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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Abstsract. 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 (PGE_2) in the condition medium were also reduced to 30% after siRNA transfection. Transfection of COX-2 siRNA exhibited a more potent antiproliferative 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

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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 overexpressed 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 antitumor effect of COX-2 inhibitors. For example, NS398 is found to inhibit the oral carcinoma cell line independent of prostaglandin E2 (PGE_2) 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 strepto-mycin (Invitrogen). Cultures were maintained in a humidified incubator at 37˚C with 5% CO_2.

NS398 (Cayman, Ann Arbor, MI) was used as the selective inhibitor of COX-2. The IC_{50} 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 instruction. Oligonucleotides used for generating siRNA against COX-2 (siCOX-2) were sense 5'-AATGTCCGGGTACAATCGCACCCTGTCTC and antisense strand, 5'-AAGTGCGATTGTACCCGGACACCTGTCTC (nucleotide position, 255-275, NM_000963). The underlined nucleotide sequence was annealed to the T7 pro-moter 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 containing sequences specific for COX-2 (5'-GATCCGTGCGATTGTACCCGGACATTCAAGAGATGTCCGGGTACAATCGCACTTTTTTGGAAA-3' and 5'-GCACGCTAACATGGGCCTGTAAGTTCTCTACAGGCCCATGTTAGCGTGAAAAAACCTTTTCGA-3') were synthesized and annealed. COX-2 siRNA expression vector (pCOX-2), which expresses hairpin siRNA under the control of human H1 RNA polymerase III promoter, was constructed by inserting pairs of the annealed DNA oligonucleotides into the p*Silencer*-3.1-H1 neo siRNA expressing vector that was digested with BamH1 and HindIII (Ambion). A p*Silencer*-3.1-H1 neo (pNEG) that expressed a hairpin siRNA with limited homology to any known sequences in the human, mouse, and rat genomes was used as a negative control (Ambion).

COX-2 siRNA transfection. MKN45 cells were plated at a density of 50000 cells/well in 6-well plates and grown to 50% confluence. After 24 h, the cells were transfected with siRNA in oligofectamine reagent (Invitrogen) according to the manufacturer's instructions. Briefly, for each well, 4 μl of oligofectamine reagent was incubated with 11 μl of Opti-MEM I Reduced Serum Medium (Invitrogen) for 15 min. 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/μl 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'-TTCAAATGAGATTGTGGGAAAATTGCT-3' (forward) and 5'-AGATCATCTCTGCCTGAGTATCTT-3' (reverse) (22). Expression of *COX-2* was normalized to cDNA loading for each individual sample using *ß-actin* (primer sequence: forward, 5'-GCATTTGCGGTGGACGATGGAGG; reverse, 5'-GGTCACCCACACTGTGCCCATCTA) as an internal standard. Hot start PCR was initiated at 94˚C for 6 min and followed by 35 cycles of denaturation at 94˚C for 30 sec, annealing at 60˚C for 25 sec and extension at 72˚C for 25 sec.

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 (Bio-Rad Laboratories). Fifty micrograms of protein were separated on 10% SDS-polyacrylamide gel electro-phoresis 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 striped and reprobed with anti-ß-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, 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
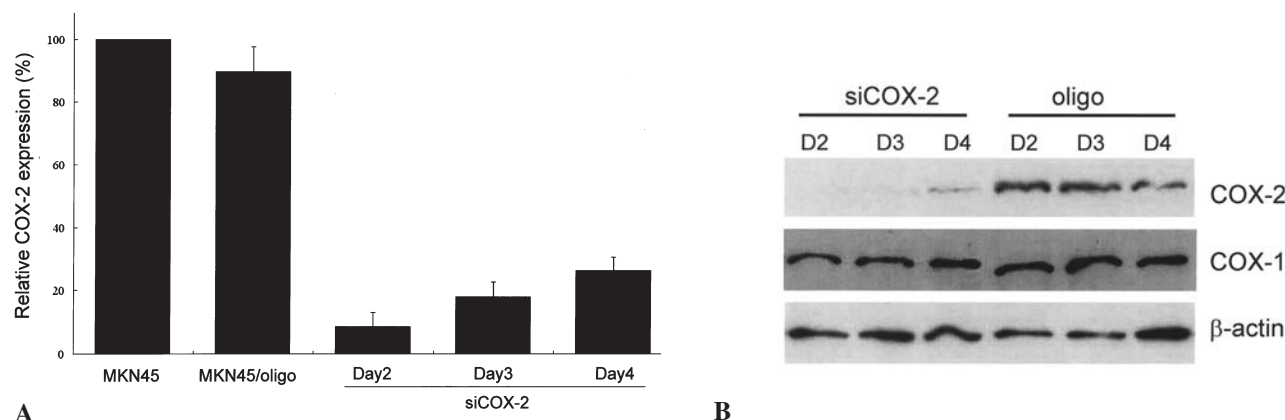
Figure 1. Specific knockdown of COX-2 by siRNA in MKN45 gastric cancer cells. Cells were transiently transfected with 400 nM siRNA against COX-2. COX-2 mRNA and protein levels were analyzed by real-time RT-PCR (A) and Western blot (B) on days 2-4 after transfection. COX-2 levels deceased to 10% of the original on day 2 after transfection, while levels of COX-1 and ß-actin (loading control) remain unchanged, suggesting that the siRNA was gene specific. In the control experiment (oligofectamine only), the levels of both COX-2 and COX-1 showed no change.

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.

PGE$_2$ production by ELISA assay. PGE$_2$ was measured using the Biotrak PGE$_2$ 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 1×10^4 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 PGE$_2$ analysis. The PGE$_2$ 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, 5×10^5 cells were seeded onto 10-cm diameter plates. Cells were transfected the following day with siCOX-2 expressing plasmid (pCOX-2) or empty vector (p*Silencer*-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 Labortories, 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 (1×10^6 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 $(\pi \times L \times W \times H)/6$ (23). At the end of the experiment, all mice were sacrificed by cervical dislocation.

Results

Inhibition of COX-2 expression and PGE$_2$ 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 ß-actin remained unchanged (Fig. 2), suggesting that our siRNA was specific.

We then investigated the effects of COX-2 siRNA on PGE$_2$ production in MKN45 cells (Fig. 2). Maximal suppression of PGE$_2$ 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 PGE$_2$ production on day 1 with doses as low as 1 μM ($P < 0.05$ vs. control).
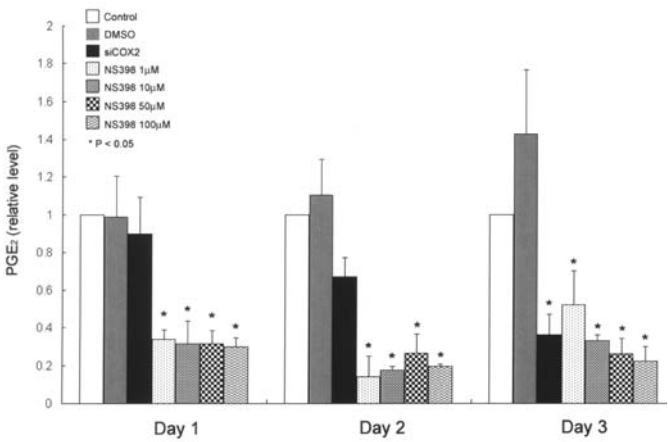
Figure 2. Relative levels of PGE_2 in MKN45 cells after treatment with COX-2 siRNA or NS398. Cells were either transiently transfected with 400 nM siRNA or treated with various concentrations of the COX-2 specific inhibitor NS398. On different days, levels of PGE_2 in the condition media were measured 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 ($P<0.05$).
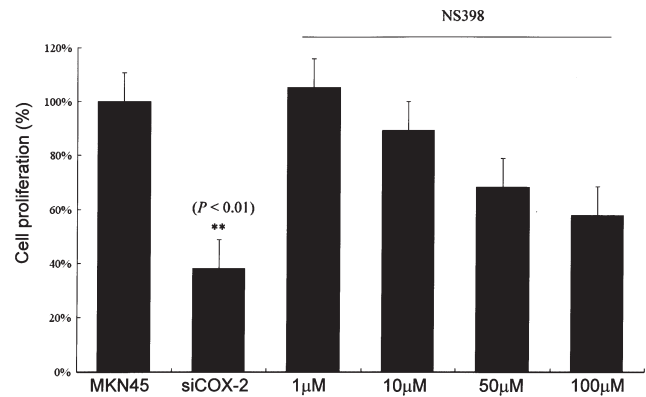

Figure 3. Effects of COX-2 siRNA on cell proliferation. Cells were transfected with either 400 nM of COX-2 siRNA or various concentrations of NS398 on a 96-well plate. The proliferation was determined by BrdU incorporation assay. COX-2 siRNA demonstrated a significant growth inhibition of 62% on MKN45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

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 treated with either NS398 or siRNA against COX-2. Proliferation was then determined by BrdU incorporation. Specific inhibition of COX-2 by siRNA reduced cell growth by 62% as compared to the control (vehicles only; $P<0.01$). NS398 showed a dose-dependent inhibition of cell proliferation. However, only 10% growth reduction was observed in MKN45 cells treated with 10 μM of NS398 and the inhibitory effect of 100 μM NS398 was still less than that of siRNA (Fig. 3).
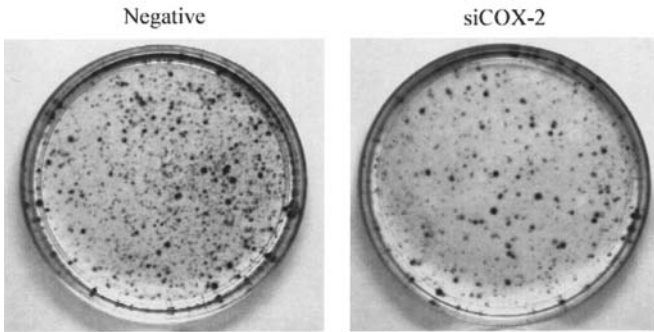

Figure 4. Effects of COX-2 siRNA on the colony formation on MKN45 cells. Cells were transiently transfected with either plasmid expressing COX-2 siRNA (siCOX-2) or scramble siRNA (negative control). Colonies were formed after 14 days of G418 selection. Cells transfected with COX-2 siRNA showed a significant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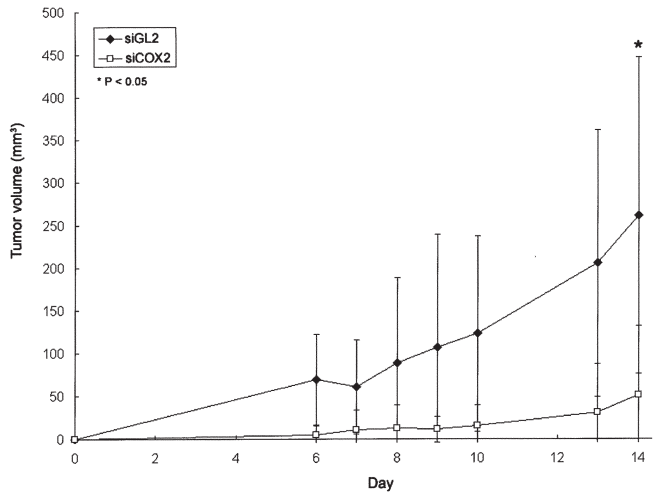

Figure 5. Effects of COX-2 siRNA on tumor growth *in vivo*. MKN45 cells transiently transfected either with COX-2 siRNA (open box) or luciferase siRNA (black box) were injected subcutaneously into athymic nude mice. Tumor volumes were measured after day 6. At day 14, the tumor with COX-2 siRNA had a 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
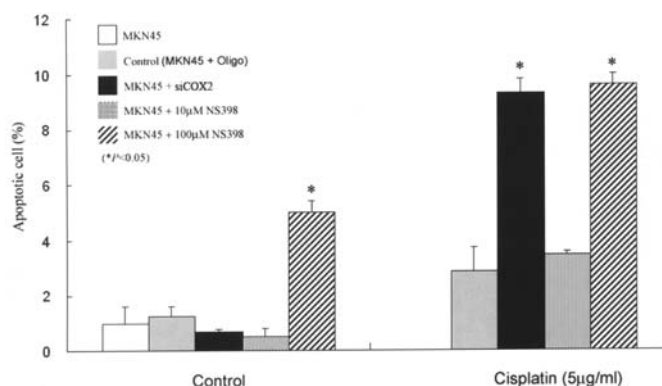
Figure 6. Analysis of apoptotic cells by flow cytometry. MKN45 cells treated with COX-2 siRNA or NS398 were subjected to a cisplatin-induced chemosensitivity assay as determined by the flow cytometry. COX-2 siRNA and 100 μM of NS398 significantly increased the apoptosis of MKN45 cells in combination with cisplatin treatment. Data were expressed as mean ± SD (n=3) (*P<0.05 as compared to control, oligofectamine only).

in tumor volume between cells transfected with siCOX2 and siGL2 (51.2±79.8 mm^3 vs. 261.3±185.8 mm^3, P<0.05).

Effect of COX-2 siRNA on cisplatin-induced apoptosis in MKN45 cells. The effects of COX-2 siRNA on apoptosis was determined by flow cytometry (Fig. 6). Treatment with 100 μM of NS398 significantly increased apoptosis (P<0.05). On the other hand, transfection with COX-2 siRNA or treatment with 10 μM of NS398 showed no significant induction of apoptosis as compared to the control MKN45 cells. Notably, COX-2 siRNA significantly potentiated the effects of cisplatin-induced (5 μg/ml) apoptosis on gastric cancer cells (P<0.05, Fig. 6). The pro-apoptotic effect was comparable to treatment with 100 μM of NS398, suggesting that COX-2 siRNA enhances the chemosensitivity of MKN45 cells to cisplatin.

Discussion

Overexpression of COX-2 is associated with gastric carcinogenesis. Several earlier studies, including ours, have investigated the role of COX-2 in human gastric carcinomas (9,10,12,25,26). These studies provide the basis for the use of COX-2 inhibitors on chemoprevention and the treatment of gastric cancer. However, the existence of possible COX-2 independent effects may raise concerns about the role of 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.

Our data show that COX-2 siRNA can specifically suppress COX-2 mRNA and protein expression levels in the MKN45 gastric cancer cell line. Moreover, PGE$_2$ production was effectively suppressed by siRNA to a level comparable to treatment with NS398. COX-2 siRNA resulted in significant growth inhibition of MKN45 both *in vitro* and *in vivo*. Unlike treatment with high doses of NS398, the anti-proliferative effect of COX-2 siRNA was not associated with a significant induction of apoptosis. Although several reports have demonstrated that NS398 induces growth inhibition in a dose-dependent manner (29-31), the inhibitory effect of NS398 at

high concentrations is associated with the induction of apoptosis via COX-2 independent pathways (13,32). Our results are also in keeping with a previous report by Denkert *et al*, which demonstrates that the growth inhibitory effect of COX-2 siRNA is not associated with the induction of apoptosis (33). Together, the results imply that the specific function of COX-2 in gastric cancer therapy is not linked to apoptotic machinery.

Defects in apoptosis are important in tumorigenesis as well as in the treatment of cancer. A number of chemotherapeutic agents have been shown to induce apoptosis. However, most gastric cancers are not responsive to chemotherapy, suggesting that these tumors are intrinsically resistant to apoptosis-inducing agents (34,35). We therefore examined siRNA to see if it could enhance the sensitivity of MKN45 towards chemotherapeutic agents. While COX-2 siRNA alone had no significant effect on apoptosis induction, cells transfected with siRNA experienced a significant increase in cisplatin-induced apoptosis. A similar synergistic effect, however, can only be observed at high concentrations of NS398, which is likely medicated through COX-2 independent pathway. Previous reports have shown that 100 μM of NS398 can enhance various chemotherapeutic drug-induced apoptosis in hypopharyngeal carcinoma (31).

Recently, several reports were published demonstrating 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

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