Coexpression of Cyclooxygenases (COX-1, COX-2) and Vascular Endothelial Growth Factors (VEGF-A, VEGF-C) in Esophageal Adenocarcinoma


Abstract
Cyclooxygenases (COX), especially COX-2, are considered to be involved in carcinogenesis. Our study was initiated to test whether expression of COX isoforms (COX-1 and COX-2) is linked to expression of potent inducers of angiogenesis [vascular endothelial growth factor (VEGF)-A] and lymphangiogenesis (VEGF-C) in esophageal adenocarcinoma. One hundred twenty-three esophageal adenocarcinomas were investigated by means of quantitative reverse transcription-PCR for expression of COX-1, COX-2, VEGF-A, and VEGF-C. Additionally, COX-2 protein expression was determined using immunohistochemistry. Three esophageal cancer cell lines (OE-33, OSC-1, and OSC-2) were treated with COX-inhibiting substances (diclofenac, rofecoxib, and SC-560) and the effect on expression of the four genes was determined. COX-2 protein expression was found in all carcinomas under analysis. RNA expression levels of COX-1 and COX-2 varied markedly in carcinoma tissues and correlated significantly with each other (P < 0.001, r = 0.726). Furthermore, COX expression correlated with expression of VEGF-A (COX-1: P < 0.001, r = 0.753; COX-2: P < 0.001, r = 0.764) and VEGF-C (COX-1: P < 0.001, r = 0.778; COX-2: P < 0.001; r = 0.613). Exposure of esophageal cancer cell lines OE-33, OSC-1, and OSC-2 with three COX-inhibiting substances (diclofenac, rofecoxib, and SC-560) resulted in significantly reduced expression of VEGF-A and VEGF-C. In conclusion, our data suggest that both COX isoforms may be involved in the pathogenesis of esophageal adenocarcinoma, as they are linked to the expression of important modulators of angiogenesis (VEGF-A) and lymphangiogenesis (VEGF-C). (Cancer Res 2005; 65(12): 5038-44)

Introduction
Barrett’s esophagus is the precursor lesion of distal esophageal adenocarcinomas, an entity of increasing clinical importance due to a vastly unexplained, rapidly increasing incidence during the previous decades (1). Carcinomas in Barrett’s esophagus develop under the chronically damaging effect of gastroesophageal reflux, by which the metaplastic epithelium is promoted through a (low-grade/high-grade) intraepithelial neoplasia sequence toward invasive cancer (2). Chances for cure are limited to surgical resection in early tumor stages before systemic generalization of the disease (3).

Despite intensive research, the molecular changes underlying the development of Barrett’s cancer are largely unknown. A central role is attributed to the enzyme cyclooxygenase (COX), which converts arachidonic acid into prostaglandins. The involvement of COX into gastrointestinal carcinogenesis has at first been indicated by epidemiologic studies, demonstrating a decreased cancer risk following a regular intake of nonsteroidal anti-inflammatory drugs (NSAIDs; ref. 4). This has also been proven for esophageal cancer in population-based studies and meta-analyses (5). The cancer protective effect of NSAIDs is currently mainly attributed to the inhibition of COX (6–8). Previous work revealed that COX-2, an isoform of COX, is overexpressed in Barrett’s carcinomas (9). More recently, a negative prognostic impact of COX-2 protein overexpression has been shown in this tumor type (10). COX-1, the other isoform of cyclooxygenase, until lately, has been assumed to be constitutively expressed and to be of minor impact for carcinogenesis. However, reports on murine models of lung carcinogenesis (11) and colorectal carcinogenesis (12) as well as on some human tumor entities [i.e., ovarian cancer (13), cervical cancer (14), and breast cancer (15)] suggest that COX-1 may be important for carcinogenesis as well. However, the role of COX-1 expression for the development of gastrointestinal cancers has not been investigated thus far.

Apart from being important for the regulation of apoptosis and immune surveillance, COX has been recognized to be strongly involved into the regulation of angiogenesis (16). Cell culture experiments using breast cancer (17), colon cancer (6), and ovarian cancer cell lines (13) indicate that one main mechanism is induction of proangiogenic growth factors of the vascular endothelial growth factor (VEGF) family, in particular VEGF-A, a central regulator of blood vessel neoformation. This concept has been further substantiated on colon cancer samples (18) that showed that microvessel density in tumors increased with increasing COX-2 expression levels. Consequently, the antitumor effect of NSAIDs is considered as at least partially antiangiogenic in nature. More recently, studies on lung adenocarcinoma samples indicate that through induction of the lymphangiogenic factor VEGF-C, COX-2 may also be involved in the regulation of lymphangiogenesis (19).

Against this background, the current study was initiated to find out whether cyclooxygenases are involved in the regulation of VEGFs in primary esophageal adenocarcinomas, thus providing insights in the mechanisms involved in angiogenesis and lymphangiogenesis in this tumor type.

Materials and Methods
Samples. One hundred twenty-three cases of resected esophageal adenocarcinomas that had developed in Barrett’s esophagus (Barrett’s
cancer) were randomly selected from our tissue database. Patients had been treated by radical surgical resection—either transthoracic or transhiatal esophagectomy—between 1991 and 2002. None of the patients had received neoadjuvant chemotherapy or radiochemotherapy. One hundred eleven patients were male, the age ranged between 33 and 84 (median: 65). Tumor characteristics—according to the current tumor-node-metastasis classification, Union Internationale Contre le Cancer 2002—were as follows: 58 pT1 carcinomas, 21 pT2 carcinomas, 44 pT3 carcinomas. Seventy-two cases were nodal negative, whereas the remaining 51 cases had positive lymph nodes. Sixty-four tumors were well or moderately differentiated (G1 or G2), whereas 59 tumors showed poor differentiation (G3). Invasion of lymphatic vessels by tumor cells, as determined by light microscopic examination by one of the authors (M. Sarbia), was present in 34 cases, whereas no lymphatic vessel invasion was detectable in 89 cases.

Immunohistochemical evaluation of cyclooxygenase-2 protein expression. Expression of COX-2 protein was determined by immunohistochemistry in a subset of 40 (randomly selected) patients. For each of the carcinomas, one representative paraffin block that included central and peripheral portions of the tumors was selected. All tumors had been fixed in 10% buffered formalin immediately after esophageal resection. After pressure cooker pretreatment in citrate buffer (pH 6.0) for 7 minutes, 4 µm sections of the tumors were incubated at room temperature for 1 hour with a monoclonal COX-2 antibody (Cayman Chemical Company, Ann Arbor, MI; dilution: 1:50), respectively. After incubation with an appropriate secondary antibody, the slides were incubated with labeled LSAB-alkaline phosphatase. Reaction products were visualized by immersing the slides in fast red. Finally, the slides were counterstained with hemalun. Each experiment included samples with known COX-2 expression as positive control. Negative controls were obtained by incubation with a blocking peptide for COX-2 (Cayman Chemical Company) according to the manufacturer's instruction. Immunostaining for COX-2 could be completely abolished by preincubation with COX-2 blocking peptide. The expression of COX-2 in the tumor cells was determined by one senior pathologist (M. Sarbia) according to the methods described previously (9). Briefly, the percentage of positive tumor cells was assigned into one of the following categories: 0 (0–4% positive tumor cells), 1 (5–24%), 2 (25–49%), 3 (50–74%), and 4 (75–100%). The intensity of immunostaining was determined as 0 (negative), 1+ (weak), or 2+ (strong). Additionally, an immunoreactive score was calculated by multiplication of the percentage of positive cells and staining intensity (9).

Preparation of samples for messenger RNA extraction. From representative paraffin blocks of tumor tissue, two 10 µm sections were deparaffinized (xylene for 2 × 10 minutes) and rehydrated (ethanol 100%/90%/80%/70% ethanol for 5 minutes each). Following short hemalun staining, a minimum of 2,000 tumor cells was scraped off the glass slides using a sterile blade under light microscopic control by a senior pathologist (M. Sarbia). Hemorrhagic or necrotic areas were excluded. Tissue samples were homogenized in 200 µL RNA lysis buffer [10 mmol/L Tris-HCl (pH 8.0), 0.1 mmol/L ethylenediamine tetraacetic acid (pH 8.0); 2% SDS (pH 7.3)]. After addition of 10 µL proteinase K (500 µg/mL), the material was incubated until the tissue was completely solubilized (60°C for at least 24 hours).

RNA extraction, cDNA synthesis, and quantitative PCR. Extraction of RNA from formalin-fixed and paraffin-embedded tumor samples and subsequent cDNA synthesis were done according to a protocol previously published by our group (20). PCR reactions were done at least in duplicate for each sample, using TaqMan Universal PCR Master Mix (15 µL, Applied Biosystems; Roche Diagnostics, Mannheim, Germany) and 5 µL of the cDNA preparation. Experiments were done using the ABI Prism 7700 Sequence Detection System instrument and software (Perkin-Elmer Applied Biosystems, Inc., Foster City, CA). Optimial primer and probe concentrations were defined according to subtle testing and varied between the different assays. After initial incubation at 50°C for 2 minutes and 95°C for 10 minutes, samples were amplified for 50 cycles of 95°C for 15 seconds, followed by 60°C for 1 minute.

Assays for the determination of cyclooxygenase-1, cyclooxygenase-2, vascular endothelial growth factor-A, and vascular endothelial growth factor-C mRNA expression. Expression of COX-1, COX-2, and VEGF-A were determined using primers and probes supplied by Applied Biosystems (Roche Diagnostics, Mannheim, Germany). Assay details are as follows: VEGF-A (spanning exon 1/exon 2; ID: Hs00173626_m1); COX-1 (spanning exon 2/exon 3; ID: Hs00153133_m1); and COX-2 (spanning exon 5/exon 6; ID: Hs00153333_m1). Primer and probes were added to the reaction volume following the manufacturer's recommendations. For investigation of VEGF-C expression, primers and probes were designed, using the Primer Express software (Perkin-Elmer Applied Biosystems). The sequences of forward and reverse primer were 5'-GCTGTGGTAAAGGAGGAAAGTTCCAC-3' and 5'-AGGCTTCTTGGCGGTTCG-3', respectively, thus spanning exon 6 and exon 7 of the VEGF-C gene. The sequence of the probe was 5'-FAM-TCTGGTGCACAGACTTGCCCTC-TAMRA-3'. The specificity of the PCR products was verified by repeated examination of ethidium bromide–stained agarose gels and by sequencing of a subset PCR products (data not shown).

For quantitative analyses, target gene expression (VEGFs and COXs) was normalized against mRNA expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S RNA, because the measurement of absolute amounts of target gene mRNA may be influenced by various factors (i.e., quality of mRNA and amount of input cDNA; ref. 20). Because expression of GAPDH mRNA and 18S RNA were highly correlated with each other (r = 0.908; Spearman r test), target gene expression is subsequently presented as the ratio between target gene expression and GAPDH expression. For each tumor sample under investigation, the amount of target gene mRNA and GAPDH mRNA was determined from a standard curve. The latter was constructed with 5-fold serial dilutions of an esophageal squamous cell carcinoma cell line (OSC-1; 200–0.0128 ng) that has been previously established in our laboratory.

Cell culture experiments. To further test the relationship between expression of COXs and VEGFs, permanent esophageal cancer cell lines were treated with COX-inhibiting substances. Therefore, the esophageal squamous cell cancer cell lines OSC-1 and OSC-2, in which we had previously shown COX-1 and COX-2 mRNA expression (9), and the esophageal adenocarcinoma cell line OE-33 (European Collection of Cell Cultures no. 96070808) were selected. Approximately 250,000 cells were seeded in six-well plates (Sarstedt, Nümbrecht, Germany) and incubated in DMEM (Life Technologies, Cergy, France) supplemented with 10% FCS for 24 hours at 37°C in a humidified incubator. Subsequently, the following COX-inhibiting drugs were added to the medium (each at final concentration of 10 µmol/L): the nonselective COX-inhibitor diclofenac (Voltaren, Novartis Pharma GmbH, Nürnberg, Germany), the COX-2–selective inhibitor rofecoxib (Vioxx, MSD, Haar, Germany), and the COX-1–selective inhibitor SC-560 (Cayman Chemical Company). Cells were harvested after 6, 12, and 24 hours of incubation. RNA was extracted as described above. All experiments were done in triplicate.

Expression of the four genes under analysis (COX-1, COX-2, VEGF-A, VEGF-C) as well as of the control gene (GAPDH) was determined using the same primers, probes, and PCR conditions as described above. Expression levels of COX-1, COX-2, VEGF-A, and VEGF-C in drug-treated samples were expressed in relation to the expression levels of the corresponding control samples (without treatment) after normalization with GAPDH expression (arbitrarily designated as 1.00).

Statistical analysis. Correlation analysis between expressions of target genes in clinical tumor samples was done using the Spearman r test. Correlation between expression of target genes and categorical variables (i.e., pT category, pN category, presence of lymphatic vessel invasion, and tumor differentiation) was determined using the Mann-Whitney test. For statistical evaluation of cell culture experiments, mean values and SD of the triplicate experiments were calculated. Gene expression levels under treatment with COX inhibitors were compared with the respective expression levels without drug treatment using Student’s t test. P values smaller 0.05 were considered as statistically significant.
Results

Expression of Cyclooxygenase-1, Cyclooxygenase-2, Vascular Endothelial Growth Factor-A, and Vascular Endothelial Growth Factor-C in Esophageal Adenocarcinomas

Using immunohistochemistry, expression of COX-2 protein was found in all tumor samples under analysis (Fig. 1). All tumors showed immunoreactivity for COX-2 in 75% to 100% of the tumor cells (category 4) in combination with strong staining intensity (category 2+), thus resulting in an immunoreactive score of 8 in all tumors. Nonneoplastic esophageal squamous epithelium adjacent to the cancer tissues was present in 12 of the samples under analysis. In all cases, expression of COX-2 protein of weak staining intensity was found in the basal layer of the squamous epithelium. Percentage of immunoreactive cells as well as staining intensity was weaker than in the adjacent adenocarcinoma cells. Regarding stromal cells, immunoreactivity for COX-2 was found in endothelial cells (strong intensity), smooth muscle cells, lymphocytes, and fibroblastic cells of the tumor stroma (all weak intensity).

Using quantitative PCR, mRNA expression was found in all 123 tumors (100%) for COX-1, COX-2, and VEGF-A as well as in 91 of 123 tumors (74%) for VEGF-C. The median relative target gene expression levels in the tumor samples were as follows: COX-1 4.269 (range: 0.011-108.109), COX-2 10.701 (0.049-300.792), VEGF-A 2.761 (range: 0.076-104.565), and VEGF-C 0.002 (0.000-0.357; Table 1).

Expression levels of COX-1 and COX-2 mRNA correlated significantly with each other (r = 0.726). Furthermore, COX-expression correlated with expression of VEGF-A (COX-1: P < 0.001, r = 0.753; COX-2: P < 0.001, r = 0.764; Figs. 2A and 3A) and VEGF-C (COX-1: P < 0.001, r = 0.778; COX-2: P < 0.001, r = 0.613; Figs. 2B and 3B). The proangiogenetic factors VEGF-A and VEGF-C also showed strong positive correlation regarding their gene expression levels (P < 0.001; r = 0.877). All results of correlations of the relative gene expression levels of the target genes under investigation are given in Fig. 4.

Correlation of Messenger RNA Expression with Clinicopathologic Parameters

Patients with lymphatic vessel invasion by tumor cells showed significantly increased expression of VEGF-A (P = 0.048) and VEGF-C (P = 0.003). Tumors with lymph node metastases showed significantly higher VEGF-C expression levels than tumors without metastases (P = 0.029). Patients with locally advanced tumors (pT3) had significantly higher expression of VEGF-A (P = 0.004) and VEGF-C (P = 0.003) compared with locally limited tumors (pT1/2).

In contrast, target gene expression failed to show significant correlation with tumor grade (well/moderately versus poorly differentiated; data not shown). The expression level of none of the genes under investigation showed a significant impact on survival (univariate analysis with the method of Kaplan and Meier, stratification according to mean and median gene expression levels, data not shown).

Cell Culture Experiments

Gene expression in cell lines without treatment. All four target genes under investigation (COX-1, COX-2, VEGF-A, VEGF-C) were found to be expressed in the esophageal cancer cell lines OSC-1, OSC-2, and OE-33. Relative gene expression levels were calculated similar to investigation of human tumor specimen as target gene/GAPDH ratios. In accordance to previously reported data (9), OSC-2 showed relatively high expression of COX-2 (416.168 ± 27.042), in comparison with OSC-1 (26.179 ± 11.602) and OE-33 (37.959 ± 13.665). In contrast, COX-1 expression levels did not differ substantially between OSC-1 cells (0.596 ± 0.299), OE-33 cells (0.480 ± 0.237), and OSC-2 cells (0.278 ± 0.013).

The relative gene expression levels of the proangiogenetic factors VEGF-A (OSC-1: 10.417 ± 3.876; OSC-2: 16.518 ± 1.078; OE-33: 15.013 ± 3.306) and VEGF-C (OSC-1: 0.460 ± 0.248; OSC-2: 0.435 ± 0.028; OE-33: 0.346 ± 0.186) were similar in all three cell lines under investigation. All results of gene expression analysis in the cell lines without exposition to COX inhibitors are summarized in Table 2.

Figure 1. Strong cytoplasmic expression of COX-2 (red reaction product) in an esophageal adenocarcinoma (A). Inset, magnification showing COX-2 immunoreactivity also in stromal cells (i.e., fibroblasts and endothelial cells). Complete blockade of immunoreactivity after preincubation of a serial section of this tumor with a COX-2 blocking peptide (B).
Gene expression levels of the four genes under analysis did not significantly change under exposition to cell culture medium without addition of COX inhibitors after 6, 12, or 24 hours.

**Gene Expression Under Exposition to Cyclooxygenase Inhibitors**

**OSC-1.** In the cell line OSC-1 (see Table 3A), COX-1 expression was induced under exposition to the COX-2–selective inhibitor rofecoxib: This effect was significant after 12 hours ($P = 0.018$) and 24 hours ($P = 0.041$). Under treatment with the COX-1–selective inhibitor SC-560, COX-1 expression was significantly diminished ($P = 0.045$) after 6 hours. Under exposition to the nonselective COX-inhibitor diclofenac, COX-1 expression was significantly elevated ($P = 0.025$) after 12 hours. COX-2 expression was not altered significantly by COX inhibition with any of the substances. VEGF-A expression was reduced during treatment with rofecoxib and during exposition with diclofenac. This effect was statistically significant after 6 hours ($P = 0.027$) and 24 hours ($0.016$) of incubation with rofecoxib and after 6 hours of treatment with diclofenac ($P = 0.033$), rofecoxib (after 12 hours, $P = 0.015$; after 24 hours, $P = 0.008$), and diclofenac (after 12 hours, $P = 0.009$ and 24 hours, $P = 0.010$).

**OSC-2.** In the cell line OSC-2 (see Table 3B), originally expressing relatively high levels of COX-2 and low levels of COX-1, COX-2 expression was reduced during exposure with SC-560 (after 6 hours, $P = 0.003$; after 24 hours, $P = 0.001$), during exposure with rofecoxib (after 24 hours, $P < 0.001$) and during exposure with diclofenac (after 24 hours, $P = 0.002$). COX-1 expression was diminished under treatment with rofecoxib for 6 hours ($P = 0.003$). VEGF-A expression tended to be decreased under COX-inhibition, although in OSC-2 this was only statistically significant with diclofenac treatment for 6 hours ($P = 0.016$). Results for rofecoxib and SC-560 marginally failed to achieve statistical significance ($P = 0.055$ and $P = 0.059$, respectively). VEGF-C expression also tended to become decreased under exposition to the COX-inhibiting substances. The effect was significant after 24 hours with SC-560 ($P = 0.009$) and after 6 hours with rofecoxib ($P = 0.019$) but marginally failed to achieve statistical significance with diclofenac after 24 hours ($P = 0.068$).

**OE-33.** In the cell line OE-33 (see Table 3C), COX-1 expression tended to get induced due to COX inhibition with rofecoxib and diclofenac (similar to OSC-1), an effect that was statistically significant after 24 hours of exposure to rofecoxib ($P = 0.045$). A significantly decreased COX-1 expression ($P = 0.007$) was—similarly to OSC-1—noted after 6 hours of exposition to SC-560. In OE-33, expression was reduced during treatment with rofecoxib and during exposition with diclofenac. This effect was statistically significant after 6 hours ($P = 0.027$) and 24 hours ($0.016$) of incubation with rofecoxib and after 6 hours of treatment with diclofenac ($P = 0.033$), rofecoxib (after 12 hours, $P = 0.015$; after 24 hours, $P = 0.008$), and diclofenac (after 12 hours, $P = 0.009$ and 24 hours, $P = 0.010$).

**Table 1.** Relative mRNA expression levels of the target genes COX-1, COX-2, VEGF-A, and VEGF-C, following normalization against the mRNA expression of the housekeeping gene GAPDH in 123 esophageal adenocarcinomas

<table>
<thead>
<tr>
<th>Gene</th>
<th>Median</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-1</td>
<td>4.269</td>
<td>0.011</td>
<td>108.109</td>
</tr>
<tr>
<td>COX-2</td>
<td>10.701</td>
<td>0.049</td>
<td>300.792</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>2.761</td>
<td>0.076</td>
<td>104.565</td>
</tr>
<tr>
<td>VEGF-C</td>
<td>0.002</td>
<td>0.000</td>
<td>0.357</td>
</tr>
</tbody>
</table>

**Figure 2.** Correlation between the relative gene expression levels showing significant positive correlation between expression of COX-1 and VEGF-A ($A; P < 0.001, r = 0.753$) and VEGF-C ($B; P < 0.001, r = 0.778$) in 123 esophageal adenocarcinomas.
COX-2 expression was not significantly altered under treatment with COX inhibitors; only after 12 hours of exposure to diclofenac, COX-2 level was increased ($P = 0.023$). VEGF-A was diminished under exposition to COX-inhibiting substances, an effect that was significant after 6 hours of treatment with rofecoxib ($P = 0.031$), but failed significance after 12 and 24 hours ($P = 0.084$). The expression of VEGF-C was significantly diminished under exposition to SC-560 (after 6 hours, n.s.; after 24 hours, $P < 0.001$), under exposition to rofecoxib (after 6 hours, $P = 0.001$; after 12 hours, $P = 0.018$; and after 24 hours, $P = 0.008$), as well as under exposition to diclofenac (after 6 hours, $P = 0.025$).

Discussion

The current study shows highly variable mRNA expression levels of both cyclooxygenase isoforms (i.e., COX-1 and COX-2) in primary esophageal adenocarcinomas. Furthermore, the relative amount of COX-1 as well as of COX-2 mRNA expression is strongly correlated with the expression level of two angiogenetic key molecules (i.e., VEGF-A and VEGF-C). Furthermore, we could show that exposition of esophageal cancer cell lines to COX-inhibiting substances results in a significantly decreased expression of VEGF-A and VEGF-C mRNA. Altogether, these findings indicate that both COX isoforms may be involved in the processes of angiogenesis and lymphangiogenesis in esophageal adenocarcinoma.

Until recently, COX-2 has been regarded as the only inducible form of COX, whereas COX-1 was assumed to be constitutively expressed at a relatively constant level. COX-2 was shown to be absent or present in minimal amounts in most normal tissues, but to be inducible upon cell activation by mitogens, cytokines, and tumor promoters (21). However, our findings demonstrating that mRNA expression of both COX isoforms varies greatly in esophageal adenocarcinoma are in accordance with recent studies showing overexpression of COX 1 in a subset of ovarian cancers (13), cervical cancers (14), and breast cancers (15). Moreover, our findings on the mRNA level are at least partially corroborated by our immunohistochemical investigations. Thus, we found a strong expression of COX-2 protein in all carcinomas under analysis. Although expression of COX-2 was found also in variety of stromal cell (e.g., endothelial cells, smooth muscle cells), immunohistochemical analyses suggest that the COX-2 mRNA signals from carefully dissected tumor samples are indeed primarily derived from cancer cells and only to a minor amount from contaminating stromal cells. On the other side, our immunohistochemical analyses indicate intertumoral heterogeneity of COX expression at a much lesser degree than quantitative mRNA analyses. This may at least partially be due to the known...
Reduced expression of VEGF-A in resected esophageal adenocarcinomas, but also by the positive correlation between expression levels of COX-2 and induction of VEGF-A. This conclusion is not only supported by fact that immunohistochemistry does not provide a reliable tool for gene expression quantification.

Besides the discussion whether both COX isoforms are important in carcinogenesis, our data support the current concept that COX-2 may be involved in the regulation of angiogenesis by induction of VEGF-A. This conclusion is not only supported by a positive correlation between expression levels of COX-2 and VEGF-A in resected esophageal adenocarcinomas, but also by the results of our cell culture experiments. Thus, we could show a reduced expression of VEGF-A of three esophageal cancer cell lines after exposure to the COX inhibitors diclofenac (nonselective) and rofecoxib (selective for COX-2). A similar, although not significant, effect was also seen after exposure of the OE-33 cell line to the COX-1 inhibitor SC-560. Nonetheless, one has to point out that inhibition of VEGF-A production was incomplete in all three cell lines indicating either incomplete COX inhibition or to the presence of alternative molecular pathways to bypass the effect of COX inhibition on expression of VEGFs. As observed in our cell culture experiments, incomplete suppression of COX enzymes in tumor cells may partly be explained by an induction of new COX mRNA expression, probably as an effort of tumor cells to regulate against COX inhibition. This assumption is in line with our observation that expression of COXs and VEGFs varies temporally in esophageal cancer cell lines treated with COX-inhibiting compounds.

Besides the role of COXs in angiogenesis, a potential involvement of COX-2 in the process of lymphangiogenesis has been recently concluded from the observation of a significant association of COX-2 expression with expression of the lymphangiogenic molecule VEGF-C in lung adenocarcinomas (19). These findings are

### Table 2. Relative gene expression levels of the esophageal cancer cell lines OSC-1, OSC-2, and OE-33, used for cell culture COX inhibition experiments (relative target gene/GAPDH ratio, determined similar to analysis of human tumor specimen)

<table>
<thead>
<tr>
<th></th>
<th>COX-1</th>
<th>COX-2</th>
<th>VEGF-A</th>
<th>VEGF-C</th>
</tr>
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<tbody>
<tr>
<td>OSC-1</td>
<td>0.596 ± 0.299</td>
<td>26.179 ± 11.602</td>
<td>10.417 ± 3.876</td>
<td>0.460 ± 0.248</td>
</tr>
<tr>
<td>OSC-2</td>
<td>0.278 ± 0.013</td>
<td>416.168 ± 27.042</td>
<td>16.518 ± 1.078</td>
<td>0.433 ± 0.028</td>
</tr>
<tr>
<td>OE-33</td>
<td>0.480 ± 0.237</td>
<td>37.959 ± 13.665</td>
<td>15.013 ± 3.306</td>
<td>0.346 ± 0.186</td>
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### Table 3. Effects of incubation of the selective COX-1 inhibitor SC-560, the selective COX-2 inhibitor rofecoxib, and the nonselective COX inhibitor diclofenac (each 10 μmol/L) on the esophageal cancer cell lines OSC-1, OSC-2, and OE-33: mRNA expression of COX-1, COX-2, VEGF-A, and VEGF-C in relation to untreated control cells, where expression was arbitrarily set as 1.00

<table>
<thead>
<tr>
<th></th>
<th>COX-1</th>
<th>COX-2</th>
<th>VEGF-A</th>
<th>VEGF-C</th>
</tr>
</thead>
</table>
| A. OSC-1
SC-560  | 6 h 0.55 ± 0.08 | 1.00 ± 0.07 | 0.99 ± 0.12 | 0.98 ± 0.01 |
   24 h 1.63 ± 0.57 | 0.97 ± 0.19 | 1.51 ± 0.58 | 0.38 ± 0.17 |
Rofecoxib 6 h 1.83 ± 0.31 | 0.94 ± 0.25 | 0.57 ± 0.10 | 1.16 ± 0.18 |
      12 h 2.87 ± 0.36 | 0.89 ± 0.25 | 0.72 ± 0.09 | 0.24 ± 0.13 |
      24 h 1.68 ± 0.20 | 0.81 ± 0.31 | 0.65 ± 0.06 | 0.14 ± 0.11 |
Diclofenac 6 h 1.30 ± 0.33 | 1.32 ± 0.35 | 0.63 ± 0.08 | 0.39 ± 0.30 |
        12 h 1.80 ± 0.18 | 0.84 ± 0.24 | 0.81 ± 0.17 | 0.20 ± 0.11 |
      24 h 1.33 ± 0.16 | 0.69 ± 0.33 | 0.73 ± 0.17 | 0.17 ± 0.12 |
B. OSC-2
SC-560  | 6 h 1.03 ± 0.31 | 0.80 ± 0.11 | 0.73 ± 0.18 | 0.96 ± 0.43 |
   24 h 0.98 ± 0.39 | 0.37 ± 0.10 | 1.04 ± 0.30 | 0.60 ± 0.25 |
Rofecoxib 6 h 0.56 ± 0.24 | 0.86 ± 0.47 | 0.60 ± 0.27 | 0.68 ± 0.20 |
      24 h 0.97 ± 0.59 | 0.32 ± 0.07 | 0.84 ± 0.21 | 0.75 ± 0.26 |
Diclofenac 6 h 1.22 ± 0.41 | 1.01 ± 0.25 | 0.63 ± 0.15 | 1.15 ± 0.38 |
      24 h 1.48 ± 0.57 | 0.22 ± 0.15 | 0.79 ± 0.28 | 0.66 ± 0.24 |
C. OE-33
SC-560  | 6 h 0.36 ± 0.08 | 1.05 ± 0.38 | 0.74 ± 0.10 | 0.92 ± 0.31 |
   24 h 1.24 ± 0.21 | 0.84 ± 0.36 | 0.90 ± 0.12 | 0.28 ± 0.02 |
Rofecoxib 6 h 1.34 ± 0.32 | 1.41 ± 0.37 | 0.65 ± 0.09 | 0.61 ± 0.02 |
      12 h 1.31 ± 0.21 | 1.40 ± 0.41 | 0.79 ± 0.24 | 0.39 ± 0.12 |
      24 h 1.76 ± 0.24 | 0.99 ± 0.23 | 0.88 ± 0.06 | 0.54 ± 0.06 |
Diclofenac 6 h 1.14 ± 0.22 | 1.70 ± 0.60 | 0.81 ± 0.18 | 0.34 ± 0.15 |
       12 h 1.23 ± 0.20 | 1.58 ± 0.13 | 0.90 ± 0.21 | 0.30 ± 0.38 |
       24 h 1.30 ± 0.23 | 1.17 ± 0.39 | 0.96 ± 0.20 | 0.46 ± 0.31 |

NOTE: Values printed in bold are significantly different from untreated controls (P values are mentioned in the text).
now supported by our result that expression of COX-1 and COX-2 is significantly correlated with expression VEGF-C in esophageal adenocarcinoma. Again, our results based on resection specimens are corroborated by our cell culture experiments, where we could show that expression of VEGF-C in three esophageal cancer cell lines can be significantly suppressed by exposure to selective (SC-560, rofecoxib) and nonselective (diclofenac) COX inhibitors. These findings provide evidence for the first time in gastrointestinal cancers that expression of both COX isozymes may not only be involved in the regulation of angiogenesis but also in lymphangiogenesis.

Besides the implications for the understanding of the molecular pathogenesis of esophageal adenocarcinoma, our findings may be also relevant for future concepts on chemoprevention of this tumor type. Because epidemiologic data indicated that the regular intake of NSAIDs may reduce the prevalence of gastrointestinal tumor type. Because epidemiologic data indicated that the regular intake of NSAIDs may reduce the prevalence of gastrointestinal carcinomas (4), research on chemoprevention has largely been focused on the role of COX-2 (16). This focus was mainly driven by the assumption that COX-2 but not COX-1 is involved in the propagation (i.e., angiogenesis and lymphangiogenesis). This conclusion is supported by the findings of a recently published chemoprevention trial that showed that the development of esophageal adenocarcinoma in rats could be suppressed more effectively using a nonselective COX inhibitor (Sulindac) than using a selective COX-2 inhibitor (MF tricyclic: refs. 7, 8). For the planning of future chemoprevention trials, it will be of importance to determine if the apparent induction of COX-1 in a subset of esophageal adenocarcinomas already takes place in very early lesions of esophageal carcinogenesis (i.e., in low-grade and/or in high-grade dysplasias). If this is the case, the use of nonselective COX inhibitors in the prevention of esophageal adenocarcinomas will probably have to be considered with greater emphasis.

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