Cyclooxygenase-1 Is Overexpressed in Multiple Genetically Engineered Mouse Models of Epithelial Ovarian Cancer

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Abstract

Cyclooxygenases-1 and -2 (Cox-1 and Cox-2) are two distinct isoforms that catalyze the conversion of arachidonic acid to prostaglandins. The role of Cox-2 in a variety of cancers is well recognized, but the contribution of Cox-1 remains much less explored. We have previously shown that human epithelial ovarian tumors have increased levels of Cox-1, but not Cox-2. We also observed that Cox-1 is highly expressed in a mouse model of epithelial ovarian cancer (EOC), which lacks p53 but overexpresses c-myc and K-ras or c-myc and Akt. More importantly, a Cox-1-selective inhibitor, SC-560, attenuates EOC growth. In the present investigation, we used various genetically engineered mouse models of EOC to determine whether Cox-1 overexpression is unique to specific genetic and oncogenic alterations or is widespread. These models include: (a) deletion of both p53 and Rb, (b) induction of the transforming region of SV40 under the control of Mullerian inhibitory substance type II receptor, or (c) activation of K-Ras in the absence of Pten in the ovarian surface epithelium. We found that these three models, which produce spontaneous EOC, also show up-regulated expression of Cox-1, but not Cox-2. The results provide further evidence that Cox-1 overexpression is common in various models of EOC. Thus, Cox-1 serves as a potential marker of EOC and is a possible target for the prevention and/or treatment of this deadly disease. (Cancer Res 2006; 66(5): 2527-31)

Introduction

The genetic and molecular mechanisms underlying ovarian cancer remain largely unknown, and treatment options for patients with advanced disease are limited. Epithelial ovarian cancers (EOC) originating from ovarian surface epithelial (OSE) cells comprise 90% of ovarian cancers (1, 2). EOCs are morphologically and biologically heterogeneous, causing difficulty in defining molecular events underlying the disease development and progression (1). Research primarily focusing on colorectal cancer has provided strong evidence that nonsteroidal anti-inflammatory steroids (NSAID) are effective in both cancer prevention and treatment of established tumors (3). NSAIDs interfere with prostaglandin biosynthesis by inhibiting cyclooxygenases-1 and -2 (Cox-1 and Cox-2), the enzymes that catalyze the rate-limiting step in prostaglandin biosynthesis from arachidonic acid. These drugs encumber colorectal tumor growth primarily by attenuating Cox-2 activity, although there is a possibility that other non-Cox-2 targets are also affected. In addition, Cox-2 is overexpressed in a variety of extracolonic cancers, and selective Cox-2 inhibitors show potent antineoplastic effects in vivo in preclinical models of various solid malignancies (2). However, epidemiologic studies examining whether NSAIDs prevent or delay development of ovarian cancers remain inconclusive (reviewed in refs. 3–5). The question is further exacerbated by many published reports of Cox-2 expression in ovarian cancer rather than Cox-1 (reviewed in refs. 4, 5). Many of these studies used immunologic techniques to differentiate between Cox-2 and Cox-1, or did not examine Cox-1 expression. The nonspecificity of many commercially available Cox-2 antibodies provoked us to use multiple approaches to examine Cox isoform expression in EOC. We previously showed that Cox-1, but not Cox-2, is highly expressed in human EOC (4), and in a mouse EOC model lacking p53 but overexpressing c-myc and K-ras or c-myc and Akt (5, 6). Other groups have also recently questioned the importance of Cox-2 in ovarian tumorigenesis (7, 8). In recent years, several independent laboratories have developed mouse models of EOC employing strategies for genetic or functional inactivation of tumor suppressor genes and/or activation of oncogenes specifically in the epithelial compartment of the ovary (9–11). In this study, we used these various mouse models to examine the expression of Cox isoforms to determine whether Cox-1 overexpression is common to EOC arising from various manipulations of tumor suppressor genes and/or oncogenes.

Materials and Methods

Genetically engineered mouse models of EOCs. EOCs used in the present investigation were induced either by (a) inactivation of p53 and Rb, (b) induction of activated K-ras in the absence of Pten, or (c) induction of the transforming region of SV40 T antigen (Tag) under transcriptional control of a portion of the murine Mullerian inhibiting substance type II receptor (MISHIR) gene promoter locally in the OSE as previously described (9–11). These are existing mouse models that are maintained independently in the laboratories of the investigators who generated them (9–11). Our previous experiments using AdCre and Rosa26SOPfpfoxP1A mice showed that recombination occurs in 60% to 80% of cells (10). Using microdissection-PCR assay, it was also shown that all neoplastic cells lack wild-type alleles of p53 and Rb from the earliest morphologically detectable lesions (10). Tumors were graded based on the established histopathologic criteria. The diagnosis of well and poorly differentiated neoplasms reflects the degree of structural and cytologic atypia as well as their proliferative activity as described in previous publications (9–12).

RT-PCR. Total RNA was isolated with Trizol according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). After DNase

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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treatment (Ambion, Austin, TX), 1 μg of total RNA was reverse transcribed with Superscript II (Invitrogen). PCR was done as previously described (13, 14). The primers for Cox-1 were 5'-AGGAGAGTTGGCCT-GAGTTGG-3' (sense) and 5'-AATCTGACTTCTGAGTTGCC-3' (antisense) and those for Cox-2 were 5'-ACACACTCATCAGGCAC-3' (sense) and 5'-TTCAGGAAAGGTTCGTTGC-3' (antisense).

Hybridization probes. cDNA clones for Cox-1 and Cox-2 have been previously described (15). In brief, a cDNA fragment encoding Asp178-Glu226 was used to generate the Cox-1 probe, whereas a cDNA fragment encoding Met246-Glu292, including 55 nucleotides at the 5'-untranslated region was used to generate the Cox-2 probe. For in situ hybridization, sense and antisense 35S-labeled cRNA probes were generated using Sp6 and T7 polymerases, respectively.

In situ hybridization. In situ hybridization was done as previously described (14). Sections hybridized with sense probes did not exhibit any positive signals and served as negative controls. Furthermore, day 1 and day 4 pregnant mouse uterine sections were used as positive controls for Cox-1 and Cox-1, respectively (ref. 16; Supplemental Fig. S1).

Western blot analysis. Tissue samples were prepared as previously described (5). After measuring protein concentrations, extracts (20 μg protein) were boiled for 5 minutes in SDS sample buffer. The samples were run on 10% SDS-PAGE gels under reducing conditions and transferred onto nitrocellulose membranes. The membranes were blocked with 10% milk in TBST, and probed with Cox-1 (1:1,000, kindly provided by David DeWitt, East Lansing, MI) or Cox-2 (1:30,000, Cayman, Ann Arbor, MI) for 16 hours at 4°C (5, 17). After washing, blots were incubated in peroxidase-conjugated donkey anti-goat IgG or donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Protein signals were detected using chemiluminescent reagents (Amersham, Piscataway, NJ). The antibodies to Cox-1 and Cox-2 are highly specific as determined by Western blot analysis.

Immunohistochemistry. Immunohistochemistry was done as previously described (5). In brief, formalin-fixed paraffin-embedded tumor sections (6 μm) were subjected to immunostaining using Cox-1 or Cox-2 antibodies, as described above. After deparaffinization and hydration, sections were subjected to antigen retrieval by autoclaving in 10 mmol/L sodium citrate solution (pH 6.0) for 15 minutes. A Histostain-Plus kit (Zymed, San Francisco, CA) was used to visualize the antigen; reddish-brown deposits indicate sites of positive immunostaining.

Primary culture of OSE cells and gene array. Primary culture of OSE cells was done as previously described (10). In brief, individual ovaries were dissected and digested in DMEM/F12 (Ham's) medium containing collagenase-dispase at 5% CO2 for 1 hour. After brief washing, the collected OSE cells were plated in 12-well plates, covered with 0.1% gelatin, maintained in Ham's medium supplemented with 5% fetal bovine serum, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 10 ng/mL epidermal growth factor, 500 ng/mL hydrocortisone, 5 μg/mL insulin, 5 μg/mL transferrin, and 5 mg/mL sodium selenite. The cells were passaged and exposed to 200 multiplicity of infection of either AdCMVlacZ or AdCMVCre for 2 hours to establish OSE control or knockout cells, respectively. Treated cells at the indicated passages after infection were used for microarray studies using Affymetrix mouse GeneChip U74Av2.

Results and Discussion
Cox-1 is expressed in ovarian cancer arising from surface epithelium lacking p53 and Rb. We evaluated differential expression of Cox isoforms in mouse EOC generated by deleting p53 and Rb in OSE cells of mice carrying p53floxP/floxP/RbfloxP/floxP genes by local delivery of adenovirus-mediated Cre (AdCMVCre). These mice primarily develop well-differentiated (39%) and poorly differentiated (45%) serous epithelial neoplasms, although some develop mixed types (10). We analyzed Cox-1 and Cox-2 expression in five independent EOC samples. Analysis of RT-PCR results shows that Cox-1 mRNA levels are high in well-differentiated serous epithelial neoplasms, whereas levels of Cox-2 mRNA are low to undetectable. However, both Cox-1 and Cox-2 mRNAs are detected in poorly differentiated neoplasms (Fig. 1A). This pattern is also reflected at the protein level as determined by Western blotting (Fig. 1B). We next examined cell-specific expression of Cox-1 and Cox-2 in tumor sections by in situ hybridization (Fig. 1C). The expression of Cox-1 is high in all tumor samples, whereas Cox-2 expression is low to undetectable, except for some focal expression in poorly differentiated neoplasms. Immunohistochemistry also detected high levels of Cox-1 protein with low levels of Cox-2 in poorly differentiated neoplasms (Fig. 1D). Collectively, these results provide evidence that Cox-1 and not Cox-2 is primarily expressed in EOC originating from OSE missing p53 and Rb.

A microarray study comparing global gene expression between p53floxP/floxP/RbfloxP/floxP OSE cells (controls) or cells lacking both p53 and Rb genes after local delivery of AdCMVCre found that Cox-1 expression is >50-fold higher in cells missing both genes when compared with control OSE cells. In contrast, Cox-2 expression was insignificant when compared with intact OSE cells (Fig. 2A). Comparative RT-PCR results of these samples more or less followed the pattern of microarray data (Fig. 2B). This

Figure 1. Differential expression of Cox-1 and Cox-2 in ovarian tumors induced by inactivation of p53 and Rb in the surface epithelium. Results from representative samples. A, RT-PCR results. β-Actin is a housekeeping gene. B, Western blot analysis. Actin serves as a control. C, in situ hybridization results. Arrowheads, sites of higher signal intensity. H&E staining; bar, 200 μm; p, poorly differentiated; w, well-differentiated; m, mixed. D, immunohistochemistry. Red deposits, sites of positive immunostaining; bar, 200 μm.
Cox-1 is expressed in ovarian cancer arising from the surface epithelium expressing LSL-K-ras\(^{G12D/+}\) in the absence of Pten. Dinulescu et al. have recently shown that endometrioid ovarian carcinomas, as characterized by metaplastic squamous differentiation and notable glandular morphology, develop after activation of K-ras with simultaneous deletion of Pten in the OSE in LSL-K-ras\(^{G12D/+}/PTEN\text{fl}^{+/+}\) mice by local delivery of adenovirus-mediated Cre (11). Using this model, we examined whether endometrioid EOCs differentially express Cox isoforms in eight independent samples. In situ hybridization and Western blotting were done in these well-differentiated tumor specimens. Again, we observed abundant expression of Cox-1 with low to undetectable Cox-2 expression. Once again, Cox-2 expression was spotty and restricted to small areas (Fig. 3A). The results of Western blotting on Cox-1 and Cox-2 protein levels are consistent with their mRNA expression (Fig. 3B). These results suggest that not only serous, but also endometrioid EOCs, express higher levels of Cox-1, but not Cox-2.

**Cox-1 is expressed in EOC derived by expression of the SV40 TAg in the ovaries of mice.** Connolly et al. have previously shown that female mice expressing SV40 TAg under the transcriptional control of the MISIR gene promoter (TgMISR\(\text{Tag}\)) develop poorly differentiated ovarian tumors (9). We also used this model to examine differential expression of Cox-1 and Cox-2 in 11 poorly differentiated independent samples. Using RT-PCR, we found that Cox-1 expression was higher in all tumors, whereas that of Cox-2 was low to undetectable (Fig. 4A). Western blot analysis showed similar results (Fig. 4B). We also examined cell-specific expression of Cox-1 and Cox-2 in tumor sections by in situ hybridization (Fig. 4C). Again, the expression of Cox-1 was high in all tumor samples. In contrast, Cox-2 expression was low to undetectable, except for minor focal expression. These results suggest that EOC originating from an oncogenic insult also abundantly express Cox-1, but not Cox-2.

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**Figure 2.** A, comparative microarray analysis of Cox-1 and Cox-2 expression in OSE. OSE cells carrying p33/\text{fl}^{+/+}\text{Rb}^{-/-}\text{LacZ} or p33/\text{fl}^{+/+}\text{Rb}^{-/-}\text{LacZ} were treated with either AdCMVCre or AdCMV\text{LacZ} recombinant adenovirus resulting in p33/\text{fl}^{+/+}\text{Rb}^{-/-}\text{LacZ} or control cells, respectively. Cells were collected after 3 passages (p33/\text{fl}^{+/+}\text{Rb}^{-/-}\text{LacZ} and control) and after 26 passages (p33/\text{fl}^{+/+}\text{Rb}^{-/-}\text{LacZ} and control) of AdCMVCre infection. Cox-1 gene (mean of log2 signal ± SE): 3.69 ± 0.42 (p33/\text{fl}^{+/+}\text{Rb}^{-/-}\text{LacZ} and control), 3.73 ± 0.89 (p33/\text{fl}^{+/+}\text{Rb}^{-/-}\text{LacZ} and control), 8.28 ± 0.11 (Rb\(^{-/-}\)), 9.03 ± 1.29 (p33/\text{fl}^{+/+}\text{Rb}^{-/-}\text{LacZ} and control), and 10.05 ± 0.31 (p33/\text{fl}^{+/+}\text{Rb}^{-/-}\text{LacZ} and control). B, Pair-wise comparison of test samples versus control (n = 3) by t test yielded two-tailed P = 0.9658 (p33/\text{fl}^{+/+}\text{Rb}^{-/-}\text{LacZ} and control), 0.0005 (Rb\(^{-/-}\)), 0.0169 (p33/\text{fl}^{+/+}\text{Rb}^{-/-}\text{LacZ} and control), and 0.0017 (p33/\text{fl}^{+/+}\text{Rb}^{-/-}\text{LacZ} and control). The results of Western blotting were done in these well-differentiated tumor specimens. Again, we observed abundant expression of Cox-1 with low to undetectable Cox-2 expression. Once again, Cox-2 expression was spotty and restricted to small areas (Fig. 3A). The results of Western blotting on Cox-1 and Cox-2 protein levels are consistent with their mRNA expression (Fig. 3B). These results suggest that not only serous, but also endometrioid EOCs, express higher levels of Cox-1, but not Cox-2.

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**Cox-1 and Epithelial Ovarian Cancer**

**Figure 3.** Differential Cox-1 and Cox-2 expression in endometrioid ovarian tumors induced by inactivation of Pten and activation of K-ras in the surface epithelium. Results of representative samples. A, In situ hybridization results. H&E staining; bar, 500 μm. B, Western blot analysis. Actin serves as a control.
The highlight of the present investigation is that Cox-1 is the predominant isoform expressed at high levels in a variety of mouse models of EOC. The present results are exciting, and these models are clinically relevant because Cox-1 is also overexpressed in human EOC (4). The current realization that Cox-1 could be as important as Cox-2 in certain normal and pathologic situations provides new insight concerning the role of Cox-derived prostaglandins in pathophysiology (4, 5, 15). Indeed, we have shown previously that Cox-1-derived prostaglandins, particularly prostaglandin E₂, stimulates the expression of proangiogenic factors in human OVCAR3 cells which is inhibited by a Cox-1-selective inhibitor, SC-560 (4). More recently, we have shown that Cox-1-derived prostacyclin stimulates cell proliferation with attenuation of apoptosis in a mouse model of EOC. More importantly, SC-560 inhibited tumor growth in this model (5). It is also interesting to note that Cox-1-derived prostaglandin E₂ stimulates cell motility during zebrafish gastrulation (18).

Mutation or loss of tumor suppressor genes, amplification of growth-stimulatory factors, and/or suppression of death signaling pathways cause increased susceptibility to various cancers, including breast and ovarian cancers in women (19). For example, mutations in several tumor suppressor genes including p53, BRCA1, or BRCA2, and/or activating mutations or amplification of proto-oncogenes such as c-myc, K-ras, and Akt are considered contributors to human EOC. Therefore, the mouse models of EOC in which specific tumor suppressor genes were deleted with amplification of oncogenes serve as relevant models to better understand the etiology and progression of EOC. Our previous and present work showing predominant expression of Cox-1, not Cox-2, in EOC of both humans and in four different mouse models places the Cox-1 isoform as an important candidate for further investigation.

Although a wealth of information is available regarding regulation of Cox-2 in pathophysiologic situations, very little is known regarding the regulation of Cox-1 expression. Traditionally, Cox-1 is considered a housekeeping gene. There is now evidence that two Sp1 sites in the human Cox-1 promoter direct its constitutive expression in human umbilical vein endothelial cells (8). A role for histone deacetylase is also implicated in regulating Cox-1 promoter activity in astrocytes (20). We have previously shown that Cox-1 expression is influenced by estrogen and/or progesterone in the mouse uterus (16). However, it is not yet clear how Cox-1 is overexpressed in EOC. Our preliminary data suggests that an inhibitor of histone deacetylase influences Cox-1 expression in mouse EOC cells in vitro. It is possible that chromatin remodeling is important for Cox-1 expression in EOC, which is currently under investigation in our laboratory.

Nonetheless, the present investigation forms the basis for the independent laboratories to examine the efficacy of Cox-1 inhibition or dual inhibition of both Cox-1 and Cox-2 to prevent and/or treat EOC growth in these mouse models. Because Cox-1 expression is heightened in different types of EOC in mouse models, this isoform may serve as a potential marker for the early detection of ovarian cancer and could be a promising target for EOC treatment.

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