SPANDIDOS PUBLICATIONS

Promotion of hematogenous metastatic potentials in human KB carcinoma cells with overexpression of cyclooxygenase-2

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Abstract. To understand the role of cyclooxygenase (COX)-2 in metastatic potential of oral cancer, COX-2 overexpressing KB/COX-2 cells were inoculated orthotopically into the masseter muscle or injected into the left cardiac ventricle of nude mice. KB/COX-2 showed about 4-fold increase of COX-2 protein expression as compared to KB/Neo which was a mock transfected control. In orthotopic inoculation, metastasis to the regional lymph nodes occurred in 2 out of 15 mice, and metastasis to the lung in 3 out of 15 mice. On the other hand, in intra-cardiac injection, hematogenous metastasis to the lung and bone occurred in 8 out of 10 mice in KB/COX-2, but no metastasis occurred except for only one metastasis to the femur bone out of 10 mice in KB/Neo. Treatment of KB/COX-2 with COX-2 small interfering RNA (siRNA) inhibited the colony formation but not cell growth *in vitro*, and suppressed tumorigenicity and hematogenous metastasis in nude mice. When expression of adhesion molecules such as E-cadherin, α-catenin, ß-catenin and CD44 was examined, there was no difference in α- and ß-catenin between the cells. However, expression of E-cadherin was detected in KB/Neo, but not in KB/COX-2. In contrast, expression of CD44 was markedly increased in KB/COX-2 as compared to KB/Neo. Treatment with COX-2 siRNA resulted in suppression of CD44 expression and detectable expression of E-cadherin in KB/COX-2. These findings suggested that overexpression of COX-2 increased hematogenous metastasis, at least in KB cells, via down-regulating E-cadherin and up-regulating CD44 expression.

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Abbreviations: COX-2, cyclooxygenase-2; siRNA, small interfering RNA; PG, prostaglandin; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases

Key words: CD44, COX-2, metastasis, oral cancer, siRNA

Introduction

Cyclooxygenase (COX)-2 is an enzyme induced by mitogens, cytokines and growth factors of epithelial cells and plays an important role in prostaglandin (PG) production. Overexpression of COX-2 has been reported in a variety of cancers (1-4) including head and neck cancer (5-8). Recently, a human colon cancer cell line transfected with a COX-2 expression vector acquired increased invasiveness and metastatic potential with activation of matrix metalloproteinase (MMP) (9). A correlation between COX-2 overexpression and hematogenous metastasis of colorectal cancer was also demonstrated (10). These findings suggest that COX-2 overexpression is involved in colon cancer metastasis. In head and neck cancer, however, the significance of COX-2 overexpression in metastasis is poorly understood.

We have already shown that KB/COX-2, which was established by stable transfection with full length COX-2 cDNA to human KB carcinoma cells, showed elevated PGE_2 production, cell migration and invasion as compared to mock transfected control, KB/Neo. Furthermore, it was found that KB/COX-2 increased tumorigenicity, tumor growth and local tumor invasion in nude mice. These effects were apparently modulated by up-regulation of MMPs and Rho family small guanosine triphosphatases (GTPases) and down-regulation of tissue inhibitor of metalloproteinase (TIMP) activities (11).

The aim of this study was to investigate the metastatic potential in nude mice using KB/COX-2. In addition, to assess the molecular and cellular effects on COX-2 gene silencing, we knocked down COX-2 using small interfering RNA (siRNA) in KB/COX-2 cells, and examined tumorigenicity, tumor growth, invasion and metastasis. KB/COX-2 cells were inoculated in the masseter muscle as an orthotopic spontaneous metastasis model, and intra-cardiac injection was performed as a hematogenous metastasis model in nude mice.

Materials and methods

Cell lines. Two cell lines, KB/COX-2 and KB/Neo, were used in this study as described previously (11). We isolated a clone which highly expressed COX-2 and designated as KB/COX-2, and a mock-transfected clone, KB/Neo as a

control. These cell lines were routinely subcultured in Dulbecco's modified Eagle's MEM (DMEM, Nissui Pharmaceutical Co., Tokyo, Japan).

Transfection of siRNAs. KB/COX-2 was plated in DMEM supplemented with 10% FBS for 24 h, then transfected with 5 nM of siRNA using Lipofectamine™ 2000 (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's protocol. SMART pool siRNA targeting COX-2 (M-004557-00) and control siRNA, Lamin A/C (D-001050-01-20), were purchased from Dharmacon Inc. (Lafayette, CO, USA). We used the cells cultured for 24 h after transfection of siRNAs.

Animals and animal care. Female BALB/c nu/nu mice (6 weeks old) were purchased from Oriental Yeast Ltd. (Osaka, Japan). The mice were maintained under pathogen-free conditions and were handled in accordance with the Guidelines for Animal Experiments of Hyogo College of Medicine.

Tumorigenicity and metastasis assays. The cells were trypsinized, washed once with DMEM and resuspended in 0.1 ml of Mg^{2+}- and Ca^{2+}-free phosphate-buffered saline [PBS(-)] for transplantation in nude mice. For subcutaneous or orthotopic inoculation, cells were inoculated into the flank or the masseter muscle of mice. For intra-cardiac injection, cells were slowly injected into the left cardiac ventricle of mice. Then, mice were examined for the development of tumors and measured for body weight once a week. The tumor size was measured by using calipers. Tumor volume was calculated by the following formula: volume (mm^3) = $a^2xb/2$, where a is the width in mm and b is the length in mm (12). Mice were sacrificed and dissected 7 weeks after cell inoculation. Tumors formed at inoculated site, regional lymph nodes, lungs and other organs were subjected for metastatic analysis.

Western blot analysis. Cell lysates were submitted to Western blot analysis as described previously (13). The primary antibodies used were goat polyclonal antibody against COX-2 and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal antibody against E-cadherin (Chemicon International Inc., Temecula, CA, USA) and CD44 (Thermo Scientific, Fremont, CA, USA) and rabbit polyclonal antibody against α-catenin and ß-catenin (Santa Cruz Biotechnology). The secondary antibodies used were anti-goat, anti-mouse or anti-rabbit IgGs conjugated with alkaline phosphatase (Santa Cruz Biotechnology). Actin was used as an internal control.

RNA preparation and real-time PCR. Tumor which became about 5 mm in diameter was snap-frozen. Total RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) and cDNAs were generated by using a First-Strand cDNA Synthesis kit (Amersham Biosciences, Piscataway, NJ, USA) with 2 μg of total RNA and oligo(dT) (Amersham Biosciences). All reagents required for real-time PCR were from Applied Biosystems (Foster City, CA, USA). Oligonucleotide primer pairs and fluorescent probes for COX-2 and GAPDH were designed using a primer design program (Primer Express, Applied Biosystems) and were obtained from Integrated DNA Technologies (Coralville, IA, USA). The real-time PCR was carried out using the TaqMan Gene Expression Assays products on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) according to the manufacturer's protocol as described previously (14).

Histological studies. Tissues specimens were fixed in 10% formalin, embedded in paraffin and cut into 4-μm-thick sections according to conventional procedures. Histological examination was performed by H&E stain and evaluated by light microscopy for the number of regional lymph nodes or distant metastases in the largest sagittal cross section.

Clonogenic assay. Clonogenic assay was performed by plating an appropriate number of cells (10^2-10^3 cells/ml) into 100-mm culture dishes. After 14 days, cells were fixed and stained with 1% crystal violet, and colonies containing >50 cells were counted.

PGE_2 immunoassay and motility assay. PGE_2 immunoassay was performed as indicated in a protocol of Prostaglandin E2 EIA kit (Cayman Chemical, Ann Arbor, MI, USA) as described previously (15). Wound healing assay was performed using 60-mm fibronectin-coated dishes (Asahi Techno Glass Co., Tokyo, Japan) and cell invasion assay was carried out using BioCoat Matrigel Invasion Chambers (Becton-Dickinson Labware, Bedford, MA, USA) as described previously (11).

Statistical analysis. Statistical analysis was performed by using the Student's t-test and Fisher's exact test. Differences were considered significant at p-value <0.05.

Results

Suppression of COX-2 expression in KB/COX-2 cells by treatment with siRNA of COX-2. We examined the suppressive effect of COX-2 siRNA on COX-2 protein and mRNA expression in KB/COX-2 cells. COX-2 siRNA treatment markedly suppressed the expression of COX-2 protein in KB/COX-2 cells, and this suppressive effect continued significantly until day 7 (Fig. 1A). Using real-time PCR, expression level of COX-2 mRNA was also examined. KB/COX-2 cells showed about a 50-fold increase of COX-2 mRNA expression as compared to KB/Neo, whereas COX-2 siRNA-treated KB/COX-2 showed about 98% decrease of COX-2 mRNA expression as compared to control siRNA-treated KB/COX-2 (Fig. 1B). KB/COX-2 cells treated with siRNA of either COX-2 or control showed a similar growth rate, suggesting no contribution of COX-2 in cell growth of KB/COX-2.

In the PGE_2 production, COX-2 siRNA-treated KB/COX-2 cells showed about 85% inhibition as compared to control siRNA-treated (Fig. 2A). In addition, COX-2 siRNA-treated KB/COX-2 cells showed decreased cell migration and filled the scratch wound slightly slower than the control siRNA-treated cells (Fig. 2B). COX-2 siRNA-treated KB/COX-2 cells demonstrated a significant decreased ability to migrate through Matrigel invasion chambers as compared to control siRNA-treated cells (Fig. 2C).

Increased CD44 expression by COX-2 overexpression. Since we confirmed the suppressive effect of COX-2 siRNA on

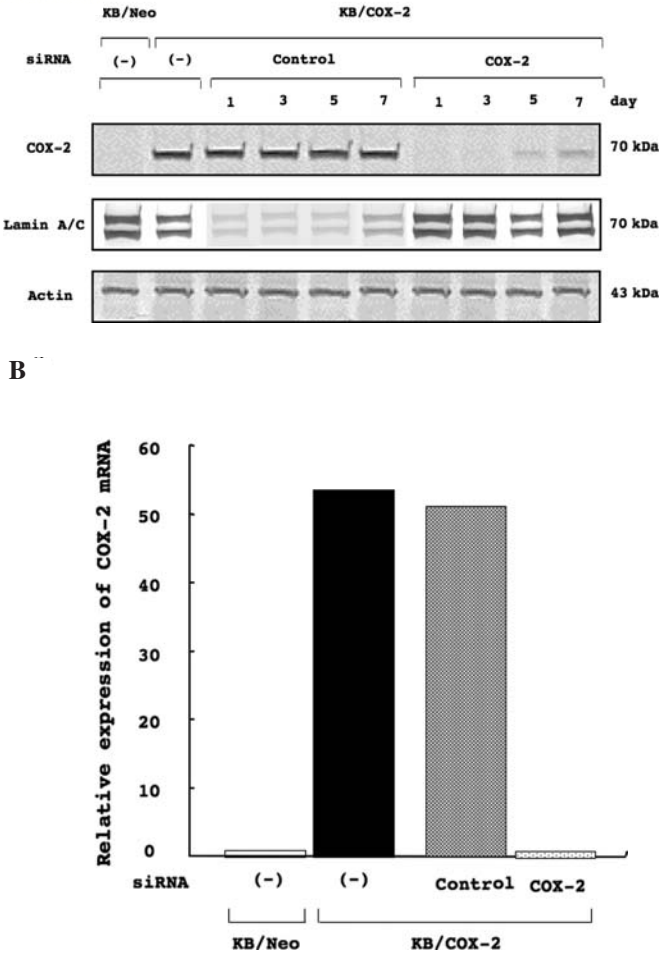

Figure 1. Expression of COX-2 in KB/Neo and KB/COX-2 cells and inhibition by COX-2 siRNA treatment. (A) Expression of COX-2 protein in Western blot analysis. Time course study with COX-2 siRNA in KB/COX-2 was performed. (B) Expression of COX-2 mRNA in real-time PCR.

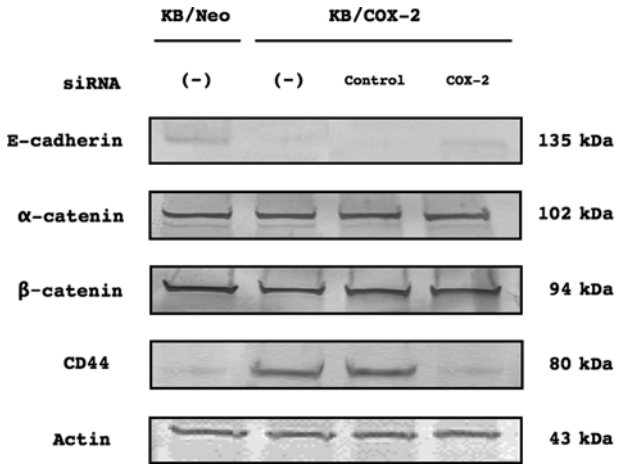

Figure 3. Expression of four proteins as representative adhesion molecules was examined in both cells by Western blotting.

COX-2 expression *in vitro*, we next examined the expression of adhesion molecules in both cell types (Fig. 3). When expression of E-cadherin, α-catenin, ß-catenin and CD44 was examined, there was no difference in α- and ß-catenin between KB/ COX-2 and KB/Neo. However, expression of E-cadherin was detected in KB/Neo, but not in KB/COX-2. In contrast, expression of CD44 was markedly increased in KB/COX-2 as compared to KB/Neo. Although treatment with COX-2 siRNA showed no effect on expression of α- and ß-catenin, the increased expression of CD44 was completely suppressed by COX-2 siRNA treatment as compared to control siRNA treatment. In addition, expression of E-cadherin in KB/COX-2 became detectable by COX-2 siRNA treatment.

Stimulation of tumor growth by COX-2 overexpression. As COX-2 overexpression resulted in increased CD44 expression,

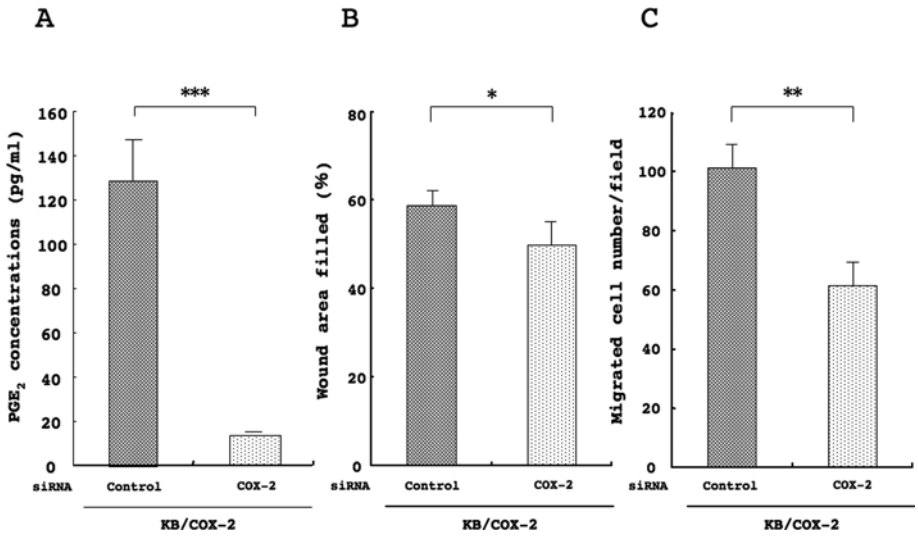

Figure 2. Inhibitory effect of COX-2 siRNA treatment on PGE_2 production and cell motility. (A) PGE_2 immunoassay was performed as indicated in a protocol of prostaglandin E2 EIA kit (Cayman Chemical) as described previously (16). (B) Wound healing assay was performed using 60-mm fibronectin-coated dishes (Asahi Techno Glass Co.) and (C) invasion assay was carried out using BioCoat Matrigel Invasion Chambers (Becton-Dickinson Labware) as described previously (11). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$.

Table I. Onset of tumor formation and tumor growth by subcutaneous inoculation of cells in nude mice.

Cells	siRNA	Onset of tumor formation (days)		Tumor volume at day 35 (mm^3)	
KB/Neo	(-)	17.5±3.8	**	61.2±53.4	*
KB/COX-2	(-)	10.5±3.8		378.1±150.6	
	Control	11.7±3.6	*	355.6±100.8	*
	COX-2	18.6±3.6		140.8±82.5	

Cells [10^6/0.1 ml PBS (-)] were inoculated into the flank of 6-week-old female nude mice (BALB/C nu/nu, n=6). Tumor formation at the inoculated sites was observed for 7 weeks. *P<0.05, **P<0.01.

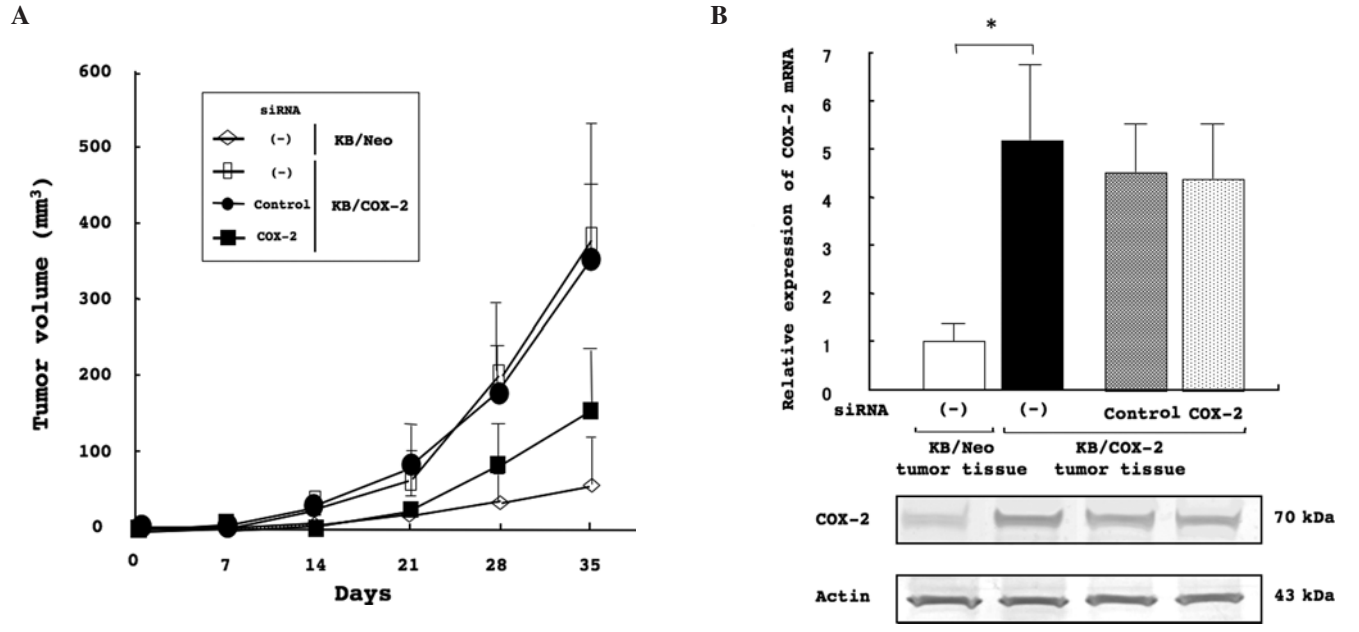

Figure 4. Growth curves of tumors produced by subcutaneous inoculation of cells in nude mice and expression of COX-2 in each tumor. (A) Tumor growth curves produced by subcutaneous inoculation of cells in nude mice. Data represent mean ± SD of 6 mice. (B) Expression of COX-2 protein and mRNA in each tumor tissue that was obtained by subcutaneous inoculation. *P<0.005.

we next examined the inhibitory effect on COX-2 *in vivo*. The onset of tumor formation and tumor growth in nude mice were examined by subcutaneous inoculation with $1x10^6$ cells/0.1 ml PBS (-). Although all nude mice developed tumors, the onset of tumor formation was 7 days earlier in the mice inoculated with KB/COX-2 than in those with KB/Neo, and COX-2 siRNA treatment delayed the onset of tumor formation for 7 days as compared to control siRNA treatment. In addition, tumor volume of KB/COX-2 at day 35 after inoculation showed about 6-fold increase as compared to KB/Neo, and that of COX-2 siRNA-treated KB/COX-2 showed about 60% decrease as compared to control siRNA (Table I, Fig. 4A).

Expression of COX-2 mRNA in KB/COX-2 tumor tissue formed in nude mice showed about 5-fold increase as compared to KB/Neo tumor tissue. Although tumor growth was retarded by COX-2 siRNA treatment, expression of COX-2 mRNA in COX-2 siRNA-treated tumor tissue was almost the same as control siRNA-treated tissue (Fig. 4B).

Elevated metastatic potential by COX-2 overexpression and inhibition with COX-2 siRNA. We examined the metastatic potential to the regional lymph nodes in nude mice by orthotopic inoculation to the masseter muscle with $1x10^5$ cells in 0.1 ml PBS (-) (Table II). All nude mice developed tumors at inoculated sites. In KB/COX-2 inoculation, metastasis to the regional lymph nodes occurred in 2 out of 15 mice, and metastasis to the lung in 3 out of 15 mice. In KB/Neo inoculation, metastasis to the lymph nodes occurred in only one out of 15 and no metastasis to the lung was found. Then, we examined the inhibitory effect of COX-2 siRNA on metastatic potential to the lymph nodes in KB/COX-2 cells. In control siRNA-treated KB/COX-2 inoculation, metastasis to

Metastases to lymph nodes and lung by orthotopic transplantation of cells in nude mice.

Cells	siRNA	Lymph node metastases (%)	Lung metastases (%)
KB/Neo	(-)	1/15 (7)	0/15 (0)
	(-)	2/15 (13)	3/15 (20)
KB/COX-2	Control	1/10 (10)	2/10 (20)
	COX-2	0/10 (0)	0/10 (0)

Cells [10^5/0.1 ml PBS (-)] were inoculated into the masseter mascle of nude mice. After 7 weeks, mice were sacrificed and metastatic lesions were examined macroscopically and confirmed microscopically.

Table III. Distant metastases by intra-cardiac injection of cells in nude mice.

Cells	siRNA	Lung metastases (%)	
KB/Neo	(-)	1/10 (10)	*
	(-)	8/10 (80)	
KB/COX-2	Control	5/10 (50)	*
	COX-2	0/10 (0)	

Cells [10^5/0.1 ml PBS (-)] were injected into the left cardiac ventricle of nude mice. After 7 weeks, mice were sacrificed and metastatic lesions were examined macroscopically and confirmed microscopically. *$P<0.01$.

the lymph nodes occurred in 1 out of 10, and metastasis to the lung occurred in 2 out of 10. In COX-2 siRNA-treated KB/COX-2 inoculation, no metastasis to the lymph nodes or lung was found.

In the orthotopic inoculation mentioned above, the number of mice with lung metastasis was greater than that with regional lymph node metastasis. This fact suggests that metastasis to the lung may occur via hematogenous but not lymphatic route. Therefore, we examined the hematogenous metastatic potential of cells using intra-cardiac injection model with $1x10^5$ cells in 0.1 ml PBS (-). In KB/COX-2 injection into the left cardiac ventricle of mice, the mice developed metastases to multiple organs; lung, stomach, femur, mandible, kidney, pancreas and adrenal gland. In KB/Neo injection, no metastases occurred except for only one metastasis to femur bone out of 10 mice.

Then, we examined the inhibitory effect of COX-2 siRNA on hematogenous metastatic potential in KB/COX-2 cells. In control siRNA treatment, the mice developed metastases in 5 out of 10, whereas no metastases was found in COX-2 siRNA treatment (Table III).

Increased tumorigenicity and colony forming ability by COX-2 overexpression. It is essential that the cells directly injected into a blood stream adhere and grow in the distant metastatic sites such as the lung and bone. Thus, we speculated that COX-2 overexpression confer the increased ability to adhere and grow. The tumorigenicity by inoculation with various cell number was examined (Table IV).

When $1x10^6$ cells were inoculated subcutaneously into the flanks of nude mice, all mice developed tumors at inoculated sites. When $1x10^5$ cells were inoculated, KB/COX-2 produced tumors in all mice, but KB/Neo produced no tumors. Then, we examined the inhibitory effect of COX-2 siRNA on tumorigenicity of KB/COX-2. When $1x10^5$ cells were inoculated subcutaneously into the flanks of mice, control siRNA-

Table IV. Tumorigenicity by subcutaneous inoculation of cells in nude mice.

		No. of cells inoculated				
Cells	siRNA	$1x10^4$ (%)	$3x10^4$ (%)	$1x10^5$ (%)	$5x10^5$ (%)	$1x10^6$ (%)
KB/Neo	(-)	0/3 (0)	0/3 (0)	0/6 (0)	2/6 (33)	6/6 (100)
	(-)	0/3 (0)	1/3 (33)	6/6 (100)	6/6 (100)	6/6 (100)
KB/COX-2	Control			3/6 (50)	5/6 (83)	6/6 (100)
	COX-2			0/6 (0)	2/6 (33)	6/6 (100)

Cells were inoculated into the flank of nude mice at cell number indicated. Tumor formation at inoculated sites was observed for 7 weeks.

treated KB/COX-2 produced tumors in half of the mice, but COX-2 siRNA-treated KB/COX-2 produced no tumor.

In addition, clonogenic assay showed that although the colony forming ability of KB/COX-2 was higher than that of KB/Neo (KB/COX-2; 82.1±9.6%, KB/Neo; 44±6.5%, P<0.005), COX-2 siRNA treatment significantly inhibited colony formation by 50% in KB/COX-2 cells (control siRNA; 17.2±2.9%, COX-2 siRNA; 8.3±1.4%, P<0.05).

Discussion

Many clinical and experimental studies have shown that COX-2 expression is associated with not only carcinogenesis but also local invasion and metastasis. However, few studies have been reported concerning the relation between COX-2 expression and metastasis of tumor cells in head and neck carcinoma (16-18). The significance of COX-2 expression in relation to tumor cell migration, invasion and metastasis of head and neck carcinoma is still poorly understood.

In this study, we have demonstrated a significant reduction of COX-2 levels overexpressed in KB/COX-2 using siRNA strategy, which can knock down the targeted genes with great specificity. We have also shown that treatment of KB/COX-2 with COX-2 siRNA has a marked inhibitory effect on cell migration, cell invasion, tumorigenicity, tumor growth and metastasis. We focused on the role of COX-2 in metastatic potential by using orthotopic inoculation or injection into the left cardiac ventricle of nude mice with KB/COX-2 and KB/Neo. Orthotopic inoculation of tumor cells in nude mice is well known as a spontaneous metastasis model (19,20). On the other hand, metastasis to liver by injection of tumor cells to portal vein or spleen (21,22) and metastasis to lung by injection of tumor cells to tail vein (20) are also reported as experimental metastasis models. Although an intra-cardiac injection of tumor cells (23,24) is easier to make metastasis than other models, it is uncommon because of the technical difficulty. When injected into the left cardiac ventricle of mice, KB/COX-2 developed metastases to multiple organ such as lung, stomach, femur and so on whereas KB/Neo was hardly found to develop metastases. On the other hand, the number of metastasis to lymph nodes was less than that to the lung in orthotopic inoculation. These findings suggested that metastasis to the lung occurred via hematogenous but not lymphatic route.

In the present study, we confirmed that COX-2 overexpression promotes hematogenous metastasis, but the precise mechanisms are still unclear. Since KB/COX-2 showed very high tumorigenic activity as compared to KB/Neo, we supposed that KB/COX-2 has a clear advantage exceeding KB/Neo in formation of metastasis by injection of small number of cells into a blood stream. As metastasis-related molecules, CXCR4 is a well-known chemokine which is a receptor for stromal cell-derived factor-1α (SDF-1α) and has been shown to be primarily involved in the distant metastasis of several types of cancer (25-27). We have recently reported that CXCR4 expression and metastatic potential are closely related in adenoid cystic carcinoma (ACC) (28). When we examined the expression of CXCR4 in KB/COX-2 and KB/Neo, there was no difference though CXCR4 was expressed in both cells (data not shown). In addition, we also speculated that expression of the adhesion molecules in tumor cells may be relevant to metastasis formation in other organs.

It has been reported that many adhesion molecules contribute to metastasis. In the present study, we examined the expression of E-cadherin, α-catenin, ß-catenin and CD44 as representative molecules. Consequently, we found that expression of CD44 was markedly increased in KB/COX-2 as compared to KB/Neo, and expression of E-cadherin was detected in KB/Neo, but not in KB/COX-2. Treatment with COX-2 siRNA resulted in suppression of CD44 expression and detectable expression of E-cadherin in KB/COX-2. E-cadherin is a direct mediator of cell-cell adhesive interactions, and the cytoplasmic tail of E-cadherin is tethered, via α-catenin and ß-catenin, to the actin cytoskeleton to form cell junctions. Therefore, loss of E-cadherin causes detachment of tumor cells to lead to early invasion and metastasis (29). CD44 is also a cell surface adhesion molecule that mediates diverse function. Experimental evidence suggests that ligation of CD44 on the cell surface induces cell detachment and migration in a variety of cells (30,31). In addition, mounting evidence suggests that CD44 is aberrantly expressed in many human tumors (32). In certain cases, such as with colorectal carcinomas, the expression of CD44 confers metastatic potential *in vivo* (33,34). Furthermore, CD44 serves to induce co-clustering with MMPs and can promote MMPs activity and tumor invasion (35-37). Kinugasa *et al* reported that the COX-2 inhibitor NS-398 and COX-2 antisense oligonucleotide suppressed the invasiveness of oral squamous cell carcinoma cell lines in a Matrigel invasion assay via down-regulation of MMP-2, MT1-MMP and CD44 (38). We also reported previously that KB/COX-2 increased cell migration and invasion via up-regulation of MMP-9, MMP-2 and MT1-MMP and down-regulation of TIMP-1 and TIMP-2 (11).

In conclusion, our results demonstrated that overexpression of COX-2 increased tumorigenicity and hematogenous metastasis via down-regulating E-cadherin and up-regulating CD44 expressions in KB cells. The positive correlation of metastatic potential and COX-2 overexpression indicates that COX-2 may become a target molecule for regulating metastases of oral cancer.

Acknowledgements

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