGCTTGG-3' and antisense, 5'-GATGCCGGTCTCGATGGGGTAC-3'. The PCR conditions were as follows: Pre-denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 10 sec and annealing/extension at 58°C for 20 sec. The amplification specificity was checked by melting curve analysis. The expression of the genes of interest was determined by normalization of the threshold cycle (Ct) of these genes to that of the β-actin control.

Western blot analysis. The cells were harvested and lysed in Triton X-100 in Heps buffer (150 mM NaCl, 50 mM Heps, 1.5 mM MgCl₂, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS) and a protease inhibitor cocktail (Sigma)). Western blot analysis was performed using conventional protocols. Briefly, the protein concentration of the extracts was determined using a bicinchoninic acid kit (Sigma) with bovine serum albumin used as the standard. The total protein samples (40 µg) were separated by 8% SDS-PAGE, proteins were transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA), immunoblotted with specific primary antibodies and incubated with corresponding horseradish peroxidase (HRP) -conjugated secondary antibody. The other primary antibodies used in the western blots were as follows: Antibodies against COX-2, β-actin, VEGF (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); MMP-9 and MMP-2 (Sigma Aldrich, St. Louis, MO, USA); secondary antibodies used for immunodetection were as follows: HRP-conjugated goat anti-mouse immunoglobulin (Ig) G and goat anti-rabbit IgG (Amersham Biosciences, Uppsala, Sweden). All the immunoblots were visualized by enhanced chemiluminescence (Pierce Biotechnology, Inc., Rockford, IL, USA). All the assays using COX-2 knockdown SKVO3 cells were performed after the third day of siRNA transfection.

Cell viability assay. At 24, 48 and 72 h post-transfection with COX-2 siRNAs, the cells were seeded in quadruplicate into 96-well plates (5,000 cells/well in 100 ml of medium). At the indicated times, the cells were incubated with 1 mg/ml MTT in normal culture medium for 6 h at 37°C. The medium was then aspirated and the formazan was dissolved in 200 ml dimethyl sulfoxide. The absorbance was measured at 570 nm by a STAKMAX™ microplate reader (Molecular Devices, Sunnyvale, CA, USA) and the experiments were repeated five times.

Cell cycle analysis. The SKVO3 cells were treated with siRNA for 24 h in Dulbecco's modified Eagle's medium (DMEM) containing 5% FBS. All the cells were collected, and 1x10⁶ cells were centrifuged, resuspended in ice-cold 70% ethanol and stored at -20°C until further analysis. Washed cells were stained by 0.1% Triton X-100 in 0.01 M phosphate-buffered saline (pH 7.2) with 50 µg/ml propidium iodide (Sigma-Aldrich) and 1 mg/ml RNase A (Invitrogen), and incubated at 37°C for 30 min in the dark. Samples of the
cells were then analyzed for their DNA content using FACScan flow cytometry (Beckman, Miami, FL, USA), and cell cycle phase distributions were analyzed by the Cell Quest acquisition software (BD Biosciences, Franklin Lanes, NJ, USA). All experiments were performed in duplicate and repeated twice.

Cell migration assay. The migration assay was performed using a 12-well Boyden Chamber (Neuro Probe, Inc., Gaithersburg, MD, USA) with an 8 µm pore size. In total, ∼1x10⁶ cells were seeded into upper wells of the Boyden Chamber and incubated for 6 h at 37°C in medium containing 1% FBS. DMEM with 10% FBS was used as a chemoattractant in the bottom wells. The cells that did not migrate through the pores of the Boyden Chamber were manually removed with a rubber swab. The cells that migrated to the lower side of the membrane were stained with hematoxylin and eosin and photographed using an inverted microscope.

Transwell invasion assay. The invasiveness of SKVO3 cancer cells was assessed using 24-well Transwell plates (Corning, Lowell, MA, USA). In brief, 2x10⁵ cells in DMEM with 0.5% FBS were added to the upper chamber containing a 8-mm pore polycarbonate coated with 1 mg/ml Matrigel; the lower chamber was filled with media containing 5% FBS. Subsequent to 16 h incubation, the upper surface of the membrane was scrubbed with a cotton-tipped swab. The invading cells on the lower surface of the membrane were fixed and stained with 0.5% crystal violet dye. A total of five random fields per membrane were photographed at magnification, x40 for calculating the cell number using an inverted phase-contrast microscope (Leica, Solms, Germany). In addition, cells were quantified by measuring the absorbance of dye extracts at 570 nm in 100 ml of Sorenson's solution (9 mg trisodium citrate, 305 ml distilled water, 195 ml 0.1 N HCl and 500 ml 90% ethanol). All experiments were performed in triplicate and repeated four times.

Determination of prostaglandin E2 (PGE2) synthesis by ELISA. PGE2 synthesis was determined according to methods that were previously described (22). In brief, the SKVO3 cells were grown in 12-well plates overnight. The culture media of the cells were changed to new DMEM for 30 min prior to harvesting of culture media and then these culture media were centrifuged to remove cell debris. Cell-free culture media were collected at indicated time periods, then PGE2 levels were determined using a Human PGE2 ELISA kit according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA) and an ELISA reader (µQuant; Biotek Instruments, Inc., Winooski, VT, USA).

Statistical analysis. The values were expressed as the mean ± standard deviation. The data were analyzed by one-way analysis of variance and P<0.05 was considered to be statistically significant.

Results

Downregulation of COX-2 mRNA and protein expression levels by COX-2-siRNA. A recombinant vector was designed and constructed, which expresses three siRNAs targeting the COX-2 gene in tandem, and transfected it into SKOV3 cells. Subsequent to 72 h transfection, the cells were harvested and the COX-2 mRNA levels were analyzed by qPCR. COX-2 expression was significantly downregulated in SKOV3 cells transfected with siRNA compared with the cells transfected with the control vector and untransfected cells (P<0.01). There was no significant difference between the untransfected and empty vector groups (P>0.05) (Fig. 1A). The results indicated that most of the COX-2 mRNA was degraded by COX-2 siRNA in the SKOV3 cells.

The effect of COX-2 siRNA treatment on protein expression was assessed by western blot analysis. As shown in Fig. 1B, there was no difference between the untransfected and empty vector group (P>0.05), while the band density clearly decreased in the COX-2 siRNA group as compared with the untransfected and empty vector group. These results demonstrated that siRNA targeting COX-2 significantly silenced COX-2 protein expression in SKOV3 ovarian cancer cells (P<0.01).

COX-2 silencing affects SKVO3 cell proliferation and the cell cycle. To elucidate the effects of the siRNA on SKOV3 ovarian cancer cell proliferation, the MTT assay was used and cell proliferation was determined by counting the number of viable cells. SKOV3 ovarian cancer cells transfected with
COX-2-siRNA proliferated at a much lower rate compared with the control cells at 24, 48 and 72 h post-transfection. Compared with the untransfected cells, the proliferation properties of SKOV3 cells were significantly inhibited (Fig. 2A). These data demonstrate that the inhibition of COX-2 by RNAi can inhibit the proliferation of SKOV3 cells.

In addition, to determine the effects of SKOV3 cell cycle progression following COX-2 silencing, flow cytometry was performed in the present study. In the siRNA therapy group, the percentage of cells in G0/G1 phase was significantly increased as compared with the scrambled-treated and control cells. These results indicated that COX-2 silencing can induce cell cycle arrest in G0/G1 phase in SKVO3 cells (Fig. 2B).

COX-2 silencing inhibits SKVO3 cell invasion and cell migration. To analyze whether siRNA affects SKVO3 cell migration, migration assays were performed using Boyden chambers. RNAi-mediated COX-2 silencing significantly inhibited SKVO3 cell migration as compared with the control (untransfected) and negative control-transfected (Vector) groups (Fig. 3A). On the other hand, COX-2 knockdown in SKVO3 cells markedly inhibited invasion in vitro as compared with the vector-treated and control groups (Fig. 3B). The control cells and vector cells remained invasive and no statistically significant differences were observed.

Silencing of COX-2 decreases levels of PGE2 and other proteins in SKVO3 cells. PGE2 levels in SKVO3 cells were significantly decreased after silencing COX-2 as compared with the control cells. "P<0.05 and ""P<0.01 vs. the control. (B) Protein expression levels of VEGF, MMP-9 and MMP-2 were detected by western blot analysis after COX-2 silencing (left lane, control; middle lane, vector; right lane, siRNA group). PGE2, prostaglandin E2; VEGF, vascular endothelial growth factor; MMP, metalloproteinase; COX-2, cyclooxygenase-2.
determined by ELISA analysis. As shown in Fig. 4A, the PGE2 levels in the siRNA therapy groups were significantly lower compared with those in the untransfected and empty vector groups. In addition, VEGF protein expression levels were determined following COX-2 silencing. It was found that COX-2 silencing was found to significantly inhibit VEGF expression in the SKOV3 tumor cells compared with the control and empty vector cells (Fig. 4B).

In order to investigate the mechanisms involved in the inhibition of the invasion and migration ability by down-regulation of COX-2 silencing of SKOV3 cells, western blot analysis was performed to evaluate the activity of MMP-2 and MMP-9. COX-2 silencing caused a significant decrease in MMP-9 and MMP-2 levels compared with the control cells as determined by western blot analysis (Fig. 4B).

Discussion

RNAi is a fundamental cellular mechanism used for silencing gene expression (16,23-25). RNAi has been widely used in cancer therapy to silence the expression of oncogenes and growth factors or their receptors, which may result in inhibition of the cell cycle, cell proliferation and tumor angiogenesis as well as induction of cell apoptosis (26-28). To date, RNAi has been regarded as an effective and useful approach for therapeutic applications of cancer (26,39). In the present study, RNAi strategies were used to reduce the expression of COX-2 in the ovarian cancer cell line SKOV3 and to evaluate the role of COX-2 in SKOV3 cells. A total of three siRNA sequences were designed to target the COX-2 coding region and inserted into the pGenesil-1 vector in tandem to efficiently suppress the expression of COX-2. The present study demonstrated that this vector was efficiently silenced COX-2. Downregulation of COX-2 reduced proliferation, cell cycle, cell migration and invasion in SKOV3 cells. The results were consistent with those of previous studies revealing that the number of viable cells was significantly decreased following transfection with COX-2 siRNA (28,30,31).

COX-2 is known to be involved in multiple pathophysiological processes, including inflammation and tumorigenesis (32,33). COX-2 is undetectable in numerous normal tissues, yet it is commonly overexpressed in various human cancers, including ovarian cancer (14), with its downstream product being PGE2, which is linked to more aggressive behavior of tumors and thus contributing to ovarian cancer progression (34,35). PGE2 is a significant mediator in tumor-promoting inflammation (36). Additionally, PGE2 promotes tumor cell proliferation, induces VEGF upregulation and inhibits tumor cell apoptosis as well as immune function (37). The present study revealed that downregulation of the COX-2 gene by silencing decreased the levels of PGE2 and VEGF (Fig. 4B) and inhibit tumor cell proliferation and the cell cycle. These results implied that the major mechanism of COX-2 in stimulating tumorigenesis is via its product PGE2.

The poor prognosis of ovarian cancer is mainly due to dissemination caused by the aggressive migration activity of the cancer cells (38). In the present study, siRNA-mediated downregulation of COX-2 expression in human ovarian cancer cells lead to a significant decrease in SKOV3 cell invasion and migration. These results were consistent with previous studies (28,30,31), demonstrating that COX-2 mediates the invasive and metastatic potential of ovarian cancer cells.

In conclusion, the vector expressing three siRNAs targeting the COX-2 gene in tandem was able to silence the expression of COX-2 in ovarian cancer cells. The COX-2 knockdown not only resulted in a decrease of cell proliferation and inhibition of the cell cycle in ovarian tumor cells, but it also suppressed cell migration and invasion of ovarian cancer cells. These results indicated that COX-2 is a potential therapeutic target for the prevention or treatment of ovarian cancer.

Acknowledgements

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References