Cyclooxygenase 2 (COX-2) and COX-2-derived prostaglandins (PG) are up-regulated in the majority of human tumors and are believed to have an important role in cell proliferation, tumor growth, cell motility, and tumor angiogenesis (1, 2). Overexpression of COX-2 in human tumors is associated with poor prognosis and poor response to radiation therapy (3–7). COX-2 is considered as one of the crucial targets for cancer therapy (2, 8). During the past decade, several preclinical and clinical studies were initiated to evaluate the antitumor potency of COX-2 inhibitors either alone or in combination with radiation (1, 2, 9–12). These studies showed the effectiveness of pharmacologic intervention of COX-2 in vivo (8, 13–15), as well as in vitro (2, 9, 16–18), or in both models (16, 19–22). The in vitro studies indicated that, in general, COX-2-specific inhibitors were effective at concentrations that were higher than those at which PG synthesis was inhibited and also required prolonged exposure to the drugs. At these concentrations, the effects were seen even in cells that did not express COX-2 (23, 24). Therefore, it seems that COX-2 inhibitors affect several cellular targets in addition to COX-2. Some of the COX-2-independent effects of these drugs include inhibition of kinases, transcription factors, inhibition of sublethal damage repair and cyclins, and changes in cell cycle distribution (13, 16, 21, 25, 26).

The mechanism by which COX-2 inhibitors inhibit tumor growth and enhance the radiosensitivity in vivo is complex and inhibition of PGE2 with subsequent inhibition of angiogenesis seems an important event (9). COX-2 inhibitors could also inhibit the COX-2 expressed in tumor stroma and vasculature and may thus indirectly affect the tumor growth (16, 17, 27). On the other hand, some of the in vitro studies suggested that COX-2 inhibitors directly increase the radiosensitivity of tumor cells (2, 20, 22).

The purpose of this study was to evaluate whether the radiosensitivity of cancer cells is determined by cellular COX-2. To avoid the COX-2-independent effects associated with the pharmacologic inhibitors of COX-2, we silenced the COX-2 expression using small interfering RNA (siRNA) targeted against COX-2 gene in human prostate and cervical carcinoma cells.
The radiosensitivity of transfected cells was determined by clonogenic cell survival assay. These studies showed that the inhibition of COX-2 by using siRNA targeted against COX-2 in vitro did not alter the radiosensitivity (enhancement ratio = 1.1) of the tumor cells.

**Materials and Methods**

**Cells and reagents.** PC3 human prostate carcinoma cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, glutamine, and antibiotics. HeLa human cervical carcinoma cells were grown in MEM–α supplemented with 10% fetal bovine serum, glutamine, and antibiotics. Tissue culture reagents were purchased from Life Technologies, Inc. (Grand Island, NY). COX-2 goat polyclonal antibody was purchased from Cayman Chemicals (Ann Arbor, MI). HIF-1α mouse monoclonal antibody was purchased from Transduction Laboratories (San Diego, CA). Topoisomerase I (topo-I) and horseradish peroxidase–conjugated goat and mouse antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-actin antibody was from Chemicon (Temecula, CA). NS 398 was purchased from Cayman Chemicals.

**Small interfering RNA and transfection reagents.** SMARTpool siRNA targeting COX-2 (siCOX-2; SMARTpool, M-004557-00) and positive and negative control siRNAs were purchased from Dharmacon (Lafayette, CO). We used three negative controls in this study. They were vehicle control, siCONTROL nontargeting siRNA pool (D-001206-13-20, siNT), and siCONTROL RISC free (siRF) siRNA (D-001220-01-20). Lamin A/C (siLamin) was used as a positive silencing control to ascertain the transfection efficiency in each experiment. Cells were transfected with siRNAs using oligofectamine reagent in Opti-MEM (Invitrogen, Carlsbad, CA).

**Transfection of small interfering RNAs.** Cells were plated in antibiotic-free RPMI at a density of 200,000 cells/5 mL in 60-mm dishes. After 20 hours, cells were transfected with 100 nmol/L siRNA in oligofectamine reagent according to the manufacturer’s instructions. Briefly, 10–μL oligofectamine was diluted 7.5-fold in Opti-MEM and incubated at room temperature for 10 minutes. In a separate tube, 5–μL of 50 μmol/L siRNA were diluted in 425 μL of Opti-MEM. Diluted oligofectamine (75 μL) was added to the diluted siRNA and the complex was incubated for 20 minutes at room temperature. Cells were washed with Opti-MEM and 2–μL Opti-MEM was added to each dish. siRNA + oligofectamine complex (500 μL) was added gently to the dish. The final concentration of the siRNA was 100 nmol/L. After 6 hours, 1.25 mL of 3× serum medium was added to the dish without removing the transfection mix. In addition to medium control (cells in Opti-MEM) and vehicle control (oligofectamine), cells were transfected with positive (siLamin A/C) and negative control (siRF, siNT) siRNAs as described. For vehicle control, 75 μL of diluted oligofectamine was added to 425 μL of Opti-MEM and this was added to dishes.

**Western blot analysis.** Whole cell extracts were prepared as described previously (28). Protein samples (25 μg) were separated on 10% gel, transferred to polyvinylidene difluoride membrane, and probed with COX-2 antibody. Membranes were processed by enhanced chemiluminescence method (Santa Cruz Biotechnology). The membranes were stripped using Re-Blot Plus stripping solution from Chemicon and probed for actin. Protein bands were visualized by autoradiography and were scanned using a Hewlett-Packard Scanjet 5470c (Palo Alto, CA) scanner. Signal intensities were quantitated using ImageQuant (5.2 version) software (Molecular Dynamics, Sunnyvale, CA). COX-2 values were normalized to their loading control actin and expressed as fold change compared with medium control.

**Prostaglandin enzyme immunoassay.** PC3 cells were transfected with vehicle, siNT, and siCOX-2 in six-well plate in duplicate for each condition. At 72 hours, media were removed and 1 mL of Opti-MEM with and without 30 μmol/L arachidonic acid was added. After 15 minutes of incubation at 37°C, media were collected, centrifuged at 4°C at 2,000 rpm × 10 minutes to remove debris, and stored at −70°C. To see the effect of COX-2 inhibitors on transfected cells, cells were pretreated with 25 μmol/L NS 398 before adding arachidonic acid. Cells from each well were trypsinized and counted. PGE2 concentration was determined by ELISA (Oxford Biomedical Research, Oxford, MI) according to the manufacturer’s instructions and expressed as ng PGE2 secreted per 10^6 cells.

**Cell cycle analysis.** PC3 cells were transfected as above with vehicle, siNT, siRF, and siCOX-2. Forty-eight hours after transfection, medium was removed and fresh medium was added. After 24 hours, cells were analyzed for changes in cell cycle distribution. Data were collected on FACS Calibur cytometer (BD) Immunochemistry (Fullerton, CA) and analyzed using CellQuest/MOD-Fit software.

**Irradiation.** Cells were irradiated in a PANTAK high frequency X-ray generator (East Haven, CT), operated at 300 V and 10 mA with Al filtration, at a dose of 1.6 Gy/min.

**Clonogenic cell survival assay.** Cells were transfected as above with vehicle, siNT, siRF, and siCOX-2. Seventy-two hours after transfection, cells were trypsinized, counted, and appropriate number of cells were plated in six-well plates and allowed to attach for 6 hours. After 6 hours, cells were irradiated and incubated for 12 days. Colonies were stained with crystal violet and colonies of ≥50 cells were counted. To study the effect of COX-2-specific inhibitor NS 398 on the radiosensitivity of COX-2-depleted cells, cells were transfected with siRNAs as above and treated with 100 μmol/L NS 398 in fresh medium after 48 hours. After 24 hours of treatment with NS 398, cells were irradiated with 4 Gy and plated for clonogenic assay.

**Statistical analysis.** Data are expressed as mean ± SE. Data were analyzed by t test. P < 0.05 was considered significant.

**Results**

To ascertain that RNA inhibition conditions were optimal and transfection efficiency was satisfactory, Lamin A/C siRNA was used as a positive control in each experiment. Lamin A/C is a housekeeping gene that is abundantly expressed in most mouse and human cells. In Western blot analysis, Lamin A/C is seen as a double band; the lower band (Lamin C) is more prominent than the upper band (Lamin A). In cells transfected with Lamin A/C siRNA, the upper band was completely inhibited and the lower band was significantly reduced confirming high transfection efficiency (Fig. 1A). In cells transfected with siRF or siNT controls, the lamin expression was comparable with the vehicle control.

COX-2 protein levels in PC3 cells transfected with small interfering RNA targeted to COX-2 and control small interfering RNAs. Figure 1B shows Western blot analysis of COX-2 protein expression in PC3 cells (top) at 72 hours following transfection with siRNAs. COX-2 protein levels of cells transfected with vehicle, siRF, and siLamin were comparable with the COX-2 in nontransfected cells. In cells transfected with 100 nmol/L siCOX-2, the COX-2 protein was significantly reduced compared with the nontransfected cells. Unexpectedly, in PC3 cells transfected with negative control siNT, there was a significant increase in COX-2 protein compared with the nontransfected cells. Densitometric analysis of Western blots (bottom) showed that in cells transfected with siCOX-2, the COX-2 protein was reduced by 75% and in cells transfected with negative control siNT there was 2-fold increase in COX-2 protein (Fig. 1B, top and bottom).

Prostaglandin levels in the medium secreted by PC3 cells transfected with COX-2 small interfering RNA. Figure 1C shows arachidonic acid–induced PGE2 levels in medium secreted by
PC3 cells transfected with vehicle, control siNT, and siCOX-2. The basal PGE2 levels were very low because the assay media were collected only 15 minutes following media change. Treatment of cells with 30 μmol/L arachidonic acid induced PGE2, which was secreted in the medium. In vehicle controls, the basal and arachidonic acid–induced PGE2 concentrations (mean ± SE, n = 3) were 0.17 ± 0.03 and 4.81 ± 0.85, respectively. As seen in Fig. 1C, there was ~50% inhibition in PGE2 synthesis in cells that were transfected with siCOX-2 compared with the PGE2 increase in cells transfected with vehicle alone. On the contrary, control siNT that induced COX-2 protein also increased PGE2 secretion by ~2-fold in comparison with cells transfected with vehicle alone. A 15-minute pretreatment with 25 μmol/L NS 398 prevented the up-regulation of arachidonic acid–induced PGE2 in all transfected cell types (data not shown).

COX-1 levels remained unchanged in PC3 cells transfected with vehicle, siNT, siRF, siCOX-2, or siLamin (Fig. 1D). The data on Western blot analysis together with PGE2 ELISA analysis thus confirmed that transfection of PC3 cells with siRNA targeted against COX-2 resulted in the specific inhibition of COX-2 at both protein level and functional level.

Control siNT that induced COX-2 protein in PC3 cells did not increase any of the other proteins analyzed in this study including Lamin A/C, COX-1, HIF-1α (data not shown), actin, or topo-1 suggesting that the induction of COX-2 by siNT was a specific response. We included this negative control in all experiments with PC3 cells, because it allowed us to evaluate responses of cells expressing almost 10-fold differences in COX-2 protein.

Cell growth and cell cycle analysis. PC3 cells were counted at 72 hours after transfection to monitor the cell growth after transfection. In general, the number of cells in dishes transfected with siCOX-2 was lower by ~15% to 20% compared with vehicle-treated cells. However, the plating efficiencies of cells were comparable. Table 1 shows the cell cycle distribution of PC3 cells transfected with vehicle, siNT, and siCOX-2. Cells were stimulated with 30 μmol/L arachidonic acid for 15 minutes and PGE2 levels were determined by ELISA as described in Materials and Methods. Columns, means of three separate experiments; bars, ± SE.

Radiosensitivity of PC3 cells transfected with COX-2 small interfering RNA. The plating efficiencies of unirradiated cells (mean ± SE, n = 3) were 0.66 ± 0.02 (control), 0.60 ± 0.04 (vehicle), 0.56 ± 0.04 (siNT), 0.58 ± 0.02 (siRF), and 0.53 ± 0.53 (siCOX-2). The difference in the plating efficiency of
siCOX-2 cells compared with vehicle or siNT or siRF cells was not statistically significant. Figure 2 shows the radiation survival curves of untreated control cells and cells transfected with siRNAs for 72 hours. The radiation survival curves of cells transfected with vehicle and siRF were identical. The differences in the radiosensitivity of cells expressing different levels of COX-2 were small. Compared with the vehicle-treated control, at 10% survival level, the radiosensitivity of cells transfected with siCOX-2 was enhanced by a factor of 1.1 and the radiosensitivity of cells transfected with siNT was enhanced by a factor of 1.17. Cells transfected with COX-2 siRNA expressed almost 10-fold less COX-2 protein compared with cells transfected with control NT siRNA. However, the difference in the radiosensitivity of these two transfected cell lines was only 1.06-fold.

In addition to PC3 cells, we wanted to examine the radiosensitivity of other prostate carcinoma cells after silencing the COX-2. However, DU-145 cells did not express COX-2 protein (28) and LNCaP cells were not amenable to in vitro clonogenic survival studies. Therefore, we used HeLa cells that constitutively expressed COX-2 protein as the second tumor line to examine the radiosensitivity after silencing COX-2.

**COX-2 protein and radiosensitivity of HeLa cells transfected with COX-2 small interfering RNA.** Figure 3A shows the Western blot analysis (top) of COX-2 protein in HeLa cells transfected with vehicle, negative controls siNT and siRF, and COX-2 siRNA at 48 hours after transfection. COX-2 protein levels were comparable in cells transfected with vehicle, siNT, and siRF. In HeLa cells transfected with siCOX-2, COX-2 protein was reduced by 80% on day 2 compared with the vehicle (bottom). Unlike PC3 cells, transfection with siNT-negative control did not increase COX-2 protein in HeLa cells.

Figure 3B shows the radiosensitivity of HeLa cells transfected with vehicle, siRF, and siCOX-2. The plating efficiencies of unirradiated transfected cells were (mean ± SE, n = 4) 0.79 ± 0.06 for vehicle, 0.77 ± 0.04 for siRF, and 0.78 ± 0.09 for siCOX-2. The radiation survival curves of HeLa cells transfected with vehicle, siRF, and siCOX-2 were almost identical.

**Effect of NS 398 on COX-2 protein and radiosensitivity in transfected cells.** Figure 4 shows the Western blot analysis of PC3 and HeLa cells treated with 100 μmol/L NS 398 for 24 hours. Treatment with NS 398 increased COX-2 protein in all transfected cells. However, the COX-2 protein in cells transfected with siCOX-2 was much lower compared with the COX-2 in cells transfected with vehicle or control siRNAs.

Table 2 shows the effect of NS 398 on the radiosensitivity of PC3 and HeLa cells expressing different levels of COX-2. NS 398 reduced the plating efficiency of COX-2-depleted siCOX-2 cells as well as COX-2-overexpressing siNT cells. Pretreatment with 100 μmol/L NS 398 enhanced the radiosensitivity of vehicle control, siRF control, and siNT control. However, the plating efficiency of siCOX-2 cells treated with NS 398 plus 4 Gy was comparable with siCOX-2 cells treated with 4 Gy alone. Thus, NS 398 enhanced the radiosensitivity of PC3 cells that expressed COX-2 but had no effect on cells in which COX-2 was significantly reduced. Treatment with NS 398 did not reduce the plating efficiency of HeLa cells. However, pretreatment with 100 μmol/L NS 398 significantly reduced the clonogenic survival of irradiated HeLa cells transfected with vehicle or siRF control as well as COX-2 siRNA. Thus, NS 398 enhanced the radiosensitivity HeLa cells irrespective of their COX-2 status.

**Discussion**

We have had a long-standing interest in the use of nonsteroidal anti-inflammatory drugs to enhance the efficacy of radiation (28–31). We focused primarily on ibuprofen and prostate cancer, because our initial observations indicated radiosensitization of prostate cancer cells with ibuprofen in vitro and in vivo (29, 31). Subsequently, a number of potential mechanisms of action were identified, which required higher concentrations of the drug (28, 29, 32), questioning what mechanisms will be operative in the clinic. These studies also raised a concern about the toxicity associated with...
nonsteroidal anti-inflammatory drugs should such high doses be needed in the clinic. Over the last few years, COX-2 inhibitors have become of interest due to their lower gastrointestinal toxicity profile. However, recent reports (33) on cardiovascular toxicity of COX-2-specific inhibitors may affect their potential use for cancer prevention and treatment.

Thus, in the present study, we investigated the role of COX-2 in radiosensitization. We used RNA interference technique to disrupt the COX-2 gene in PC3 and HeLa cells and investigated the radiation responses of cells expressing different levels of COX-2. Transfection of cells with siRNA targeted for COX-2 resulted in significant inhibition of COX-2 expression and activity, as assessed by PG production. Unexpectedly, transfection with a nontargeting control siRNA induced COX-2 protein and activity in PC3 cells but not in HeLa cells. The difference in COX-2 expression between these two transfectant PC3 cell lines was 10-fold. A comparison of the radiation survival curves of cells expressing different levels of COX-2 showed no significant differences in the radiosensitivity of PC3 or HeLa cells.

Extensive data are available on radiosensitization of tumor cells after pharmacologic intervention of COX-2. However, only a few studies have analyzed the radiosensitivity of cells after genetically altering cellular COX-2 levels (22, 34–37). The data presented here showed that the radiosensitivity of PC3 and HeLa cells was minimally affected by the COX-2 status of the cells. This observation is in agreement with an earlier study comparing the intrinsic radiosensitivity of rat intestinal epithelial cells (RIE) stably transfected with COX-2 in sense (RIE-S) and antisense (RIE-AS) orientation (22). The intrinsic radiation sensitivity of both cell lines, as determined by clonogenic survival, was found to be similar, although COX-2 protein levels were significantly different in the two cell lines (22). In addition, irradiation of COX-2−/− mice had no effect on the intestinal crypt stem cell survival at 3.5 days following 14 Gy total body γ-irradiation compared with wild-type mice (34). These studies suggest that the radiosensitivity does not correlate with cellular COX-2 levels.

Although our data on the clonogenic survival showed that the radiosensitivity of siRNA-transfected cells did not correlate with cellular COX-2 levels, it seems that enhancement of radiosensitivity by NS 398 in PC3 cells was dependent on the cellular COX-2.
of siCOX-2 cells in which COX-2 was obliterated. However, NS 398 enhanced radiosensitivity of COX-2-expressing cells. These data are in agreement with the earlier observations from DuBois’ group (22). In this study, they found that NS 398 enhanced the radiosensitivity of COX-2-overexpressing RIE-S cells and NCI-H460 human lung cancer cells. However, this effect was not observed in COX-2 lacking RIE-AS cells or human colon cancer HCT-116 cells. Similarly, celecoxib-mediated radiosensitization was seen in MCA-35 murine mammary tumor cells that express high COX-2 protein but not in A549 human lung carcinoma cells that expressed low COX-2 protein (23). However, our data on HeLa cells indicated that NS 398 enhanced the radiosensitivity of HeLa cells irrespective of their COX-2 levels. NS 398 increased COX-2 protein in PC3 and HeLa cells. However, the increase in cells transfected with siCOX-2 was small in comparison with the substantial increase observed in cells transfected with control siRNAs, in both PC3 as well as HeLa cells. Therefore, it seems that NS 398 acts via other targets besides COX-2 itself and the effect may be cell type dependent. Further studies are required to identify the molecular targets of COX-2 inhibitors as well as radiation to use these inhibitors effectively as radiosensitizers.

Pharmacologic intervention of COX-2 by COX-2 inhibitors results in an accumulation of cells in G0-G1 phase (37, 38) or radiosensitive G2-M phase (15). However, disruption of COX-2 by transfecting cells with COX-2 siRNA did not result in G1 accumulation (37). In the present study also, the cell cycle distribution of cells transfected with COX-2 siRNA was comparable with cells transfected with vehicle or control siRF. However, the cell cycle distribution of PC3 cells overexpressing COX-2 as a result of transfection with siNT differed significantly from the vehicle-treated cells. siNT cells showed significantly higher percentage of cells in G1 compared with the vehicle-treated cells. This observation is in agreement with an earlier study where an increase in G1 delay associated with reduction in cell cycle-related proteins was seen in COX-2 overexpressing cells that were transfected with sense COX-2 CDNA (39). Thus, the present data show that genetic silencing of COX-2 does not result in significant cell cycle changes, but overexpression of COX-2 is associated with perturbations in cell cycle. Failure of cells to accumulate in radiosensitive cell cycle stage could be one of the reasons for the lack of increase in the radiosensitivity of cells transfected with siCOX-2.

siRNA is a highly specific tool for targeted gene knockdown (40–42). siRNA-mediated gene silencing is of great value in validating gene functions and has potential to silence the tumor-specific target genes. Induction of COX-2 protein and activity in PC3 cells by siNT, a nontargeting control siRNA, in the present study is of interest. Although siRNAs are known to nonspecifically induce a significant number of genes as a cellular response to double-stranded RNA, siNT did not induce the housekeeping genes including actin, topo-I, COX-1, and lamin A/C. It also did not induce HIF-1α, a stress response protein (data not shown). Induction of COX-2 by siNT seems cell type dependent for no increase in COX-2 was seen in HeLa cells transfected with siNT. Currently, we are analyzing the specificity of siRNAs used in this study by gene expression profiling in PC3 and HeLa cells. These studies are expected to identify the COX-2-dependent genes as well as nonspecific targets.

The potential of COX-2 inhibitors for cancer treatment and prevention remains a strategy of interests; however, it is of concern in the light of the recent reports of cardiac complications (33). The present data indicate that the in vitro radiosensitivity of human carcinoma cells is not determined by the COX-2 status of the cells. Because COX-2 inhibitors affect multiple cellular targets, silencing COX-2 by RNA interference as shown in the present study may prove to be a more precise analytic approach to elucidate the role of COX-2 in cancer therapy and in optimizing the use of COX-2 inhibition strategies.

Acknowledgments

We thank Diane Milenic for her help in flow cytometry and Kiran Devisetty and Drs. David Cerna and Phil Tofilon for helpful suggestions. This research was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

Table 2. Clonogenic survival of siRNA-transfected PC3 and HeLa cells following treatment with 100 μmol/L NS 398 and 4 Gy

<table>
<thead>
<tr>
<th></th>
<th>PC3</th>
<th>HeLa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>vehicle</td>
<td>siRF</td>
</tr>
<tr>
<td>DMSO control</td>
<td>1.00 ± 0.06</td>
<td>1.00 ± 0.08</td>
</tr>
<tr>
<td>NS 398</td>
<td>0.99 ± 0.03</td>
<td>1.00 ± 0.16</td>
</tr>
<tr>
<td>DMSO 4 Gy</td>
<td>0.36 ± 0.02</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td>NS 398 + 4 Gy</td>
<td>0.31 ± 0.02†</td>
<td>0.30 ± 0.00‖</td>
</tr>
</tbody>
</table>

NOTE: PC3 and HeLa cells were transfected with siRNAs as described in Materials and Methods. At 48 hours, media were changed and cells were treated with 100 μmol/L NS 398 or DMSO (vehicle control for NS 398) for 24 hours and then irradiated with 4 Gy. Effect of NS 398 on radiosensitivity was calculated by dividing the plating efficiency of cells treated with drug plus radiation by the plating efficiency of cells treated with drug alone. Mean ± SE of three separate experiments.

†P < 0.001 (compared with DMSO control).
‖P < 0.0002 (compared with DMSO control).
*P < 0.01 (compared with DMSO 4 Gy).
*P < 0.001 (compared with DMSO 4 Gy).
*P < 0.02 (compared with DMSO 4 Gy).
*P < 0.002 (compared with DMSO 4 Gy).
References

Radiation Sensitivity of Human Carcinoma Cells Transfected with Small Interfering RNA Targeted against Cyclooxygenase-2


Updated version  Access the most recent version of this article at:  http://clincancerres.aacrjournals.org/content/11/19/6980

Cited articles  This article cites 41 articles, 17 of which you can access for free at:  http://clincancerres.aacrjournals.org/content/11/19/6980.full.html#ref-list-1

Citing articles  This article has been cited by 7 HighWire-hosted articles. Access the articles at:  /content/11/19/6980.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.