Cyclooxygenase-2 and Microsomal Prostaglandin E Synthase-1 Are Overexpressed in Squamous Cell Carcinoma of the Penis

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ABSTRACT

Purpose: Prostaglandin E$_2$ (PGE$_2$) promotes malignant growth. Cyclooxygenase (COX) catalyzes the synthesis of PGH$_2$, which is converted, in turn, by microsomal prostaglandin E synthase (mPGES-1) to PGE$_2$. One strategy for inhibiting carcinogenesis is to prevent PGE$_2$ production in premalignant and malignant tissues. It is important, therefore, to determine whether enzymes involved in PGE$_2$ biosynthesis are deregulated in neoplasia. The main purpose of this study was to determine whether amounts of COX-2 or mPGES-1 were increased in intraepithelial neoplasia or squamous cell carcinoma (SCC) arising in an HPV16 transgenic mouse.

Experimental Design: Immunohistochemistry and immunoblotting were utilized to evaluate the expression of COX-2 and mPGES-1 in benign and malignant lesions including metastases to lymph nodes. Amounts of intratumoral PGE$_2$ were quantified by enzyme immunoassay. Reverse transcription-PCR was used to determine the expression of each of the four known receptors (EP$_{1-4}$) for PGE$_2$.

Results: Immunohistochemistry demonstrated increased expression of COX-2 and mPGES-1 in dysplasia, carcinoma in situ, invasive SCC, and metastases to lymph nodes. Immunoblot analysis confirmed that COX-2 and mPGES-1 were consistently overexpressed in SCC. PGE$_2$ and all four of the PGE$_2$ receptor subtypes were detected in each of the tumor samples.

Elevated levels of COX-2 were also detected in SCC arising in an HPV16 transgenic mouse.

Conclusions: Increased amounts of COX-2 and mPGES-1 were detected in penile intraepithelial neoplasia and carcinoma. These findings provide the basis for evaluating whether inhibiting COX-2 will be useful in the prevention or treatment of penile SCC.

INTRODUCTION

Squamous cell carcinoma (SCC) of the penis accounts for ~95% of all cases of penile cancer worldwide (1). In some developing countries in South America, Asia, and Africa, where genital hygiene is poor, penile cancer accounts for 10–20% of all male malignancies (1, 2). In contrast, penile cancer accounts for <0.5% of all male malignancies in the United States.

Although invasive penile carcinomas can arise de novo, premalignant lesions have been identified. Examples of penile intraepithelial neoplasia (PIN) include Bowen’s disease and erythroplasia of Queyrat (3, 4). The evolution of low-grade PIN to invasive cancer may take as long as 15–20 years (5). Several risk factors for penile carcinoma have been identified. Poor hygiene, chronic inflammation, and human papillomavirus (HPV) infection have been linked to the development of penile SCC (6, 7). Similar to cervical cancer, high-risk HPV types are detected in the majority of cases of PIN and 40–50% of cases of penile SCC (6). Oncogenic HPV16 is the most frequently identified type (6). A variety of ablative treatments including Mohs surgery and laser ablation are used to treat both PIN and superficial penile cancers (8). Because the majority of cases occur in underdeveloped countries, the identification of relatively inexpensive and safe treatments could have significant practical implications.

One recognized strategy for inhibiting carcinogenesis is to suppress prostaglandin (PG) production in premalignant and malignant tissues. PGE$_2$ has been shown to stimulate cell proliferation (9), induce angiogenesis (10, 11), inhibit apoptosis (12), and suppress immune surveillance (13, 14). Inhibitors of PGE$_2$ synthesis including nonsteroidal anti-inflammatory drugs and selective cyclooxygenase (COX)-2 inhibitors protect against both tumor formation and growth (15, 16). It is important, therefore, to identify enzymatic pathways that are deregulated in neoplastic tissues that result in enhanced PGE$_2$ production.

The synthesis of PGE$_2$ from arachidonic acid requires two enzymes that act in sequence. COX catalyzes the conversion of arachidonic acid to PGH$_2$. There are two isoforms of COX designated COX-1 and COX-2, respectively (17). In general, COX-1 is constitutively expressed (17). In contrast, COX-2 is ordinarily not expressed in normal epithelium but is induced by cytokines, growth factors, oncogenes, and tumor promoters (17–21). Elevated levels of COX-2 have been detected in a variety of premalignant and malignant tissues (22–28). An inducible hu-
Human microsomal prostaglandin E synthase (mPGES-1) was identified and characterized recently (29). This enzyme converts COX-derived PGH \(_2\) to PGE \(_2\). Evidence is accumulating that mPGES-1 in addition to COX-2 contributes to carcinogenesis (30–36). PGE \(_2\) elicits cellular responses via interaction with four cell surface receptors, EP \(_{1-4}\). Several studies suggest that the role of specific EP receptors varies in different tumor types (37–39).

In this study, we investigated whether COX-2 and mPGES-1 were overexpressed in penile neoplasia. Notably, the expression of both enzymes was increased in PIN, SCC, and metastatic disease. PGE \(_2\) and all four of the PGE \(_2\) receptor subtypes (EP \(_{1-4}\)) were detected in each of the carcinomas that were evaluated. Given the link between HPV and penile carcinogenesis, we also evaluated the expression of COX-2 in SCC arising in the skin of an HPV16 transgenic mouse. Overexpression of COX-2 was detected in this preclinical model. Taken together, these findings provide the basis for evaluating whether nonsteroidal anti-inflammatory drugs or selective COX-2 inhibitors will be useful in preventing or treating penile SCC.

**MATERIALS AND METHODS**

**Materials.** Rabbit polyclonal anti-COX-2 antiserum (PG-27) and anti-β-actin antiserum were obtained from Oxford Biomedical Research (Oxford, MI). Rabbit polyclonal anti-human mPGES-1 antiserum was obtained from Cayman Chemical (Ann Arbor, MI). Taq polymerase and TRizol reagent were obtained from Invitrogen (Carlsbad, CA). Lowry protein assay kits, biotinylated antirabbit IgG antibody, and 3,3'-diaminobenzidine were purchased from Sigma Chemical Co. (St. Louis, MO). Streptavidin-horseradish peroxidase was purchased from DAKO Corp. (Carpinteria, CA). Enhanced chemiluminescence solution was from Perkin-Elmer Life Sciences (Boston, MA). PCR primers were synthesized by Sigma Genosys (The Woodlands, TX). PCR buffer II, MuLV Reverse Transcriptase, RNase inhibitor, deoxyxynucleoside triphosphate, and SYBR Green master mix were purchased from Applied Biosystems (Foster City, CA). RNeasy Mini-kits and RNease-Free DNase kits were obtained from Qiagen Inc. (Valencia, CA).

**Patient Samples.** Specimens used for immunohistochemistry were obtained from patients who had penile skin biopsies, excision, or penectomy at New York Presbyterian Hospital and Memorial Sloan-Kettering Cancer Center. These samples came from patients with SCC (\(n = 6\)) of the penis or normal penile skin (\(n = 1\)). Additional cases of normal skin (\(n = 3\)) were obtained from adults who underwent circumcision for non-neoplastic conditions.

Additional specimens for analysis by Western blot, reverse transcription-PCR, and enzyme immunoassay (PGE \(_2\) determination) were obtained from the tissue bank at Memorial Sloan-Kettering Cancer Center. These samples came from patients with SCC (\(n = 6\)) of the penis or normal penile skin (\(n = 1\)). Samples were frozen and stored at \(-80°C\) until analysis. The presence of SCC or normal penile skin was confirmed by routine histopathology before experimental analysis. The above studies were conducted in accordance with an Institutional Review Board-approved protocol.

**Immunohistochemistry.** Neutral buffered formalin-fixed tissue was embedded in paraffin. Tissue sections (4 μm) were prepared using a microtome and mounted on Superfrost/Plus slides. Sections were deparaffinized in xylene, rehydrated in graded alcohols, and washed in distilled water. Antigen retrieval was performed by steaming the sections in 10 mM citric acid (pH 6.0) for 30 min. Subsequently, endogenous peroxidase activity was blocked with 3% hydrogen peroxide. The slides were washed in PBS and then blocked for 20 min with 2% BSA. Slides were then incubated with antisera to COX-2 or mPGES-1 at a 1:1000 dilution (2% BSA in PBS) for 18 h at 4°C. Control sections were incubated with preimmune serum, or with COX-2 or mPGES-1 antisera preabsorbed with a 100-fold excess of blocking peptide. After being washed three times with PBS, the sections were incubated with biotinylated antirabbit antiserum (1:500 dilution) for 1 h at room temperature. The slides were again washed three times in PBS, then labeled using streptavidin-horseradish peroxidase (1:500 dilution) for 1 h at room temperature. The reaction was visualized using 3,3'-diaminobenzidine. Subsequently, the slides were rinsed in tap water and counterstained with hematoxylin. Finally, the slides were dehydrated with ethanol, rinsed with xylene, and coverslipped. Staining for both COX-2 and mPGES-1 was carried out in a single session to minimize variability.

Each immunostained slide was compared with a serial section that was H&E stained to ensure the presence of the lesion of interest. A semiquantitative method was then used to score the immunoreactivity for each case. The three most representative areas of each immunostained slide were evaluated. An estimate of the percentage of immunoreactive cells was determined using a scale of 0–4 (0, no staining; 1, 1–10% cells stained; 2, 11–50% cells stained; 3, 51–80% cells stained; 4, 81–100% cells stained). Staining intensity was rated 0–3 (0, negative; 1, weak; 2, moderate; 3, strong). Mean values for each of the two parameters (percentage immunoreactive cells and intensity) were then calculated from the three areas of each slide that were scored. Values for the quantity and staining intensity scores were then multiplied giving results that ranged from 0 to 12. The results are reported according to the following scoring criteria: negative, 0; 1–4, 1+; 5–8, 2+; and 9–12, 3+.

**Western Blotting.** Human tissue containing histologically confirmed SCC or normal penile skin was thawed in

| Table 1 | Expression of cyclooxygenase (COX)-2 and mPGES-1 in penile intraepithelial neoplasia and squamous cell carcinoma (SCC) |
|-----------------|-----------------|-----------------|-----------------|
| **Histology**   | **Number of cases** | **COX-2** | **mPGES-1** |
| Normal skin     | 3               | Negative      | Negative       |
| Dysplasia       | 1               | 2+            | 2+             |
| CIS             | 7               | 1+(4/7)       | 2+(4/7)        |
| Invasive SCC    | 6               | 2+(4/6)       | 2+(4/6)        |
| Lymph node metastasis | 2       | 2+(1/2)       | 2+(1/2)        |

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ice-cold lysis buffer [150 mM NaCl, 100 mM Tris (pH 8.0), 1% Tween 20, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml trypsin, and 10 μg/ml leupeptin]. Tissues were sonicated for 3 min on ice and then centrifuged at 10,000 × g for 10 min at 4°C to remove particulate matter. Subsequent analysis of COX-2 and mPGES-1 was carried out using methods described in previous studies (32, 33). COX-2 protein in murine tissue was assayed using a coupled immunoprecipitation/immunoblotting assay, as described previously (40).

**Analysis of EP Receptor Expression.** Expression of EP receptors in SCC was analyzed by reverse transcription-PCR. RNA was prepared from frozen tissue using the RNase-Free DNase (Qiagen), and cDNA was generated as described previously (36). Primer pairs used were: EP1 Forward 5′-TGGGCCAGCTTGTCGGTAT-3′, Reverse 5′-AGC-GCAGCATTTGTCTCAGA-3′; EP2 Forward 5′-TCCGTGTGTCTTGTCAGT-3′; EP3 Forward 5′-CAGCTTATGGA-TCATGTG-3′; Reverse 5′-TCCGTGTGTCTTGTCAGT-3′; EP4 Forward 5′-CAGATTTGCAAGGCCATCC-3′, Reverse 5′-GAGCAACTGTCTTTCTCAGGA-3′; and β-actin Forward 5′-GGTGACCCACACTGTGAGG-3′, Reverse 5′-GGATGCTCAGAGGATGTG-3′. PCR was performed using 2 μl cDNA and 200 nM upstream and downstream primer per 20 μl reaction. Thermal cycling conditions were as follows: 95°C for 10 min; 30 s at 94°C, 30 s at 62°C, and 30 s at 72°C for 40 cycles (iCycler thermal cycler; Bio-Rad Laboratories, Inc., Hercules, CA). The identity of each PCR product was confirmed by DNA sequencing.

**Animal Model.** The K14-HPV16 transgenic mouse has been described previously (41, 42). In this model, the early gene

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**Fig. 1** Cyclooxygenase (COX)-2 is overexpressed in intraepithelial neoplasia and squamous cell carcinoma (SCC) of the penis. Immunohistochemistry was used to evaluate COX-2 expression in normal squamous epithelium, penile precancerous lesions, SCC of the penis, and carcinoma metastatic to a lymph node. COX-2 was not detected in normal squamous epithelium (A, ×200). In contrast, COX-2 immunoreactivity was detected in epithelial cells in dysplasia (B, ×200), Bowen’s disease (C, ×400), carcinoma in situ (D, ×200), invasive SCC (E, ×400), and SCC metastatic to a lymph node (F, ×400). Immunoreactivity was lost when the antiserum to COX-2 was preincubated with a COX-2 blocking peptide (G, ×200).
region of HPV16, including the \textit{E6} and \textit{E7} oncogenes, are expressed under the control of the human keratin 14 promoter/enhancer. In K14-HPV16 transgenic mice, the oncogenes of HPV16 are expressed in the basal squamous epithelial cells that are the natural target of clinical HPV infection. SCC of the skin develop in this model (42).

**RESULTS**

Immunohistochemistry was used to assess the expression of COX-2 and mPGES-1 in normal penile skin, PIN, and SCC of the penis. Neither COX-2 nor mPGES-1 were detected in three samples of normal penile skin or in normal skin adjacent to penile neoplasia (Table 1; Figs. 1 and 2). In contrast, granular cytoplasmic staining for COX-2 and mPGES-1 was detected in neoplastic epithelial cells in all of the samples of squamous dysplasia (n = 1), carcinoma \textit{in situ} (n = 7), invasive SCC (n = 6), and SCC metastatic to lymph nodes (n = 2; Table 1; Figs. 1 and 2). Immunoreactivity for the two enzymes did not always localize to the same cells and was not particularly increased in koilocytes. The magnitude of overexpression of COX-2 and mPGES-1 also varied from case to case. Interestingly, in invasive SCC, the strongest staining signal for both COX-2 and mPGES-1 was observed in the invading fronts of most cases. We did not observe significant immunoreactivity for COX-2 in the stroma. In contrast, a weak signal for mPGES-1 was observed in the stroma of some cases. The staining for COX-2 and mPGES-1 was specific because immunoreactivity was lost when antisera to COX-2 or mPGES-1 were preincubated with COX-2 or mPGES-1 blocking peptides (Figs. 1 and 2). To corroborate the immunohistochemical findings, Western blotting was carried out. Consistent with the immunohistochemical results, COX-2 and mPGES-1 were detected in 6 of 6 cases of

\begin{figure}[h]
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\caption{mPGES-1 is overexpressed in intraepithelial neoplasia and squamous cell carcinoma (SCC) of the penis. Immunohistochemistry was used to evaluate the expression of mPGES-1 in normal squamous epithelium, precancerous lesions, SCC of the penis, and carcinoma metastatic to a lymph node. mPGES-1 was not detected in normal squamous epithelium (A, \times 200). In contrast, mPGES-1 immunoreactivity was detected in epithelial cells in dysplasia (B, \times 400), Bowen’s disease (C, \times 200), carcinoma \textit{in situ} (D, \times 200), invasive SCC (E, \times 200), and carcinoma metastatic to a lymph node (F, \times 200). Immunoreactivity was lost when antisera to mPGES-1 were preincubated with an mPGES-1 blocking peptide (G, \times 200).}
\end{figure}
SCC of the penis, but not in normal penile skin (Fig. 3). Once again, differences in the extent of up-regulation of COX-2 and mPGES-1 were observed. In case 1, for example, the level of COX-2 was markedly elevated compared with the other cases of penile SCC. In contrast, the level of mPGES-1 was only modestly increased in this case.

COX-2 and mPGES-1 act in sequence to synthesize PGE₂. PGE₂ was detected in the 6 carcinoma samples that were evaluated (21–192 pg/μg protein). Notably, the highest level of PGE₂ was present in case 1, which also had the greatest amount of COX-2 (Fig. 3). As mentioned above, PGE₂ elicits cellular responses via interaction with four cell-surface receptors, EP₁–⁴. Reverse transcription-PCR was carried out to determine which receptors were expressed in penile carcinoma. As shown in Fig. 4, the four known EP receptors were detected in each of the 5 cases of penile SCC analyzed.

Penile carcinoma has been associated with HPV16 infection. Hence, we were also interested in analyzing COX-2 expression in a SCC arising in the skin of a K14-HPV16 transgenic mouse. As shown in Fig. 5, COX-2 was expressed in a SCC that developed in a K14-HPV16 transgenic mouse. Similar to the human, COX-2 was not detected in normal mouse skin. Levels of mPGES-1 were not evaluated because our antibody detects murine mPGES-1 relatively poorly.

**DISCUSSION**

In this study, we showed that both COX-2 and mPGES-1 are commonly overexpressed in PIN and penile SCC. This finding is consistent with several other recent reports in which elevated levels of both enzymes were detected in human malignancies (28, 32–36). For example, overexpression of COX-2 and mPGES-1 has been observed in cancers of the colon, lung, uterus, stomach, and head and neck (32–36). The fact that these two enzymes are commonly up-regulated in tumors helps to explain the longstanding observation that amounts of PGE₂ are increased in multiple epithelial malignancies (43–45).

Although both COX-2 and mPGES-1 are commonly up-regulated in penile SCC, the extent of overexpression varied in individual tumors. Furthermore, the expression of the two enzymes did not always localize to the same cells. This suggests that the mechanisms controlling the expression of these two enzymes differ. In support of this point, COX-2 is regulated by both transcriptional and post-transcriptional mechanisms (17, 28, 46, 47), whereas transcriptional control appears to be the primary mechanism regulating the expression of mPGES-1 (48, 49). Moreover, different transcription factors are important for regulating the expression of these two genes. Activator protein-1, nuclear factor κB, NF-IL6, and PEA3 transcription factors control COX-2 gene expression (17, 28), whereas Egr-1 is critical for regulating the transcription of mPGES-1 (48). Additional studies will be needed to identify the transcription factors that are activated in PIN and SCC of the penis.

As mentioned above, COX-2 and mPGES-1 act sequentially to synthesize PGE₂. It is not surprising, therefore, that PGE₂ was detected in each of the SCCs that was analyzed. We show for the first time that all four of the EP receptors are expressed in penile SCC. Different PGE₂ receptors appear to be important in carcinogenesis in different models and tumor types (37–39). The relative role of individual EP receptors and the cellular localization in penile SCC remains to be defined.

On the basis of the findings in this study, it is important to consider existing evidence suggesting that COX-2 is a rational target for the prevention and treatment of cancer. The most specific data that support a cause-and-effect relationship between COX-2 and tumorigenesis come from genetic studies.
Multiparous female transgenic mice that were engineered to overexpress human COX-2 in mammary glands developed mammary gland hyperplasia, dysplasia, and metastatic tumors (50). These observations are consistent with the notion that, under certain conditions, increased expression of COX-2 induces tumor formation. In a related study, transgenic mice that overexpressed COX-2 in skin developed epidermal hyperplasia and dysplasia (51). Consistent with these findings, knocking out COX-2 caused an ~75% reduction in skin papillomas and intestinal tumors (52, 53). In addition to genetic evidence, numerous pharmacological studies indicate that COX-2 is a therapeutic target. Treatment with selective COX-2 inhibitors suppressed the formation and growth of numerous tumor types in experimental animals (28, 54–59) and caused a reduction in colorectal polyp burden in familial adenomatous polyposis patients (60). In this study, we found that COX-2 was overexpressed in SCC arising in an HPV16 transgenic mouse. Although SCC develops in the skin rather than the penis in this model, the pathogenesis of HPV-induced penile SCC could be similar. This finding provides the basis for future studies that will determine whether a selective COX-2 inhibitor can inhibit the formation or growth of an HPV-related SCC.

PGE\(_2\) also plays a role in cell invasiveness and metastasis (9, 61, 62). In most invasive carcinomas, we observed the strongest signals for COX-2 and mPGES-1 in the invading front of the tumor. Similarly, the highest levels of COX-2 and PGE\(_2\) have been observed at the invasive edge of other tumor types (63, 64). Possibly, selective COX-2 inhibitors will have a role in suppressing the development of metastases in patients with penile cancer as has been suggested for other tumor types (28).

The results of initial studies also suggest that mPGES-1 may represent a therapeutic target. More specifically, cells overexpressing mPGES-1 and COX-2 produced more PGE\(_2\), grew faster, and exhibited abnormal morphology compared with cells in which either COX-2 or mPGES-1 were overexpressed (30). Kamei et al. (31) reported recently that cotransfection of COX-2 and mPGES-1 into HEK293 cells resulted in cellular transformation manifested by colony formation in soft agar culture and tumor formation when injected s.c. into nude mice. cDNA array analyses revealed that mPGES-1-directed cellular transformation was accompanied by changes in the expression of a variety of genes related to morphology, proliferation, cell cycle, and adhesion (31). These results demonstrate that aberrant expression of mPGES-1 in combination with COX-2 can be associated with cellular transformation and tumor formation. Selective inhibitors of mPGES-1 are being developed. Once these compounds become available, an approach comparable with the one with cellular transformation and tumor formation. Selective inhibition of mPGES-1 in combination with COX-2 can be associated with changes in the expression of a variety of genes related to morphology, proliferation, cell cycle, and adhesion (31). These results demonstrate that aberrant expression of mPGES-1 in combination with COX-2 can be associated with cellular transformation and tumor formation. Selective inhibitors of mPGES-1 are being developed. Once these compounds become available, an approach comparable with the one used to investigate the anticancer properties of selective COX-2 inhibitors can be used to determine whether mPGES-1 is a \textit{bona fide} therapeutic target.

Finally, it is important to consider the clinical implications of the current study. On the basis of our findings, it will be reasonable to investigate whether targeting COX-2 can impact on either the natural history of PIN or established SCC of the penis. In support of this idea, a recent clinical study showed that celecoxib, a selective COX-2 inhibitor, reduced markers of proliferation and neoangiogenesis in human cervical cancer, another HPV-related tumor (65). Selective COX-2 inhibitors are more costly than aspirin or traditional nonsteroidal anti-inflammatory drugs. Because the majority of cases of penile cancer occur in underdeveloped countries in which cost is a major concern, it is likely to be more practical to evaluate the efficacy of aspirin or traditional nonsteroidal anti-inflammatory drugs.

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