Cyclooxygenase-2 Is Overexpressed in Human Cervical Cancer

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ABSTRACT

Multiple lines of evidence suggest that cyclooxygenase-2 (COX-2) is an important target for preventing epithelial malignancies. Little is known, however, about the expression of COX-2 in gynecological malignancies. By immunoblot analysis, COX-2 was detected in 12 of 13 cases of cervical cancer but was undetectable in normal cervical tissue. Immunohistochemistry revealed COX-2 in malignant epithelial cells. COX-2 was also expressed in cervical intraepithelial neoplasia. The mechanism by which COX-2 is upregulated in cervical cancer is unknown. Because the epidermal growth factor (EGF) receptor is commonly overexpressed in cervical cancer, we investigated whether EGF could induce COX-2 in cultured human cervical carcinoma cells. Treatment with EGF markedly induced COX-2 protein, COX-2 mRNA, and stimulated COX-2 promoter activity. The induction of COX-2 by EGF was suppressed by inhibitors of tyrosine kinase activity, phosphatidylinositol 3-kinase 3-kinase, mitogen-activated protein kinase kinase, and p38 mitogen-activated protein kinase. Moreover, overexpressing dominant-negative forms of extracellular signal-regulated kinase 1, c-Jun NH2-terminal kinase, p38, and c-Jun blocked EGF-mediated induction of COX-2 promoter activity. Taken together, these findings suggest that deregulation of the EGF receptor signaling pathway may lead to enhanced COX-2 expression in cervical cancer.

INTRODUCTION

Cancer of the cervix is the second leading cause of cancer deaths in women worldwide and remains a leading cause of mortality among women of reproductive age in developing countries. An estimated 500,000 new cases are diagnosed worldwide each year. This problem is not unique to developing countries. In the United States, there will be an estimated 12,800 new cases of cervical cancer and 4,600 deaths in 2000 (1). Cervical carcinoma arises in women infected with HPV3 and progresses through a multistage process of carcinogenesis (2). For example, CIN (3), a precursor lesion detected in screening programs, can progress to invasive cancer. Because the premalignant phase of cervical carcinogenesis may last for 5–10 years, it is ideally suited for chemopreventive therapy.

Recent studies have established the presence of two distinct forms of COX. One is constitutively expressed (COX-1), and the other is inducible (COX-2; Ref. 3). COXs catalyze the formation of prostaglandins from arachidonic acid. COX-1 is a housekeeping gene with essentially constant levels of expression. In contrast, COX-2 is an immediate-early response gene that is induced by growth factors, tumor promoters, oncogenes, and carcinogens (4–7).

A large body of evidence suggests that COX-2 is important in carcinogenesis. For example, COX-2 is overexpressed in transformed cells and in malignant tissues (4, 8–14). Oshima et al. (15) showed that a null mutation for COX-2 caused a marked reduction in the number and size of intestinal polyps in a murine model of familial adenomatous polyposis. COX-2 knockout mice also develop ∼75% fewer skin papillomas than control mice (16). In addition to the genetic evidence implicating COX-2 in carcinogenesis, there are supporting pharmacological data. Selective COX-2 inhibitors suppressed the formation of a variety of tumors in experimental animals (15, 17–19) and decreased the number of colorectal polyps in familial adenomatous polyposis patients (20). In this study, we investigated whether COX-2 was overexpressed in cervical cancer compared with normal epithelium. Our data show that levels of COX-2 are increased in cervical cancer. The possibility that this reflects activation of the EGFR signaling pathway is suggested by results in cultured cervical cancer cells.

MATERIALS AND METHODS

Materials. RPMI 1640, Opti-MEM, fetal bovine serum, human recombinant EGF, TGF-α, and Lipofectin were from Life Technologies, Inc. (Grand Island, NY). Wortmannin, LY294002, and secondary antibody to IgG conjugated to horseradish peroxidase were from Sigma Chemical Co. (St. Louis, MO). Nitrocellulose membranes were from Schleicher & Schuell (Keene, NH). Rabbit polyclonal antihuman COX-2 and anti-β-galactosidase were from Santa Cruz Biotechnology (Santa Cruz, CA).

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3 The abbreviations used are: HPV, human papillomavirus; CIN, cervical intraepithelial neoplasia; COX, cyclooxygenase; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; TGF, transforming growth factor; β-galactosidase; PI3K, phosphatidylinositol 3-kinase; JNK, c-Jun NH2-terminal kinase.
tobody (PG-27) was from Oxford Biomedical Research, Inc. (Oxford, MI). Western blot detection reagents (ECL) were from Amersham Pharmacia Biotech. The human COX-2 cDNA was generously provided by Dr. Stephen M. Prescott (University of Utah, Salt Lake City, UT). [32P]CTP was from DuPont NEN Life Science Products (Boston, MA). The 18S rRNA cDNA was from Ambion, Inc. (Austin, TX). Streak tissue fixative was from Streck Laboratories, Inc. (Omaha, NE). The tyramide signal and amplification kit was from DuPont NEN Life Science. The Vector Blocking kit was from Vector Laboratories, Inc. (Burlingame, CA). Plasmid DNA was prepared using a kit from Qiagen, Inc. (Chatsworth, CA). Tryptophin 23 was from Toronto Research Chemicals (Toronto, Ontario, Canada). PD98059 (2'-amino-3'-methoxyflavone) was from Biomol (Plymouth Meeting, PA) and SB202190 [4-[(4-fluorophenyl)-2-(4-hydroxyphenyl)]-5-(4-pyridyl)-1H-imidazole] was from Calbiochem (LaJolla, CA). Reagents for the luciferase assay were from Analytical Luminescence Laboratory (San Diego, CA).

**Patient Samples.** Cervical cancer samples were collected for immunoblot analysis from patients with different histological types: squamous cell carcinoma (n = 9); adenocarcinoma (n = 2); adenosquamous carcinoma (n = 1); and sarcoma (n = 1). One case of CIN III was analyzed. Histologically normal cervical tissue samples were obtained from the same individuals (paired samples) or from other patients undergoing gynecological surgery at Barnes-Jewish Hospital/Washington University School of Medicine (St. Louis, MO). Samples were stored at −80°C until analysis. Informed consent was obtained from each patient. This study was approved by the Committees on Human Rights in Research at the participating institutions.

**Tissue Culture.** CaSkI cells were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 with 25 mM HEPES supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% sodium pyruvate in a 5% CO2/water saturated incubator at 37°C. Cells were grown to 60% confluence prior to being placed in serum-free medium for 24 h. Treatments with EGF or TGF-α were carried out in serum-free medium.

**Western Blotting.** Cells lysates were prepared by treating cells with lysis buffer [150 mM NaCl, 100 mM Tris (pH 8.0), 1% Tween 20, 50 mM diethyldithiocarbamate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin]. Lysates were sonicated for 20 s on ice and centrifuged at 10,000 × g for 10 min to sediment the particulate material. The protein concentration of the supernatant was measured by the method of Lowry et al. (21). SDS-PAGE was performed under reducing conditions on 10% polyacrylamide gels as described by Laemmli (22). The resolved proteins were transferred onto nitrocellulose sheets as detailed by Towbin et al. (23). The nitrocellulose membrane was then incubated with primary antisera. Secondary antibody to IgG conjugated to horseradish peroxidase was used. The blots were probed with the ECL Western blot detection system according to the manufacturer’s instructions.

**Northern Blotting.** Total cellular RNA was isolated from cell monolayers using an RNA isolation kit from Qiagen, Inc. Ten μg of total cellular RNA/lane were electrophoresed in a formaldehyde-containing 1.2% agarose gel and transferred to nylon-supported membranes. After baking, membranes were prehybridized overnight in a solution containing 50% formamide, 5× sodium chloride-sodium phosphate-EDTA buffer (SSPE), 5× Denhardt’s solution, 0.1% SDS, and 100 μg/ml single-stranded salmon sperm DNA and then hybridized for 12 h at 42°C with radiolabeled cDNA probes for human COX-2 and 18S rRNA. After hybridization, membranes were washed twice for 20 min at room temperature in 2× SSPE-0.1% SDS, twice for 20 min in the same solution at 55°C, and twice for 20 min in 0.1× SSPE-0.1% SDS at 55°C. Washed membranes were then subjected to autoradiography. COX-2 and 18S rRNA probes were labeled with [32P]CTP by random priming.

**Immunohistochemistry.** Tissues were fixed in Streel’s solution, embedded in paraffin, cut into 4-μm sections, and mounted on slides. Sections were deparaffinized and rehydrated in xylenes and descending alcohols. Tissues were then blocked for endogenous peroxidase (3% H2O2 in methanol) and avidin/biotin (Vector Blocking kit). The sections were permeabilized in TNB-BB [0.1 M Tris (pH 7.5), 0.15 M NaCl, 0.5% blocking agent, 0.3% Triton X, and 0.2% saponin] and incubated in primary antibody overnight at 4°C. Tissues were stained with the polyclonal antisemur to COX-2 (PG-27). Immuno reactive complexes were detected using tyramide signal amplification and were visualized with the peroxidase substrate, AEC. Control sections were treated with isotype-matched controls or were preincubated with 100-fold excess of human recombinant COX-2 protein. To ensure rigid interslide consistency, all slides were stained simultaneously on an autoimmunostainer.

**Plasmids.** The COX-2 promoter constructs (−1432/+59, −327/+59) were generous gifts of Dr. Tadashi Tanabe (National Cardiovascular Center Research Institute, Osaka, Japan). The ERK1 expression vectors were obtained from Dr. Melanie Cobb (Southwestern Medical Center, Dallas, TX). The expression vectors for JNK and p38 were generously provided by Dr. Roger Davis (University of Massachusetts, Worcester, MA). The AP-1 reporter plasmid (2xTRE-luciferase), composed of two copies of the consensus TRE ligated to luciferase, was kindly provided by Dr. Joan Heller Brown (University of California, La Jolla, CA). The c-Jun expression vectors were a gift of Dr. Tom Curran (St. Jude Children’s Research Hospital, Memphis, TN). pSV-β-gal was obtained from Promega Corp. (Madison, WI).

**Transient Transfection Assays.** CaSkI cells were seeded at a density of 5 × 104 cells/well in six-well dishes and grown to 50–60% confluence. For each well, 2 μg of plasmid DNA were introduced into cells using 6 μg of Lipofectin, following the manufacturer’s instructions. After 12 h incubation, the medium was replaced with basal medium. The activities of luciferase and β-gal were measured in cellular extract as described previously (24).

**Statistical Analysis.** Comparisons between groups were made by Student’s t test. A difference between groups of P < 0.05 was considered significant.

**RESULTS**

**Levels of COX-2 Protein Are Increased in Cervical Cancer.** Immunoblotting was performed to determine whether amounts of COX-2 protein were increased in cervical cancer. Overall, COX-2 was detected in 12 of 13 cases of
cervical cancer but was undetectable in normal cervix. A representative immunoblot comparing six cases of cervical cancer and seven samples of histologically normal cervix is shown in Fig. 1A. COX-2 was detected in various histological subtypes including squamous cell carcinoma (eight of nine), adenocarcinoma (two of two), adenosquamous carcinoma (one of one), and sarcoma of the cervix (one of one). Importantly, COX-2 was also detected in a case of CIN III (Fig. 1B). Immunohistochemical analyses of squamous cell carcinomas and adenocarcinomas of the cervix localized COX-2 to both tumor cells and inflammatory cells (Fig. 1C). COX-2 was expressed in well-differentiated parts of the squamous cancers, in contrast to adenocarcinomas, where staining was more diffuse. This staining was specific for COX-2 because immunoreactivity was lost when the antisera was preincubated with human recombinant COX-2.

**Activation of the EGFR Signaling Pathway Enhances the Transcription of COX-2.** The EGFR is commonly overexpressed in cervical cancer (25, 26). It is possible, therefore, that activation of the EGFR pathway contributes to enhanced expression of COX-2 in cervical cancer. Hence, we investigated whether EGF or TGF-α, ligands of EGFR, could induce COX-2 in cultured cervical carcinoma cells. Treatment with EGF or TGF-α markedly induced COX-2 protein (Fig. 2, A–C). To further elucidate the mechanism by which EGF and TGF-α induced COX-2, Northern blotting and transient transfections were performed. Treatment with EGF or TGF-α induced COX-2 mRNA (Fig. 2D) and stimulated COX-2 promoter activity (Fig. 2E).

Experiments were also done to identify the signaling pathway by which EGF induced COX-2. Binding of EGF to its

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**Fig. 1** Levels of COX-2 protein are increased in cervical cancer. A, immunoblotting was performed on six cases of cervical cancer (T) and seven samples of normal cervix (N). Equal amounts of protein (100 μg/lane) were loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. The immunoblot was probed with antibody specific for COX-2. Purified ovine COX-2 was used as a standard. Squamous carcinoma: 1, 3, 4–6; adenosquamous carcinoma: 2. 4 and 5 represent paired samples of tumorous and normal tissue from the same patient. B, immunoblotting was performed on a case of CIN III. Fifty μg of protein were loaded onto a 10% SDS-polyacrylamide gel and analyzed as described in A. C, COX-2 is expressed in squamous cell carcinoma of the cervix. Cytoplasmic immunoreactivity was detected in neoplastic epithelial cells with an anti-COX-2 antibody (×40).

**Fig. 2** COX-2 transcription is induced by treatment with EGF or TGF-α in cervical carcinoma cells. CaSki cells were treated with EGF or TGF-α. A, EGF caused dose-dependent induction of COX-2. Treatment was for 6 h. B, peak induction of COX-2 by EGF (10 ng/ml) occurred after treatment for 6 h. C, TGF-α caused dose-dependent induction of COX-2. Treatment was for 6 h; 10 ng/ml caused maximal induction. In A–C, lysate protein (50 μg/lane) was loaded onto 10% SDS-polyacrylamide gels, electrophoresed, and subsequently transferred to nitrocellulose membranes. Immunoblots were probed with antibody specific for COX-2. Ovine COX-2 was used as a standard. D, CaSki cells were treated with vehicle (Lane 1), EGF (10 ng/ml; Lane 2), or TGF-α (10 ng/ml; Lane 3) for 4 h. Total cellular RNA was isolated; 10 μg of RNA were added to each lane. The Northern blot was hybridized with probes that recognized COX-2 mRNA and 18S rRNA. E, CaSki cells were cotransfected with 1.8 μg of human COX-2 promoter (−1432/+59) and 0.2 μg of pSV-βgal. After transfection, cells were then treated with vehicle, EGF (10 ng/ml), or TGF-α (10 ng/ml). Reporter activities were measured in cellular extract 12 h later. Luciferase activity represents data that have been normalized with βgal activity. Columns, means; bars, SD; n = 6, *, P < 0.001 versus control.
receptor stimulates receptor tyrosine kinase activity. The induction of COX-2 by EGF was inhibited in a dose-dependent manner by tryphostin 23, an inhibitor of tyrosine kinase activity, for 6 h. Fresh medium containing vehicle or EGF was then added for 6 h. Tryphostin caused dose-dependent inhibition of EGF-mediated induction of COX-2. B. Wortmannin blocked EGF-mediated induction of COX-2. C. LY294002 suppressed the induction of COX-2 by EGF. D. EGF-mediated induction of COX-2 was blocked by PD98059. E. SB202190 suppressed EGF-mediated induction of COX-2. In B–E, cells were cotreated with EGF plus inhibitor. Equal amounts of vehicle were given to inhibitor-treated, EGF-treated, and control cells.

A pharmacological approach was used to evaluate the potential role of PI3K in mediating the induction of COX-2 by EGF. Wortmannin and LY294002, compounds that inhibit PI3K by distinct mechanisms, blocked EGF-mediated induction of COX-2 (Fig. 3, B and C). Activation of EGFR/PI3K signaling stimulates MAPKs. Experiments were done to determine whether MAPKs were involved in EGF-mediated induction of COX-2. In the first experiment, we used PD98059, a specific inhibitor of MAPK kinase activity that prevents activation of ERK1 and ERK2. PD98059 inhibited EGF-mediated induction of COX-2 (Fig. 3D). Similarly, SB202190, a selective inhibitor of p38 MAPK activity, blocked EGF-mediated induction of COX-2 protein (Fig. 3E). To further investigate the importance of MAPKs in mediating the effects of EGF, transient transfections were performed (Fig. 4A). The induction of COX-2 promoter activity by EGF was blocked by transiently overexpressing dominant-negatives for ERK1, p38, and JNK, respectively; columns ERK1 DN, p38 DN, and JNK DN, received 0.9 µg each of dominant-negative forms of ERK1, p38, and JNK, respectively. Columns c-Jun and c-Jun DN, received 0.9 µg each of expression vectors for c-Jun and c-Jun DN, respectively. The total amount of DNA in each reaction was kept constant at 2 µg by using the corresponding empty expression vectors. After transfection, cells were treated with vehicle or EGF (10 ng/ml). Reporter activities were measured in cellular extract 6 h later. Luciferase activity represents data that have been normalized with β-gal. Columns, means; bars, SD; n = 6. * P < 0.01 compared with EGF.

Fig. 3 Induction of COX-2 by EGF is mediated by PI3K and ERK1/2 and p38 MAPKs. CaSkI cells were treated with vehicle (C), EGF (10 ng/ml), or EGF plus inhibitor as detailed below. Treatments with EGF were for 6 h. Cell lysate protein (50 µg/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred to a nitrocellulose membrane. Immunoblots were probed with antisera specific for COX-2. A, cells were pretreated with vehicle or tryphostin 23, an inhibitor of tyrosine kinase activity, for 12 h. Fresh medium containing vehicle or EGF was then added for 6 h. Tryphostin caused dose-dependent inhibition of EGF-mediated induction of COX-2. B, Wortmannin blocked EGF-mediated induction of COX-2. C, LY294002 suppressed the induction of COX-2 by EGF. D, EGF-mediated induction of COX-2 was blocked by PD98059. E, SB202190 suppressed EGF-mediated induction of COX-2. In B–E, cells were cotreated with EGF plus inhibitor. Equal amounts of vehicle were given to inhibitor-treated, EGF-treated, and control cells.

Fig. 4 Activation of the COX-2 promoter by EGF is mediated by MAPKs and c-Jun. CaSkI cells were transfected with 0.9 µg of a human COX-2 promoter construct ligated to luciferase (−327/+59) and 0.2 µg of pSV-βgal. A: columns ERK1, p38, and JNK, received 0.9 µg each of expression vectors for ERK1, p38, and JNK, respectively; columns ERK1 DN, p38 DN, and JNK DN, received 0.9 µg each of dominant-negative forms of ERK1, p38, and JNK, respectively. B: columns c-Jun and c-Jun DN, received 0.9 µg each of expression vectors for c-Jun and c-Jun DN, respectively. The total amount of DNA in each reaction was kept constant at 2 µg by using the corresponding empty expression vectors. After transfection, cells were treated with vehicle or EGF (10 ng/ml). Reporter activities were measured in cellular extract 6 h later. Luciferase activity represents data that have been normalized with β-gal. Columns, means; bars, SD; n = 6. * P < 0.01 compared with EGF.
factor complex, was linked to the induction of COX-2. As shown in Fig. 4B, transiently overexpressing c-Jun stimulated COX-2 promoter activity. Moreover, overexpressing a dominant-negative form of c-Jun blocked EGF-mediated induction of COX-2 promoter activity.

**DISCUSSION**

Previously, COX-2 was found to be up-regulated in normal cervix and uterus during certain stages of the estrous cycle and in pregnancy (28, 29). In this study, we report that COX-2 is overexpressed in cancers of the cervix as well as in CIN. Levels of COX-2 were increased, regardless of the histological subtype. Increased amounts of EGFR and its ligands have been observed in both CIN and cervical cancer (25, 26). We found that EGF and TGF-α, ligands of EGFR, markedly induced COX-2 in a cervical carcinoma cell line. This suggests that deregulated signaling through EGFR is likely to account, at least in part, for increased expression of COX-2 in cervical cancer. EGF is known to stimulate Ras and PI3K signaling, which leads, in turn, to activation of MAPKs and increased AP-1 activity (27).

It was important, therefore, to determine whether this signaling cascade was linked to EGF-mediated induction of COX-2. Pharmacological inhibition of PI3K, ERK1/2 activation, and p38 MAPK activity suppressed the induction of COX-2 by EGF. Furthermore, overexpressing dominant-negative forms of ERK1, JNK, and p38 MAPK blocked the induction of COX-2 promoter activity by EGF. These results are consistent with previous reports that ERK1/2, JNK, and p38 MAPKs can regulate COX-2 expression (30–33). The AP-1 transcription factor complex consists of a collection of dimers of members of the Jun, Fos, and ATF/cyclic AMP response element binding protein bZip families. MAPKs regulate AP-1 activity, both by increasing the abundance of AP-1 components and stimulating their activity (34). Treatment with EGF increased AP-1 activity; a dominant-negative form of c-Jun blocked EGF-mediated induction of COX-2 promoter activity. These data are consistent with several other studies in which AP-1 was identified as being important for stimulating COX-2 transcription (30, 33, 35, 36).

Although this study does not establish a direct link between HPV and COX-2, this possibility should be considered. HPV16 and HPV18 are often present in high-grade CINs and malignant cervical disease. It is noteworthy, therefore, that HPV16 oncogenes activate MAPKs (37) and the AP-1 family of transcription factors (38). Furthermore, HPV16 oncoprotein E6 targets the degradation of p53 (39), an effect that could also enhance levels of COX-2 (40). Additional studies are warranted to determine whether the pathology of HPV and expression of COX-2 are mechanistically linked.

COX-2 can potentially predispose to cervical cancer by several mechanisms. In other cell types, increased expression of COX-2 has been reported to inhibit apoptosis (41), suppress immune function (42), promote angiogenesis (43), and enhance the invasiveness of malignant cells (44). The relative importance of these different effects in cervical cancer needs to be defined.

Indomethacin, a nonsteroidal anti-inflammatory drug that inhibits the activities of COX-1 and COX-2, protects against chemically induced cervical carcinoma (45). Selective COX-2 inhibitors have been developed. These compounds decrease inflammation-like traditional nonsteroidal anti-inflammatory drugs but cause fewer significant side effects (46). Selective COX-2 inhibitors suppress tumorigenesis in experimental models of colon, breast, prostate, bladder, stomach, skin, and lung cancer (15, 17, 19, 42, 47–49). Recently, celecoxib, a selective COX-2 inhibitor, was found to cause a decrease in colorectal polyph burden in patients with familial adenomatous polyposis (20). Several other chemoprevention trials using selective COX-2 inhibitors are ongoing or planned. On the basis of the results of this study, it will be important to determine whether selective COX-2 inhibitors can be used alone or in combination with other agents to prevent or treat cervical cancer.

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