Correlation of Expression of Cyclooxygenase-2, Vascular Endothelial Growth Factor, and Peroxisome Proliferator–Activated Receptor δ With Head and Neck Squamous Cell Carcinoma

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Cyclooxygenase (COX) is the rate-limiting enzyme in the formation of prostaglandins from arachidonic acid. COX exists in 2 isoforms, COX-1 and COX-2. These isoforms are encoded by separate genes and demonstrate cell-specific expression and regulation. Peroxisome proliferator–activated receptor δ (PPARδ) is a nuclear transcription factor that is activated by prostacyclin. Vascular endothelial growth factor (VEGF) is a proangiogenic factor that is up-regulated in various tumors. Vascular endothelial growth factor has been shown to interact with COX-derived prostaglandins in angiogenesis. To better understand the roles of these genes in head and neck squamous cell carcinoma (HNSCCA), we examined the differential expression of the \textit{COX1}, \textit{COX2}, \textit{VEGF}, and \textit{PPARδ} genes in these tumors. Tissue samples from patients with HNSCCA were analyzed for \textit{COX-1}, \textit{COX-2}, \textit{VEGF}, and \textit{PPARδ} messenger RNAs (mRNAs) by in situ hybridization. \textit{COX-1} and \textit{COX-2} mRNAs were also evaluated with Northern blot hybridization. Immunohistochemistry was used to analyze for \textit{COX-2} and \textit{PPARδ} proteins. Results showed focal areas of accumulation for \textit{COX-2}, \textit{VEGF}, and \textit{PPARδ} but not \textit{COX-1} in human HNSCCA. Northern blot hybridization showed higher levels of \textit{COX-2} mRNA in HNSCCA than in normal tissue. This suggests a supportive role of \textit{COX-2} in development and/or progression of HNSCCA. In addition, \textit{PPARδ} may be a receptor for \textit{COX-2}–produced prostaglandins in HNSCCA. There is a potential role for selective \textit{COX-2} inhibitors in the treatment of these lesions.


Prostaglandins (PGs), a family of lipid-derived autocrine and paracrine mediators, can favor tumorigenesis by altering cell proliferation, differentiation, and adhesion and by modulating vascular responses and immune surveillance. Levels of PGs are higher in various tumors, including those of the breast, lung, colon, and head and neck. There is emerging evidence that inhibition of PG formation can protect against these forms of cancer in animals and humans.\textsuperscript{1,10} Prostaglandins are derived from arachidonic acid by cyclooxygenase (COX). COX is the rate-limiting enzyme in the conversion of arachidonic acid to PGH\textsubscript{2}, the substrate for the synthesis of other PGs.

COX exists in 2 isoforms, COX-1 and COX-2. These isoforms are encoded by separate genes and exhibit cell-specific expression and regulation. COX-1 is a constitutive enzyme that produces PGs required for normal physiological functions. COX-2 is an inducible enzyme that is not detected in most tissues under normal conditions. However, it is induced in a variety of tissues by growth factors, oncoproteins, inflammatory stimuli, and tumor promoters.\textsuperscript{11-13} The increased levels of PGs in tumors correlate with increased COX-2 expression.\textsuperscript{12-15} COX-1 and COX-2 are expressed in both the endoplasmic reticular membrane and the nuclear envelope, suggesting that PGs function via 2 different classes of receptors.\textsuperscript{12} Prostaglandins generated in the endoplasmic reticulum can exit the cell and function via G protein–coupled cell sur-
MATERIALS AND METHODS

TISSUE SAMPLES AND PROCESSING

Human samples included 14 patients with primary HNSCCA. Collection of human samples was approved through the University of Kansas Human Subjects Committee. From each subject, samples of tumor and normal buccal mucosa were obtained. Metastatic lymph nodes were present in 7 of 14 patients and were also collected immediately on surgical resection. Tissue was flash frozen in liquid nitrogen and stored at −70°C for in situ and Northern blot hybridization. Additional samples were fixed in Bouin solution for immunohistochemical staining.

NORTHERN BLOT HYBRIDIZATION

Total RNA was isolated from tumor, lymph node, and normal samples by a sodium dodecyl sulfate–phenol-chloroform procedure. For Northern hybridization, antisense complementary RNA (cRNA) probes labeled with phosphate 32 for mouse COX-1, human COX-2, and human β-actin were generated. Total RNA was denatured, separated by formaldehyde–agarose gel electrophoresis, transferred to nylon membranes, and UV cross-linked. Northern blots were prehybridized, hybridized, and washed as previously described. The blots were stripped and reused for each probe to ensure RNA integrity. Quantitation of radioactivity in hybridized bands was achieved by densitometric scanning (Personal Densitometer SI; Molecular Dynamics, Sunnyvale, Calif).

IN SITU HYBRIDIZATION

In situ hybridization was performed as previously described. Sense and antisense cRNA probes labeled with sulfur 35 were generated using appropriate polymerases from mouse-specific complementary DNA (cDNA) to COX-1– and human-specific cDNA to COX-2, VEGF, and PPARδ. Autoradiographic signals were detected using liquid emulsion (Kodak NTB-2; Eastman Kodak Company, Rochester, NY). The slides were poststained with hematoxylin–eosin. Sections hybridized with the sense probes did not exhibit any positive autoradiographic signals and served as negative controls.

IMMUNOHISTOCHEMICAL ANALYSIS

Rabbit antipeptide antibodies were used for immunolocalization of COX-2 and PPARδ. Immunostaining was performed in Bouin-fixed paraffin sections using a staining kit (Zymed-Histostain-SP kit; Zymed, San Francisco, Calif) as previously described.

face receptors that are linked to intracellular signaling pathways. In contrast, PGs produced via nuclear COX can exert their effects directly on the nucleus through peroxisome proliferator–activated receptors (PPARs), which belong to the nuclear hormone receptor superfamily and consist of PPARα, PPARδ, and PPARγ. These isoforms exhibit different expression patterns and ligand dependency. The PPARs modulate transcription by binding to DNA in a heterodimeric complex with retinoid X receptors. Peroxisome proliferator–activated receptor α is highly expressed in hepatocytes and implicated in lipid homeostasis. Peroxisome proliferator–activated receptor γ is mainly expressed in adipose tissue and the immune system. The activation of PPARγ also terminally differentiates tumor cells, suggesting a role in cell cycle regulation. Recent work has shown that COX-2–derived PGI2 is involved in uterine cell proliferation and differentiation during the process of decidualization via activation of PPARδ. This was the first noted function of PPARδ. Since decidualization is considered a pseudomalignant process, PPARδ may play a role in the accelerated cellular proliferation noted after malignant transformation. Recent findings have shown that PPARδ is involved in colorectal cancer.

Increased vascular permeability and angiogenesis, the formation of new blood vessels from existing ones, are the hallmarks of tumorigenesis. Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is a prime regulator of both processes. Vascular endothelial growth factor (40-45 kd) is a heparin-binding homodimeric glycoprotein, and its effects are mediated by tyrosine kinase receptors. The VEGF system has been shown to be up-regulated in various types of tumors, and there is evidence of interactions between this system and COX-2–derived PGs.

The present investigation examined the cell-specific expression of the COX1, COX2, VEGF, and PPARδ genes in head and neck squamous cell carcinoma (HNSCCA) and in metastatic lymph nodes. The results demonstrate that increased expression of COX-2, VEGF, and PPARδ, but not COX-1, is associated with tumor formation.

RESULTS

Tissues from 14 patients with primary HNSCCA were evaluated for the induction of COX-1 and COX-2 mRNA, VEGF, and PPARδ expression.

Figure 1. Northern blot analysis of cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), and β-actin messenger RNAs. Total RNA (6 µg per lane) was hybridized with specific complementary RNA probes. Lanes 1 through 4, head and neck squamous cell carcinoma; lane 5, metastatic lymph node; and lane 6, normal tissue; kb indicates kilobase.
ANALYSIS OF NORTHERN HYBRIDIZATION OF COX-1 AND COX-2 mRNAs

Northern hybridization was performed using total RNA from 4 tumor samples, 1 metastatic lymph node sample, and 1 normal mucosa sample. A 4.7-kb transcript for COX-2 and a 2.8-kb transcript for COX-1 were detected. The levels of COX-1 or COX-2 mRNA were compared with those of β-actin (a housekeeping gene). In 3 of 4 tumor samples, the levels of COX-2 mRNA were approximately 2- to 5-fold higher than that of the normal control. In contrast, only 1 of 4 tumor samples showed COX-1 mRNA expression, and no COX-1 mRNA was detected in the normal buccal tissue or the metastatic lymph node (Figure 1). These results correlated with those of in situ hybridization.

ANALYSIS OF IN SITU HYBRIDIZATION OF COX-1, COX-2, AND VEGF mRNAs IN NORMAL MUCOSA

Whole tissue samples are heterogeneous in cell type. Therefore, when total RNA is derived from these samples and assessed by Northern hybridization, it is not likely to provide specific information about gene expression from any one cell type because of dilution effects. In situ hybridization was performed to examine gene expression in a cell type–specific manner. Figure 2 depicts serial sections from a sample of normal buccal mucosa. A representative section stained with hematoxylin-eosin (A) is presented for morphologic orientation followed by sections showing expression of COX-1 (B), COX-2 (C), and VEGF (D) mRNAs. The expression of COX-1 mRNA was low in the epithelial layer, and no significant autoradiographic signals were observed in the stroma of these samples. In contrast, COX-2 mRNA was low to undetectable in most normal samples. However, when a modest accumulation in the normal tissue was noted, as in Figure 2C, it was in the basal epithelium and subepithelial stroma with a patchy expression pattern. The VEGF mRNA expression was more homogeneous throughout the normal squamous epithelium, albeit at modest levels.

ANALYSIS OF IN SITU HYBRIDIZATION OF COX-1 AND COX-2 mRNAs IN TUMOR TISSUE

Although some normal tissues showed a small amounts of COX-2 mRNA accumulation along the basement membrane, the tumor tissues evaluated produced distinctly elevated COX-2 mRNA expression and in patterns that were different from normal buccal mucosa. Figure 3 depicts low-power (A-B) and high-power (C-D) views from a rep-
resentative tumor sample, showing a heterogeneous expression pattern that was localized within specific foci of the tumor that was invading into the normal tissues. In one tumor sample, expression along the leading edge of the invading tumor was highly distinct (E-F). In well-differentiated squamous cell carcinoma, tumor cells still attempt to differentiate and produce abnormal areas of keratin accumulation termed keratin whorls. These areas also showed distinct accumulation of COX-2 mRNA (G-H). An interesting pattern of COX-2 expression was noted in metastatic lymph nodes. In all of the tissues examined, distinct accumulations were observed in the pericapsular and perivascular regions, contrasted by areas outside the lymph node, which showed no accumulation in perivascular regions (Figure 4A-F). In contrast to the elevated and varying patterns of COX-2 mRNA expression, the levels of COX-1 mRNA were low to undetectable in tumor tissue (Figure 5). These results correlated well with those of Northern blot hybridization. Consecutive sections of the tumor were used for COX-1 and COX-2 mRNA localization (compare Figure 5 with Figure 3A-D).

ANALYSIS OF IN SITU HYBRIDIZATION OF VEGF mRNA IN TUMOR TISSUE

The VEGF mRNA expression was evaluated in 5 tumor specimens. In all of them, the expression was heterogeneous and more intense (Figure 6A-D) than in the control tissues (Figure 2D). In general, the areas of COX-2 expression correlated with those of VEGF (compare Figure 6A-D with Figure 3A-D), suggesting that the same tumor cells could express both COX-2 and VEGF. Collectively, the results suggest that although COX-2 and VEGF are each expressed in normally proliferating cells at modest levels, the levels in areas of tumor growth are much higher with different patterns.

ANALYSIS OF IN SITU HYBRIDIZATION OF PPARδ IN NORMAL, LYMPH NODE, AND TUMOR TISSUES

The PPARδ mRNA expression was evaluated in 5 tumor specimens. The expression in normal tissues was homogeneous throughout the squamous epithelium, albeit at low levels (Figure 7A-B). In contrast, diffuse but increased expression was noted within the metastatic lymph nodes (C-D). In all 5 tumor tissues examined, PPARδ mRNA expression was heterogeneous and more intense (E-F) than that in the controls. Serial sections of tumor tissues were evaluated for COX-2 and PPARδ mRNA expression. Although the expression of COX-2 was heterogeneous and within specific localized foci, the expression of PPARδ was more diffuse throughout the tissue (data not shown). These results suggest that similar tumor cells can express both COX-2 and PPARδ.

ANALYSIS OF IMMUNOHISTOCHEMICAL STAINING OF COX-2 AND PPARδ

The cell-specific accumulation of COX-2 and PPARδ proteins was examined by immunohistochemical staining.
No noticeable accumulation of either COX-2 or PPARβ was evident in normal tissues (Figure 8). In contrast, distinct accumulation of COX-2 and PPARβ proteins was observed in tumor tissues. The accumulation of COX-2, within malignant cells, was cytoplasmic and perinuclear. The distinct nuclear immunolocalization of PPARβ was more widespread within the tumor compared with COX-2. The accumulation of both COX-2 and PPARβ proteins followed the same patterns as those observed for their mRNA localizations.

**COMMENT**

The COX-2–derived PGs are involved in a variety of physiologic and pathologic processes. The role of COX-2 in various forms of cancer is emerging as a major research focus. However, the mechanism by which the COX-2–derived PGs participate in tumor promotion is not clearly understood.

The highlight of the present investigation is that COX-2, but not COX-1, expression is elevated in HNSCCA cells. Although the increased expression of COX-2 in these cells has been reported, its site of synthesis has not been documented. Our results establish that the COX-2 expression pattern varies, depending on the nature of the tumor, suggesting that it may have multiple roles in tumor maintenance and progression. For example, COX-2 mRNA expression in the invasive border suggests the involvement of PGs in the aggressive behavior of the tumor, whereas that within the vascular cores implicates their role in vascular permeability and angiogenesis. In addition, well-differentiated tumors demonstrated the presence of COX-2 in keratin whorls, suggesting a role in differentiation. The pericapsular and perivascular expression of COX-2 in metastatic lymph nodes implies a role in immune response regulation. This is consistent with known roles of PGs in immunologic responses. Vascular permeability and angiogenesis are 2 major characteristics of tumor formation. Vascular endothelial growth factor is a potent mediator of these processes and is up-regulated in various types of tumors, including HNSCCA. Prostaglandins are also well-known proangiogenic factors that are implicated in vascular permeability changes and angiogenesis. For example, prostacyclin agonists can induce VEGF in monocyte cell lines and rat lungs. Alternatively, VEGF can stimulate prostacyclin production, which in turn can enhance vascular permeability. These observations suggest a close relationship between the VEGF and PGs in mediating vascular changes. Thus, our findings of COX2 and VEGF coexpression in HNSCCA cells suggest a relationship between these 2 genes in angiogenesis and tumorigenesis.
Recent work suggests that prostacyclin is the major COX-2-derived PG in many tissues. We have also shown that this PG is the major product in the uterus during implantation and activates PPARγ in this process. The implantation process involves massive stromal cell proliferation and polyploidy at the site of the embryo, a process known as deciduation. These cellular changes are considered a transient “pseudomalignant” state. This led us to speculate that COX-2-derived PGs could be involved in tumorigenesis by activation of PPARγ. Our results demonstrate the increased region-specific expression of COX-2 and PPARγ within tumor tissues. This would implicate a tumorigenic pathway. Indeed, there is developing evidence that COX-2–PPARγ signaling is associated with colorectal cancer. Another important aspect of the present study is the interaction between native normal tissue and the invading tumor cells, which could not be evaluated under in vitro conditions. This is an important focus, since host COX-2 recently has been shown to influence the growth of transplanted tumor in the murine model.

The results of the present investigation point toward a relationship between PGs, PPARγ, and VEGF in tumorigenesis. Further investigation of these molecular interactions will enhance our understanding of HNSCCA management and prevention. Despite recent advances in the therapy for head and neck cancer, there has been little increase in patient survival. Chemotherapeutic use of nonsteroidal anti-inflammatory drugs, as nonspecific inhibitors of COX-1 and COX-2, has shown beneficial effects in the prevention of certain forms of cancer and may prove useful in the treatment of HNSCCA. However, the broad-scale use of these drugs for cancer prevention is undesirable because the incidence of adverse effects, including peptic ulcer disease and impaired renal function, is significant. Therefore, the use of recently developed selective COX-2 inhibitors may prove advantageous in limiting the adverse effects of the drugs while maximizing the benefits.

**CONCLUSIONS**

This study has provided evidence of the potential role of COX-2 in HNSCCA. COX-2 expression was found in discrete patterns both in primary tumors and metastatic sites. The increase in COX-2 within HNSCCA suggests an autocrine and paracrine signaling mechanism that participates in cellular differentiation, angiogenesis, and/or modulation of immunologic responses. This proposal is consistent with the concomitant elevated expression of VEGF and PPARγ within similar sites of HNSCCA. The definitive involvement of COX-2 and its mode of action in HNSCCA will require further investigation.

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