The influence of cyclooxygenase-1 expression on the efficacy of cyclooxygenase-2 inhibition in head and neck squamous cell carcinoma cell lines

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We have previously observed that cyclooxygenase-2 (COX-2) inhibition blocked the production of vascular endothelial growth factor (VEGF) in some head and neck squamous cell carcinoma (HNSCC) cells. However, as some HNSCC cells showed little response to COX-2 inhibition, although they highly expressed COX-2 and prostaglandin E_2, we set out to elucidate what made this difference between them and focused on the possibility of the differential expression of COX-1. In western blotting, we found that COX-1 was expressed in SNU-1041 and SNU-1066, but not in SNU-1076 and PCI-50. Only in those cell lines without expression of COX-1 was VEGF production blocked meaningfully by small interfering RNA of COX-2. However, by cotreating with small interfering RNAs of COX-2 and COX-1, VEGF synthesis and prostaglandin E_2 were inhibited in SNU-1041 and SNU-1066, similarly in SNU-1076 and PCI-50 with high expression of only COX-2. We also found that there was no difference in the pattern of prostaglandin synthesis between COX-2 and COX-1 through enzyme-linked immunosorbent assay for various prostaglandins. Our study suggests that, as COX-1 and COX-2 express and affect VEGF synthesis in HNSCC cells, we should check COX-1 expression in investigations on cancer treatment by inhibiting COX-2-induced prostaglandins. *Anti-Cancer Drugs* **22:416–423 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

The specific physiological roles of cyclooxygenase-2 (COX-2) in several types of cancers remain unclear, and the carcinogenic action of COX-2 seems to be different even among cell lines derived from the same type of cancer. However, many reports have suggested that COX-2 plays an important role in carcinogenesis, and its inhibition may have beneficial effects in cancer management [1–3]. In the study herein, head and neck squamous cell carcinoma (HNSCC) cells were used and a possible role of COX-2 and prostaglandin E_2 (PGE_2) was observed in relation to the promotion of angiogenesis through increasing synthesis of vascular endothelial growth factor (VEGF, a key proangiogenic factor).

In addition to COX-2, COX-1, an isotype of COX, synthesizes prostaglandins from arachidonic acid. COX-1, the expression of which is not restricted to cancer cells, is involved in maintaining biological functions in many different tissues [4]. As the nonselective inhibition of COX-1 causes side effects, including gastrointestinal problems [5,6], its expression has not been thoroughly considered or studied in cancer biology. However, a number of recently published reports have challenged the notion that upregulated COX-1 positively affects cell viability and that its inhibition lowers cell proliferation in some cancers, including ovarian and colon cancers [7–10].

Upregulation of COX-1 has also been reported to play a role in the carcinogenesis of the head and neck [11]. Herein, we investigated the possible involvement of COX-1-mediated prostaglandins in the VEGF production of HNSCC cells. We observed that COX-2-specific inhibition, by using its small interfering RNA (siRNA), lowered VEGF synthesis only in some of the tested HNSCC cells that had high expression of COX-2 and PGE_2. This result can be placed in context by considering the possibility that the physiological role of COX-2 varies in each cancer cell as a result of the differences in signal transduction unique to each cell type. We assumed that the differential expression of COX-1 affects (positively or negatively) the efficiency of VEGF inhibition by blocking COX-2 activity in the tested cells. We conducted this study to determine whether the degree of COX-1 expression, relative to the expression of COX-2 in targeted cancer cells, should be analyzed in advance to establish the best therapeutic efficacy of COX-2 inhibition in the clinical field of cancer treatment.

All supplementary digital content is available directly from the corresponding author.

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Methods

Cell culture

SNU-1041, SNU-1066, and SNU-1076 (HNSCC cell lines) were obtained from the Korean Cell Line Bank (Seoul National University, Seoul, Korea), whereas PCI-1, PCI-13, and PCI-50 (HNSCC cell lines) were obtained from the Pittsburgh Cancer Institute (University of Pittsburgh, Pennsylvania, USA) [12]. The cells were maintained at 37°C in a humidified, 5% CO_2, 95% air atmosphere, and were routinely subcultured using trypsin-EDTA (0.25% w/v). Unless otherwise stated, all cell culture reagents were obtained from Gibco BRL (Grand Island, New York, USA).

Quantification of several prostaglandins and vascular endothelial growth factor production

The amounts of prostaglandins (TXB_2, $PGF_{2\alpha}$, $PGF_{1\alpha}$, PGD_2, and PGE_2) and VEGFs released by the cells were determined using prostaglandin (Cayman Chemical, Ann Arbor, Michigan, USA) and VEGF (R&D Systems, Minneapolis, Minnesota, USA) enzyme immunoassay (EIA) kits according to the manufacturer's instructions.

Transfection of cyclooxygenase-2-expressing and cyclooxygenase-1-expressing plasmids

The COX-2 complementary DNA (cDNA) was a gift from Dr William L. Smith (Department of Biological Chemistry, University of Michigan) and the COX-1 cDNA from Dr Kenneth K. Wu (Division of Hematology, University of Texas-Houston Medical School). Using these cDNAs, we established pcDNA3.1-expressing vectors (pcDNA3.1-COX-2 and COX-1). Expression and activity of plasmids were confirmed by western blotting and EIA, respectively. The cells were transfected with 0.5–1 μg of plasmid using Lipofectamine-plus according to the manufacturer's instructions (Life Technologies). After 4 h, a medium containing 10% fetal bovine serum was added and the cells were incubated for an additional 44 h. In the case of cotransfection with siRNA, cells were cotransfected with 0.5–1 μg of plasmid and 100–200 nmol/l siRNAs against COX-2 or COX-1 using Lipofectamine-plus. After 4 h, a medium containing 10% fetal bovine serum was added and the cells were incubated for an additional 44 h.

Transfection of small interfering RNA

Individual siRNAs against COX-2 (D-004557-04), COX-1 (D-004556-03), and nontargeting controls (D-001210-01) were obtained from Dharmacon RNA Technologies (Lafayette, Colorado, USA). The best conditions for siRNA's application (doses used and treatment time) were established beforehand by western blotting and EIA. The cells were plated in 6-well, 12-well, or 24-well plates and grown to 50–70% confluence. After 24 h, the cells were transfected with siRNA (100–200 nmol/l) using Lipofectamine-2000 reagent (Invitrogen, Carlsbad, California, USA) for 48–72 h according to the manufacturer's instructions.

Western blot analysis

Denatured protein lysates were resolved by 4–12% NuPAGE gels (Invitrogen, Carlsbad, California, USA) and transferred to nitrocellulose membranes (Schleicher & Schuell, Dachen, Germany). The membranes were incubated with anti-COX-2 or anti-COX-1 (Santa Cruz Biotechnology, Santa Cruz, California, USA) or monoclonal anti-α-tubulin (Sigma Chemical Co., St Louis, Missouri, USA) for 2 h at room temperature or overnight at 4°C. The membranes were then washed (four times) with Tris-buffered saline with Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, Illinois, USA) for 1 h. Immunoreactive proteins were visualized by developing with a Lumi-light western blotting substrate (Roche Diagnostics GmbH, Mannheim, Germany), followed by exposure in a LAS-3000 (Fuji Film Co., Tokyo, Japan) according to the manufacturer's instructions.

Statistical analysis

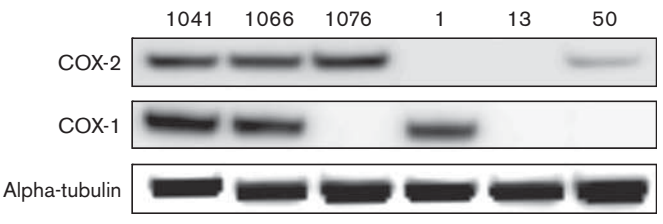
Data are presented as the mean ± standard deviation of triplicate or as a representative of three separate experiments. Statistical analysis was carried out using one-way analysis of variance (ANOVA) analysis. In the case of a significant result in the ANOVA, student's *t*-test was used for dose–response results and Dunnett's post-test for all other experiments. A P value of less than 0.05 was considered to be statistically significant.

Results

Differential expression of cyclooxygenase-2 and cyclooxygenase-1 in several head and neck squamous cell carcinoma cell lines

We investigated the expression patterns of COX-2 and COX-1 in HNSCC cells. COX-2 is expressed in most of them, except for PCI-1 and PCI-13. The expression of COX-1 is observed in SNU-1041, SNU-1066, and PCI-1 (Fig. 1). In particular, SNU-1041 and SNU-1066 express both COX-2 and COX-1, whereas SNU-1076 and PCI-50 only express COX-2.

Fig. 1

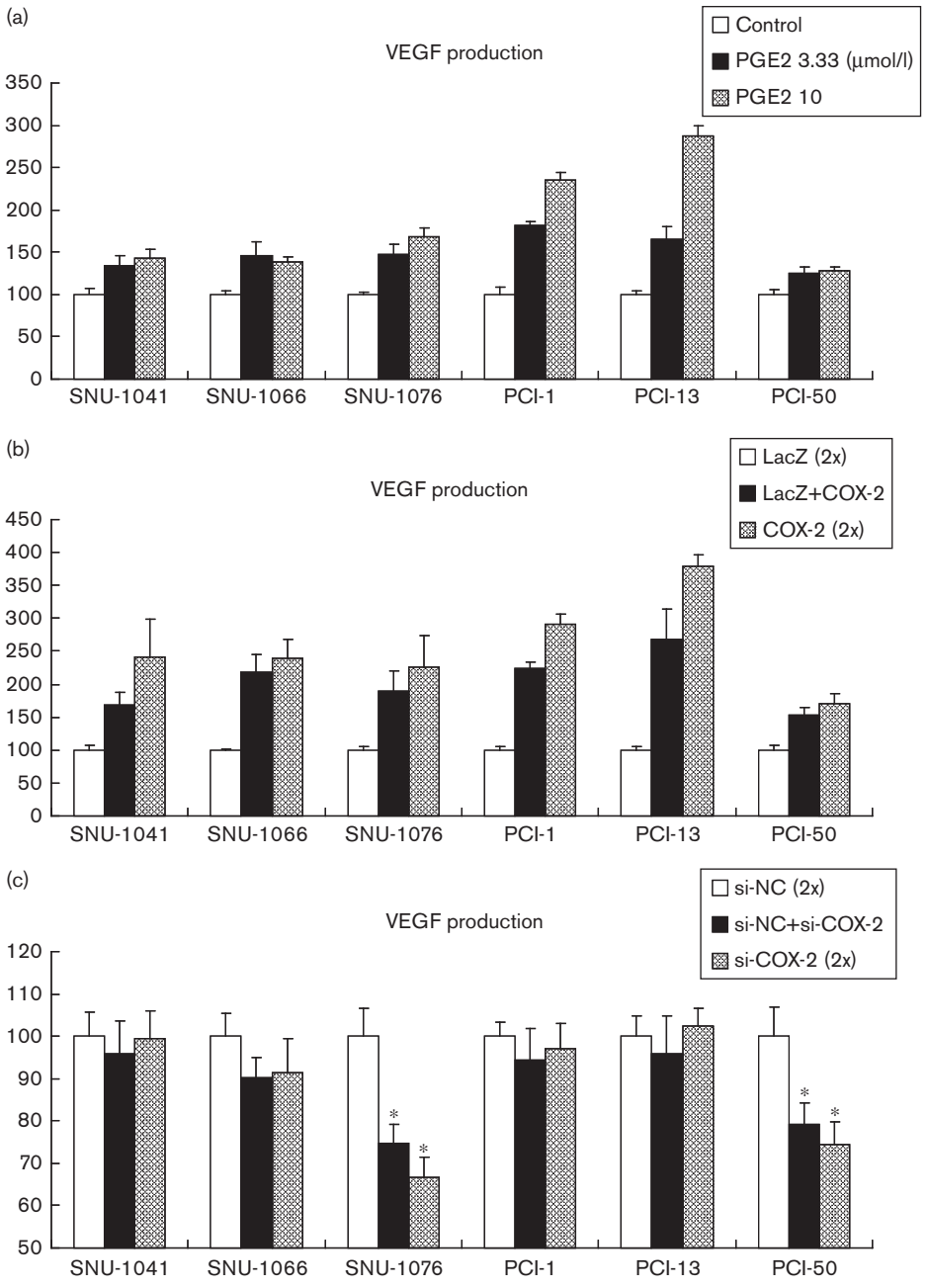


The differential expression of cyclooxygenase-2 (COX-2) and COX-1 in several head and neck squamous cell carcinoma (HNSCC) cell lines. The expression of COX-1 and COX-2 was determined for the indicated HNSCC cell lines by western blotting. Alpha-tubulin was used for loading control in western blotting.

Cyclooxygenase-2 and prostaglandin E_2 positively affect vascular endothelial growth factor production in head and neck squamous cell carcinoma cell lines

To determine whether COX-2-mediated prostaglandins affect VEGF production, we treated several HNSCC cell lines with exogenous PGE_2 (a representative of various prostaglandins). PGE_2 promoted VEGF expression by 30–300% in all tested cells, but seemed to be most effective in PCI-1 and PCI-13, with no detectable COX-2 (Fig. 2a). VEGF expression was upregulated 2-fold to 4-fold by transfecting plasmids expressing COX-2 in all HNSCC cells (Fig. 2b). The increase of PGE_2 production by transfection with plasmids expressing COX-2 in HNSCC cells was confirmed (Supplementary-1). However, in

Fig. 2



Cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2) affect VEGF production positively in head and neck squamous cell carcinoma (HNSCC) cell lines. The cells were treated with (a) exogenous PGE_2 of the indicated concentrations (μmol/l) and transfected with (b) plasmids expressing COX-2 (0.5–1.0 μg) or by (c) indicated small interfering RNA (siRNA) (100–200 nmol/l). At 48 h, the culture medium from the indicated HNSCC cell lines was subjected to vascular endothelial growth factor (VEGF) enzyme immunoassay. The plasmid-expressing LacZ gene and si-NC were used as negative controls. Results are expressed as a percentage relative to control (% of control) (*$P<0.01$).

experiments that included treatment of cells with the siRNA of COX-2, VEGF was inhibited only in SNU-1076 and PCI-50, among cells with high COX-2 expression (SNU-1041, SNU-1066, SNU-1076, and PCI-50) (Fig. 2c).

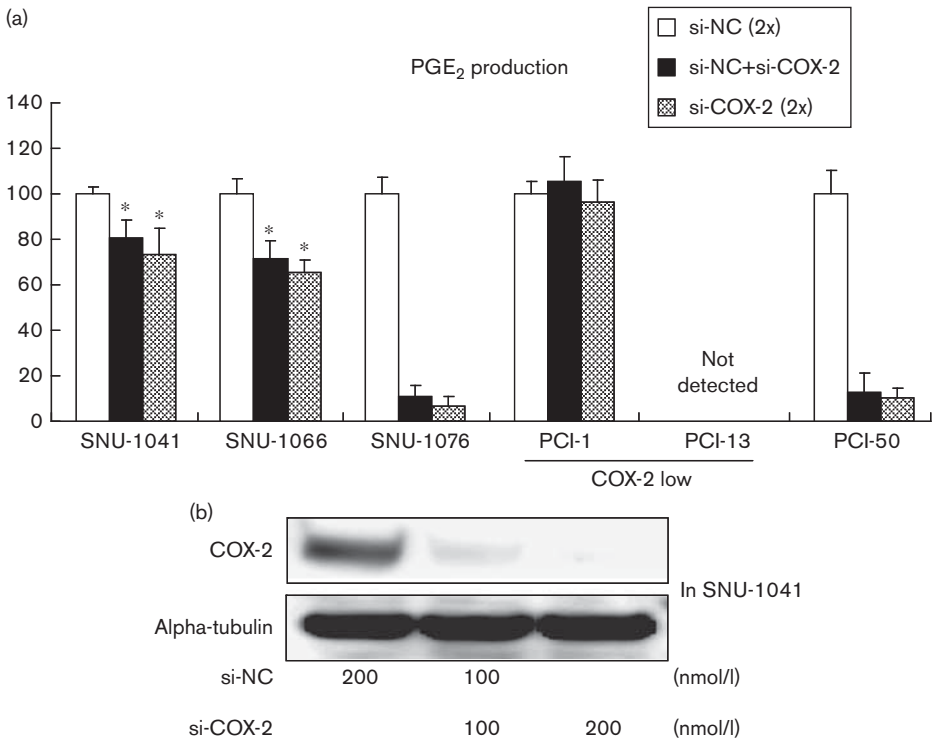
Cyclooxygenase-2 small interfering RNA inhibits prostaglandin E_2 production effectively in SNU-1076 and PCI-50 (with only cyclooxygenase-2 expression), but not in SNU-1041 and SNU-1066 (with expression of cyclooxygenase-2 and cyclooxygenase-1)

Considering the results shown in Fig. 2c, to confirm the inhibitory effect of COX-2 siRNA, we explored whether PGE_2 was blocked effectively by COX-2 siRNA. PGE_2 was not inhibited effectively by treating SNU-1041 and SNU-1066 with the siRNA of COX-2, whereas it was blocked perfectly in SNU-1076 and PCI-50 (Fig. 3a). A high level of PGE_2 was detected in PCI-1, although PCI-1 had no detectable COX-2 expression, and PGE_2 was not detected in PCI-13 even at the basal condition. Next, we examined whether siRNA against COX-2 blocked COX-2 expression effectively in SNU-1041 and SNU-1066, because it showed weak inhibition of PGE_2 in these cells. COX-2 protein was completely blocked in SNU-1041 (Fig. 3b) and SNU-1066 (data not shown). At that point, we realized that COX-1 was expressed to a greater degree than we had expected in these cells and these findings explain the different inhibitory effects of COX-2 siRNA in HNSCC cells.

Prostaglandin E_2 production is blocked effectively by coinhibition of cyclooxygenase-2 and cyclooxygenase-1 in SNU-1041 and SNU-1066 with overexpression of both cyclooxygenase-2 and cyclooxygenase-1

Considering the coexpression of COX-1, as seen in Fig. 1, we assumed that COX-2-specific inhibition by siRNA would block prostaglandins partially in cell lines such as SNU-1041 and SNU-1066 because their COX-1 activity still persists and produces prostaglandins. Indeed, treatment of siRNA against COX-2 blocked PGE_2 only by 20–30%, whereas that against COX-1 inhibited PGE_2 by over 40% in SNU-1041 (Fig. 4a, left). Perfect inhibition of PGE_2 was not seen in SNU-1041 until cells were cotreated with the siRNAs of COX-2 and COX-1. Similar findings were observed in SNU-1066 (Fig. 4a, right). The effective inhibition of COX-2 and COX-1 proteins by treating singly or combined with their respective siRNAs was checked in SNU-1066 (Supplementary-2).

Fig. 3



Cyclooxygenase-2 (COX) small interfering RNA inhibits prostaglandin E_2 production effectively in SNU-1076 and PCI-50 (with only COX-2) but not in SNU-1041 and SNU-1066 (with expression of COX-2 and COX-1). The cells were transfected with the indicated siRNA (100–200 nmol/l). At 48 h, the culture medium from the indicated head and neck squamous cell carcinoma cell lines was subjected to PGE_2 enzyme immunoassay (EIA) (a), and cell lysates of SNU-1041 to western blotting (b). The si-NC was used as a negative control. Results are expressed as a percentage relative to control (% of control) for the results of PGE_2 EIA (*$P<0.01$).

Vascular endothelial growth factor production is blocked by the coinhibition of cyclooxygenase-2 and cyclooxygenase-1 in SNU-1041 and SNU-1066, with high expression of both cyclooxygenase-2 and cyclooxygenase-1

As VEGF synthesis seemed to be inhibited proportionally to the decreased degree of PGE_2 by COX-2 inhibition, as seen in Figs 2c and 3a, we expected that significant inhibition of VEGF production would be achieved by cotreatment of respective siRNAs against COX-2 and COX-1 in both SNU-1041 and SNU-1066. We found that VEGF synthesis was blocked by 30–40% by cotreatment of both siRNAs in both cell lines (Fig. 4b). In SNU-1041, the single inhibition of COX-1 showed a slightly better inhibition of both PGE_2 (Fig. 4a, left) and VEGF (Fig. 4b, left) production, suggesting a greater activity of COX-1 than that of COX-2.

There is no difference in the pattern of prostaglandin synthesis between cyclooxygenase-2 and cyclooxygenase-1 in SNU-1041 and SNU-1066

Some groups have suggested the possibility of distinct functional coupling between upstream COX isozymes (COX-1 and COX-2) and terminal prostanoid synthases, followed by partly segregated use of the two COX isozymes in distinct phases of prostaglandin biosynthetic responses because of their different subcellular localization [13,14]. However, as far as we know, there have been few reports on different patterns of prostaglandin synthesis between COX-2 and COX-1 in cancer cells. We investigated this possibility in SNU-1041 and SNU-1066 with a high expression of both COX-2 and COX-1. In SNU-1041, whose COX-1 seems to contribute more to prostaglandin synthesis than COX-2, there was no difference in the kinds of prostaglandins produced by the two types of COXs, as expected, and COX-1 was likely to produce more prostaglandins than COX-2 (Fig. 5a). In SNU-1066, whose COX-2 seemed to contribute equally to prostaglandin synthesis with COX-1, COX-1 was likely to produce the same kinds of prostaglandins in similar quantities as COX-2 (Fig. 5b). We did not detect PGD_2 in SNU-1041 and PGD_2 or TXB_2 in SNU-1066, suggesting the possible different patterns of prostaglandin synthesis in each type of cancer cell.

Cyclooxygenase-2 and cyclooxygenase-1 cosuppression is needed to inhibit vascular endothelial growth factor production in SNU-1076 transfected with exogenous cyclooxygenase-1

To reconfirm our hypothesis in cells transfected with COX-2 and/or COX-1 exogenously, we made SNU-1076 cells express COX-1 and COX-2 through transfection of pcDNA3.1-COX-1. At the same time, by applying siRNAs of COX-2 and/or COX-1, we analyzed the influence of the degree of COX-1 expression relative to COX-2 on the VEGF-blocking efficiency of COX-2 inhibition. When SNU-1076 cells had only endogenous expression of COX-2, single inhibition of COX-2 showed effective inhibition of PGE_2 (Fig. 6a) and caused meaningful decrease of VEGF (Fig. 6b). However, when the cells were transfected with exogenous COX-1, single inhibition of COX-2 failed to block PGE_2 and VEGF effectively. Instead, coinhibition of COX-2 and COX-1 was needed to decrease PGE_2 and VEGF production (Fig. 6a and b), suggesting that we should choose cancer cells with much higher expression of COX-2 than COX-1 as targets for COX-2 inhibition therapy. The increased expression of COX-1 by transfection and the effective inhibition of COX-2 and COX-1 protein by treating with their respective siRNAs, either singly or combined, were examined in SNU-1076 through western blotting (Supplementary-3).

The inhibitory effect of cyclooxygenase-2 and cyclooxygenase-1 (co)inhibition on vascular endothelial growth factor production in PCI-13 cotransfected with exogenous cyclooxygenase-2 and cyclooxygenase-1

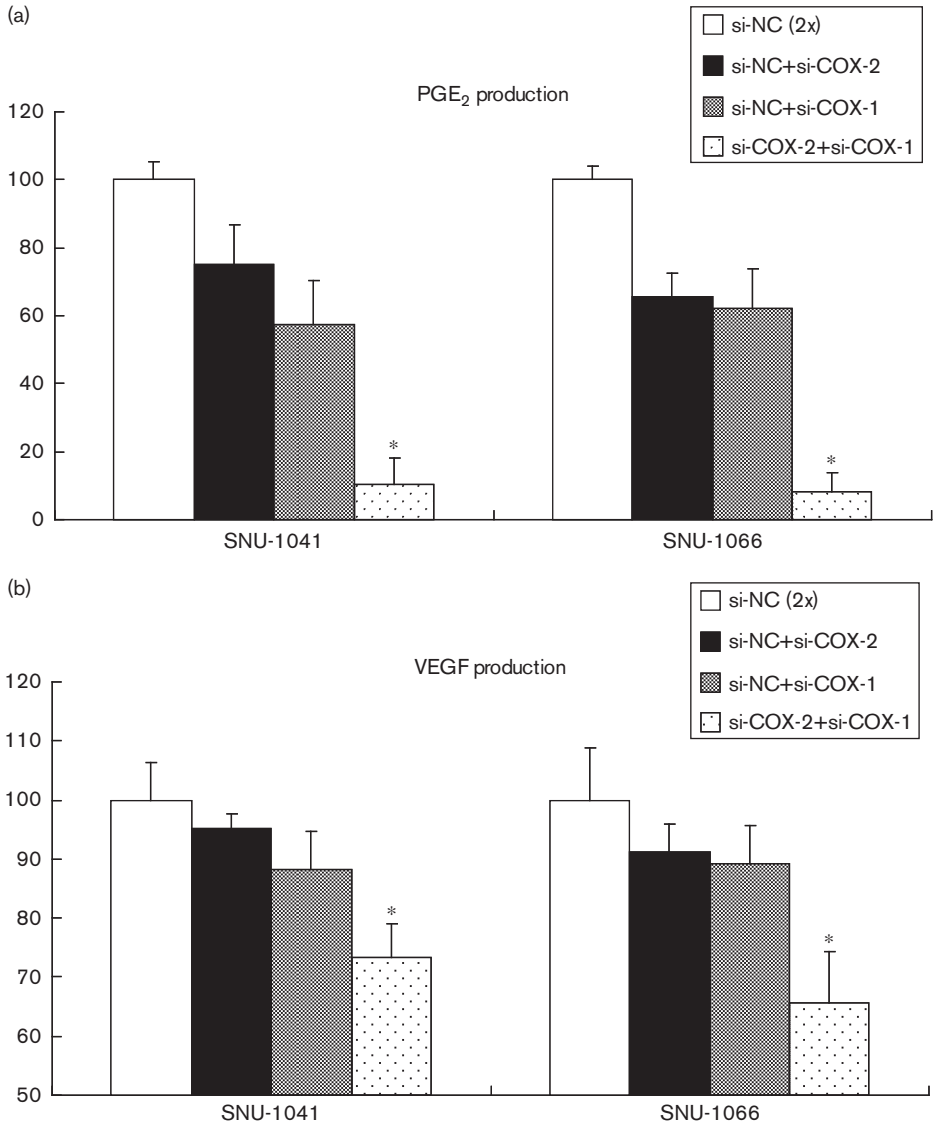
Finally, we made PCI-13 cells express only COX-2 or COX-2 plus COX-1 through transfection of pcDNA3.1-COX-2 and COX-1. At the same time, by applying siRNAs of COX-2 and/or COX-1, we analyzed the impact of the expression level of COX-1 relative to COX-2 on the VEGF-blocking efficiency of COX-2 inhibition (Fig. 7). When PCI-13 cells had only exogenous expression of COX-2, the single inhibition of COX-2 inhibited VEGF synthesis. However, when the cells were transfected with exogenous COX-2 plus COX-1, single inhibition of COX-2 failed to effectively decrease VEGF. Rather, coinhibition of COX-2 and COX-1 decreased VEGF production, suggesting that COX-1 and COX-2 both have a role in the regulation of VEGF production in HNSCC cells.

Discussion

As many studies on the roles of COX-2 in cancers have elucidated the promising effect of COX-2 inhibition as a new modality for cancer prevention and treatment, COX-2 inhibitory chemicals have been modified to remove COX-2 inhibitors' inhibitory action on COX-1, and to reduce the side effects of nonspecific COX inhibition. Application of specific blocking methods, such as siRNA, against COX-2 has also been broadly used to determine its exact action in carcinogenesis [15,16]. These efforts have moved the concerns related to the possible carcinogenic roles of prostaglandins derived from COX-1 gradually from the side to the center of this story. Recent findings on the relationship of COX-1 in the development of several cancers have suggested that the carcinogenic actions of COX-2 should be distinguished from those of COX-1 in the application of prostaglandin-blocking therapy for cancer treatment. Furthermore, production of prostaglandins in promoting the viability of cancer cells has been observed to result entirely from COX-1, not COX-2, in some cancer models [17,18].

The expression of COX-1 and COX-2 varied in the HNSCC cell lines used in this study. SNU-1076 and PCI-50 showed high expression of only COX-2, SNU-1041

Fig. 4

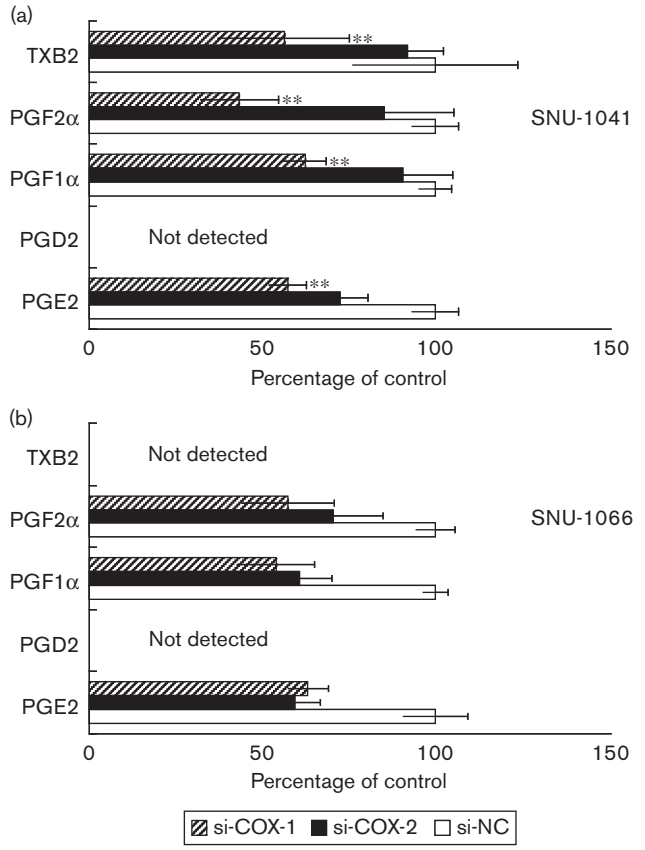


Productions of PGE_2 and vascular endothelial growth factor (VEGF) are blocked effectively by the coinhibition of cyclooxygenase-2 (COX-2) and COX-1 in SNU-1041 and SNU-1066 with overexpression of both COX-2 and COX-1. The small interfering RNAs of COX-2 and COX-1 were transfected at 100 nmol/l doses in SNU-1041 and SNU-1066. The si-NC was used for siRNA control (100–200 nmol/l). Each sample was subjected to PGE_2 enzyme immunoassay (EIA) (a) and vascular endothelial growth factor (VEGF) EIA (b) for 48 h. Results are expressed as a percentage relative to control (% of control) (one-way analysis of variance, $*P<0.01$).

and SNU-1066 showed activities of both COX-2 and COX-1, and PCI-1 showed high expression of only COX-1. In the experiment using SNU-1041 and SNU-1066 with expression of both COX-2 and COX-1, the treatment with siRNA of COX-2 completely inhibited the expression of COX-2 protein. However, siRNA against COX-2 inhibited PGE_2 synthesis by 20% at most, whereas siRNA of COX-1 blocked PGE_2 synthesis by 40–50%, showing a greater contribution of COX-1 than COX-2 to intracellular synthesis of prostaglandins in the cancer cell lines we tested. We could block PGE_2 synthesis entirely only by cotreating with the siRNAs of COX-2 and COX-1 in these cells. These findings indicate that the possible upregulation and carcinogenic role of COX-1 in HNSCC is similar to that of COX-2.

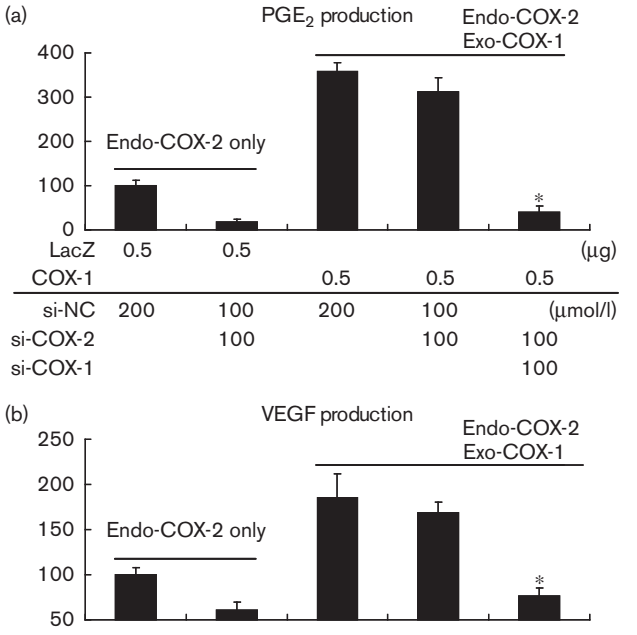
We found that targeted inhibition of COX-2 decreased VEGF production in SNU-1076 and PCI-50 meaningfully, showing high COX-2 and no detectable COX-1 activity, but coinhibition of COX-2 and COX-1 was needed to block VEGF synthesis in SNU-1041 and SNU-1066, which possess both the activities. These observations suggest that COX-1 plays a role in the regulation of angiogenesis by promoting VEGF synthesis in HNSCC,

Fig. 5



Analysis of differences in the pattern of prostaglandin synthesis between cyclooxygenase-2 (COX-2) and COX-1 in SNU-1041 and SNU-1066. The cells were transfected with the indicated small interfering RNA at 100 nmol/l doses in SNU-1041 (a) and SNU-1066 (b). At 48 h, the culture media was subjected to prostaglandin (TXB_2, $PGF_{2\alpha}$, $PGF_{1\alpha}$, PGD_2, and PGE_2) enzyme immunoassay. The si-NC was used as a negative control. Results are expressed as a percentage relative to control (% of control) (one-way analysis of variance, $^*P<0.01$).

Fig. 6



The inhibitory effect of cyclooxygenase-2 (COX-2) and COX-1 (co)inhibition on vascular endothelial growth factor (VEGF) production in SNU-1076 transfected with exogenous COX-1. The combined plasmids (0.5 μg) and siRNA (100–200 nmol/l) were transfected into SNU-1076. The plasmid-expressing LacZ gene and si-NC were used as negative controls. Forty-eight hours after transfection, the culture medium was subjected to PGE_2 enzyme immunoassay (EIA) (a) and VEGF EIA (b). Results are expressed as a percentage relative to control (% of control) (one-way analysis of variance, $^*P<0.01$) (Endo-COX-2; endogenous COX-2, Exo-COX-1; exogenously transfected COX-1).

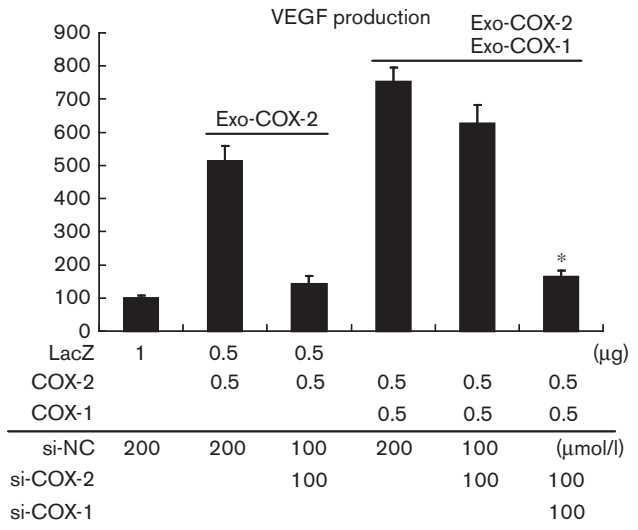
and that COX-2-specific inhibition is likely to be a promising molecular-targeted therapy for cancer treatment only in cancer cells with much lower contribution of COX-1 than COX-2 to synthesis of intracellular prostaglandins. In addition, it is possible that the shunting of arachidonic acid from COX-2 to COX-1 by COX-2-specific inhibition negatively affects the anticancer actions of COX-2 inhibition, even in cancer cells with lower expression of COX-1 than COX-2.

We also considered the possibility that the kinds of prostaglandins produced by COX-1 might be different from those produced by COX-2, even inside the same cells. Earlier observations have shown that COX-1 has different intracellular localizations from COX-2, followed by coupling with unique prostaglandin terminal synthases [13,14]. If this situation occurs in cancer cells, there would be a difference in the pattern of prostaglandin synthesis incurred by COX-2 and COX-1. In this study, which used SNU-1041 and SNU-1066, both COX-2 and COX-1 seemed to participate in the production of the same kinds of prostaglandins in each cell line, although different prostaglandins are produced in the two cell lines. Nevertheless, we assumed that the carcinogenic roles between the two COXs are different in some cancer cells, because of the variation in the kinds of prostaglandins produced by each COX. If this assumption is correct, application of COX-2 inhibition for cancer treatment must be considered after precise investigations on the relative activity of COX-1 and COX-2.

As COX-1 inhibition may cause some side effects in normal tissues, clinical trials with coinhibition of COX-2 and COX-1 may not be practical. However, the temporary inhibition of COX-1 and COX-2 may be worthy of attention in curing cancer by establishing the perfect blockage of prostaglandin synthesis in cancer cells with a significant amount of prostaglandins derived from COX-1 and COX-2.

The best way to apply COX-2 inhibition to cancers is to select cancer targets in which COX-2 clearly has key carcinogenic actions without COX-1 activity. Ultimately, our findings suggest that the expression and role of COX-1

Fig. 7



The inhibitory effect of cyclooxygenase-2 (COX-2) and COX-1 (co)inhibition on vascular endothelial growth factor (VEGF) production in PCI-13 cotransfected with exogenous COX-2 and COX-1. The combined plasmids (0.5 μg) and small interfering RNA (100–200 nmol/l) were transfected into PCI-13. The plasmid-expressing LacZ gene and si-NC were used as negative controls. Forty-eight hours after transfection, the culture media was subjected to VEGF enzyme immunoassay. Results are expressed as a percentage relative to control (% of control) (one-way analysis of variance, $*P<0.01$) (Exo-COX-1; exogenously transfected COX-1; Exo-COX-2; exogenously transfected COX-2).

in targeted cancer, as well as the expression and role of COX-2, must be considered to optimize the anticancer effect of COX-2 inhibition, although COX-1 itself seems to have less value as a molecular target for cancer treatment.

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