Targeted inhibition of COX-2 expression by RNA interference suppresses tumor growth and potentiates chemosensitivity to cisplatin in human gastric cancer cells

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Received April 20, 2007; Accepted July 6, 2007

Abstract. Although selective cyclooxygenase-2 (COX-2) inhibitors suppress cell proliferation in gastric cancer, it remains debatable whether their effect is mediated through COX-2 dependent or independent pathways. We investigated the effects of the targeted inhibition of COX-2 expression by small interfering RNA (siRNA) in human gastric cancer cells and compared it to the effects of treatment with a specific COX-2 inhibitor. COX-2 mRNA and proteins were significantly reduced by up to 80% on day 2 after COX-2 siRNA transfection to the gastric cancer cell line MKN45. Concentrations of prostaglandins E2 (PGE2) in the condition medium were also reduced to 30% after siRNA transfection. Transfection of COX-2 siRNA exhibited a more potent anti-proliferative effect on MKN45 cells than treatment with high-dose (100 μM) NS398. COX-2 siRNA also significantly reduced tumor growth in nude mice. While COX-2 siRNA transfection alone had no obvious pro-apoptotic effects, unlike low-dose (10 μM) NS398 it enhanced the apoptotic reaction of MKN45 cells to cisplatin therapy. In conclusion, our results demonstrate for the first time that COX-2 siRNA inhibits cell growth and enhances the chemosensitivity of gastric cancer cells. RNA interference may be a promising alternative to specific COX-2 inhibitors in the prevention and treatment of gastric cancer.

Introduction

Cyclooxygenase (COX) is responsible for the conversion of arachidonic acid to prostaglandins (PGs). There are two forms of COX enzyme. COX-1 is constitutively expressed in all tissues while COX-2 is induced by growth factors, oncogenes and tumor promoters (1). COX-2 is up-regulated in many malignant tumors, including gastric and breast cancers (2,3). Animal studies have found that tumor growth requires COX-2 expression (4) and that enhanced COX-2 expression is sufficient to induce mammary gland tumorigenesis (5). It has also been reported that the overexpression of COX-2 in rat epithelial cells leads to the inhibition of apoptosis (6). These data suggest that COX-2 plays an important role in tumorigenesis.

Gastric cancer is the leading cause of cancer death in China and the second most common cause of cancer death worldwide (7). We and others have previously shown that COX-2 is over-expressed in human gastric cancers and is associated with poor overall survival (2,8,9). In an animal model the use of celecoxib, a specific COX-2 inhibitor, was shown to reduce the incidence and tumor load of chemically-induced gastric cancer in rats (10). These findings suggest that COX-2 is a potential target in gastric cancer prevention and therapy.

Although non-steroidal anti-inflammatory drugs (NSAIDs) and COX-2 inhibitors have been shown to prevent and suppress cancer growth (11,12), several studies indicate that a COX-2 independent mechanism may be involved in the anti-tumor effect of COX-2 inhibitors. For example, NS398 is found to inhibit the oral carcinoma cell line independent of prostaglandin E2 (PGE2) synthesis (13) and inhibit cell growth in a COX-2-negative pancreatic tumor cell line (14). Recent data also find that COX-2 inhibitors induce apoptosis through a novel mitochondria signaling pathway (15).

In order to investigate the role of COX-2 in gastric carcinogenesis, we used small interfering RNA (siRNA) to specifically inhibit COX-2 expression in a gastric cancer cell line. siRNA are short double-stranded RNA molecules that can target complementary mRNA for degradation through a cellular process called RNA interference (RNAi) (16). Recent studies have found that siRNA are a powerful tool for the targeted inhibition of gene expression and viral propagation (17,18). In this study, we blocked COX-2 expression using siRNA in the MKN45 gastric cancer cell line. The effect of siRNA on cell growth and apoptosis induction was compared with that of the COX-2 inhibitor NS398. We also investigated
the potential induction of chemosensitivity in gastric cancer cells by a chemotherapeutic agent under treatment with siRNA or with the COX-2 inhibitor.

Materials and methods

Cell culture and drugs. Human gastric cancer cell line MKN45 (RCB1001) was obtained from the Riken Cell Bank (Tsukuba, Japan). This cell line has been shown to have strong COX-2 expression (19). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA), 50 U/ml of penicillin and 50 μg/ml of streptomycin (Invitrogen). Cultures were maintained in a humidified incubator at 37°C with 5% CO₂.

NS398 (Cayman, Ann Arbor, MI) was used as the selective inhibitor of COX-2. The IC₅₀ values for human recombinant COX-1 and COX-2 are 75 and 1.77 μM, respectively (20). Stock solution was made in 100% DMSA (Sigma, St. Louis, MO) and stored at -20°C. The drug was diluted with culture media on the day of the experiment. The concentration of DMSA for all treatments was <0.157%.

Construction of siRNA. siRNA for COX-2 was generated using the Silencer siRNA Construction Kit (Ambion, Austin, TX) according to the manufacturer's instructions. Oligonucleotides were used for generating siRNA against COX-2 (siCOX-2) were sense 5'-AATGTCCGGGTACAATCGCACCCTGTCTC and antisense strand, 5'-AAGTGCGATTGTACCCGGACACCT. The underlined nucleotide sequence was annealed to the T7 promoter sequence. The siRNA was controlled for sequence specificity by a BLAST search and did not show sequence homology to other known human genes.

Construction of siRNA expressing plasmid. To construct siCOX-2 expressing plasmid, oligonucleotides with sequences specific for COX-2 (5'-GATCCGTGCGATTGTACCCGGACATTCAAGAGATGTCCGGGTACAATCGC) and antisense strand, 5'-AAGTGCGATTGTACCCGGACACCTGTC and (nucleotide position, 255-275, NM_000963). The primer sequences for COX-2 were 5'-TTCAAAATGAGATTGAGAAATTTGAC (forward) and 5'-ATCTCTGCTCGTATCTCTT (reverse) (22). Expression of COX-2 was normalized to cDNA loading for each individual sample using β-actin (primer sequence: forward, 5'-GCACGCTAACATGGGACCCGGACATTCAAGAGATGTCCGGGTACAATCGC; reverse, 5'-GTGCCATCACGTTGAGGG; sense strand: 5'-GCACGCTAACATGGGACCCGGACATTCAAGAGATGTCCGGGTACAATCGC). The primer sequences for COX-1 and COX-2 are 75 and 1.77 μM, respectively (20). Subsequently, 10 μl of a mixture of 40 μM siRNA and 175 μl of Opti-MEM I Reduced Serum Medium was added. After incubation for 15-20 min at room temperature, 200 μl siRNA mixture was added to the well containing 800 μl of serum-free RPMI-1640 and incubated for a period of 4-5 h. As a control experiment, cells were similarly transfected with either siRNA against luciferase (siGL2) (21) or oligofectamine (vehicle only). After transfection, cells were replenished with regular medium and left untreated for different periods of time. Cells, total-RNA or protein were then harvested for further analysis.

Quantitative analysis of COX-2 mRNA expression. COX-2 mRNA levels were determined by real-time quantitative RT-PCR. Total-RNA was extracted by TRizol reagent (Invitrogen) and reverse-transcribed into cDNA by MMLV (Promega, Madison, WI). The level of COX-2 mRNA was examined by real-time quantitative PCR using ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). Each reaction contained 12.5 μl of 2x SYBR supermix (Bio-Rad Laboratories, Hercules, CA), 0.2 μl of 10 pm/ml of forward and reverse primers and 5 μl of DNA template for a total volume of 25 μl. The primer sequences for COX-2 are 5'-TTCAAAATGAGATTGAGAAATTTGAC (forward) and 5'-ATCTCTGCTCGTATCTCTT (reverse) (22). Expression of COX-2 was normalized to cDNA loading for each individual sample using β-actin (primer sequence: forward, 5'-GCACGCTAACATGGGACCCGGACATTCAAGAGATGTCCGGGTACAATCGC; reverse, 5'-GTGCCATCACGTTGAGGG; sense strand: 5'-GCACGCTAACATGGGACCCGGACATTCAAGAGATGTCCGGGTACAATCGC). The primer sequences for COX-1 and COX-2 are 75 and 1.77 μM, respectively (20). After transfection, the blots were blocked in 10% milk (fatty acid-free) with TBS-T (0.1% Tween-20, 20 mM Tris, 137 mM NaCl, and 1 M HCl). The blots were then hybridized with anti-COX-2 or anti-COX-1 goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 1:100 dilutions for 2 h at room temperature. This was followed by washing in TBS-T and then incubated with horseradish peroxidase-conjugated donkey anti-goat or rabbit anti-mouse IgG (Santa Cruz Biotechnology) at 1:2000 dilution for 2 h at room temperature. After several washings, the blots were developed using SuperSignal West Pico Western blot detection kit (Pierce, Rockford, IL).

Western blot analysis. Cells were washed with cold PBS and lysed on ice in 400 μl NET lysis buffer (1% NP-40, 50 mM Tris at pH 8.0, 150 mM NaCl, 5 mM EDTA, 10 μg/ml of aprotinin and pepstatin and 100 μg/ml of PMSF). Protein concentrations were determined using the Bio-Rad protein assay. Fifty micrograms of protein mixtures were separated on 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane at 100 V for 2 h at 4°C using a Bio-Rad transfer unit. The transfer buffers consisted of 25 mM Tris, 192 mM glycine and 20% methanol. After transfer, the blots were blocked in 10% milk (fatty acid-free) with TBS-T (0.1% Tween-20, 20 mM Tris, 137 mM NaCl, and 1 M HCl). The blots were then hybridized with anti-COX-2 or anti-COX-1 goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 1:1000 dilutions for 2 h at room temperature. As an internal control, the blots were stripped and reprobed with anti-β-actin mouse monoclonal antibody (Sigma) in 1:2000 dilution for 1 h at room temperature. This was followed by washing in TBS-T and then incubated with horseradish peroxidase-conjugated donkey anti-goat or rabbit anti-mouse IgG (Santa Cruz Biotechnology) at 1:2000 dilution for 1 h at room temperature. After several washings, the blots were developed using SuperSignal West Pico Western blot detection kit (Pierce, Rockford, IL).

BrdU proliferation assay. Cell proliferation was determined by Cell proliferation ELISA, BrdU (Roche, Mannheim, Germany) through the monitoring of BrdU (pyrimidine analogue) incorporation. Cells were plated on a 96-well plate
at a density of 3000 cells/well. After seeding overnight, cells were either transfected with siRNA against COX-2 or incubated with various amount of NS398.

PGE2 production by ELISA assay. PGE2 was measured using the Biotrak PGE2 competitive enzyme immunoassay system (Amersham, Buckinghamshire, UK) according to the manufacturer’s instructions. Cells were plated on a 96-well plate at a density of 1x10⁴ cells/well. After seeding overnight, cells were either transfected with siRNA against COX-2 or incubated with various amount of NS398. Condition medium was extracted and used for PGE2 analysis. The PGE2 level was normalized to protein amount and the relative level of that day’s control experiment was calculated. The results were expressed as mean ± standard deviation of 3 independent experiments.

Cell cycle analysis. MKN45 cells were treated with either siCOX-2 or 10 μM of NS398 and were cultured in the presence of 5 μg/ml cisplatin for 24 h. They were then collected after brief trypsinization, washed with PBS, and fixed in 70% ethanol for 24 h at 4˚C. Cells were then treated with 0.1 mg/ml RNase, stained with 10 μg/ml propidium iodide (PI) at 37˚C for 30 min and analyzed by FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ). The percentage of apoptotic cells in the sub-G1 area was analyzed using Cell Quest software (Becton-Dickinson). Cell-cycle distribution was quantified by ModFit LT software (Becton-Dickinson).

Colony formation assay. To assay colony formation, 5x10⁵ cells were seeded onto 10-cm diameter plates. Cells were transfected the following day with siCOX-2 expressing plasmid (pCOX-2) or empty vector (pSilencer-3.1-H1 neo, pNEG) using lipofectamine plus reagent (Invitrogen), according to the manufacturer’s protocol. Twenty-four hours after transfection, cells from each plate were trypsinized and re-plated equally into 3 10-cm diameter plates maintained in RPMI-1640 with 10% FBS under selection of 500 μg/ml G418 (Invitrogen) for 14 days at 37˚C. Plates were fixed with 4% formaldehyde in PBS, and colonies were visualized by staining with 0.01% crystal violet. The number of colonies formed was counted by GeneTools ver. 3.0 (SynGene Laboratories, Cambridge, UK).

In vivo tumor growth assay. Six-week-old female athymic nude mice (BALB/c nu/nu) were obtained from the Laboratory Animal Service Center of The Chinese University of Hong Kong. All mice were kept under specific pathogen-free conditions using a laminar airflow rack and had free access to sterilized food and autoclave water. All experiments were performed under a license from the government of the Hong Kong SAR and endorsed by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong.

MKN45 cells transiently transfected with either 400 nM of COX-2 siRNA or siGL2 were injected subcutaneously (1x10⁶ cells per injection) into the flank of each mouse at day 0. Tumor size was measured daily with calipers in three dimensions: length (L), width (W), and height (H). Tumor volume was calculated using the formula (π x L x W x H)/6 (23). At the end of the experiment, all mice were sacrificed by cervical dislocation.

Results

Inhibition of COX-2 expression and PGE2 production by RNAi. We first investigated the efficiency of COX-2 suppression by RNA interference. The results showed that COX-2 mRNA (Fig. 1A) and protein (Fig. 1B) was maximally inhibited by COX-2 siRNA on day 2 after transfection. There was an over 80% reduction in COX-2 mRNA expression on day 2. As shown in Fig. 1B, COX-2 protein levels gradually re-expressed on day 4 of the experiment. Protein levels of COX-1 and β-actin remained unchanged (Fig. 2), suggesting that our siRNA was specific. We then investigated the effects of COX-2 siRNA on PGE2 production in MKN45 cells (Fig. 2). Maximal suppression of PGE2 production was noted on day 3, on which it was reduced to 40% of the control (vehicles only, P<0.05). In contrast, NS398 produced a potent inhibitory effect on PGE2 production on day 1 with doses as low as 1 μM (P<0.05 vs. control).
COX-2 siRNA inhibits cell proliferation. Previous studies have found that the COX-2 inhibitor NS398 can inhibit the growth of gastric cancer cells (24). We next determined the effects of siRNA on the proliferation of the MKN45 cells (Fig. 3). Cells were either transiently transfected with 400 nM siRNA or treated with various concentrations of the COX-2 specific inhibitor NS398. On different days, levels of PGE$_2$ in the condition media were measured by ELISA assay. The relative level of PGE$_2$ was determined by comparison to the level of the control experiment on the same day. On day 3, both siRNA and NS398 showed a significant reduction of PGE$_2$ levels (P<0.01).

**Figure 3.** Effects of COX-2 siRNA on cell proliferation. Cells were transfected with either 400 nM of COX-2 siRNA or various concentrations of NS398 on a 96-well plate. The proliferation was determined by BrdU incorporation assay. COX-2 siRNA demonstrated a significant growth inhibition of 62% on MKN45 cells (P<0.01). Using NS398, a similar inhibitory effect can only be observed at high (100 μM) concentrations.

**Figure 4.** Effects of COX-2 siRNA on the colony formation on MKN45 cells. Cells were transiently transfected with either plasmid expressing COX-2 siRNA (siCOX-2) or scramble siRNA (negative control). Colonies were formed after 14 days of G418 selection. Cells transfected with COX-2 siRNA showed a significant reduction in colonies formation (P<0.05).

**Figure 5.** Effects of COX-2 siRNA on tumor growth in vivo. MKN45 cells transiently transfected either with COX-2 siRNA (open box) or luciferase siRNA (black box) were injected subcutaneously into athymic nude mice. Tumor volumes were measured after day 6. At day 14, the tumor with COX-2 siRNA had a significantly smaller volume (P<0.05). Data were expressed as mean ± SD (n=4).

COX-2 siRNA reduced cell growth in colony formation assay. To further determine the effect of COX-2 siRNA on the growth of MKN45 cells, we transiently transfected the cells with plasmid expressing COX-2 siRNA (pCOX-2). As a control experiment, plasmid expressing siRNA with no homology to human genome (scramble) was transfected into the cells. After 14 days of G418 selection, the colonies obtained were stained and counted (Fig. 4). Cells transfected with pCOX-2 showed a significant reduction in the number of colonies (82±7 colonies vs. 122±11 colonies, P<0.05).

COX-2 siRNA reduced tumor growth in vivo. To examine the effects of siCOX2 on MKN45 cells in vivo, cells transfected with either siCOX2 or siGL2 were injected subcutaneously into nude mice. Tumor growth from cells injected with siCOX2 was first observed on day 6 after the injection of the MKN45 cells (Fig. 5). After 14 days, there was a significant difference...
COX-2 inhibition (27,28). In light of these findings, we investigated the role of specific inhibition of COX-2 expression by RNAi and its effect on tumor growth. Although several reports have demonstrated that RNAi-mediated COX-2 inhibition can prevent growth in prostate and colon cancer cells (36,37). Our results demonstrate for the first time that, in gastric cancer, siRNA used against COX-2 can inhibit tumor growth and enhance cisplatin-induced apoptosis. The potential of using COX-2 as a therapeutic target for the treatment and prevention of cancer has been very encouraging. However, recent reports on cardiovascular complications related to long-term use of COX-2 inhibitors have raised safety concerns (38,39). Silencing COX-2 by RNA interference as demonstrated in this study may prove to be a more promising approach to gastric cancer therapy.

Acknowledgements

This study was supported by a direct grant from The Chinese University of Hong Kong.

References


