Up-regulation of Cyclooxygenase-2 Expression and Prostaglandin Synthesis in Endometrial Stromal Cells by Malignant Endometrial Epithelial Cells

A PARACRINE EFFECT MEDIATED BY PROSTAGLANDIN E$_2$ AND NUCLEAR FACTOR-$\kappa$B*

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We investigated the regulation of prostaglandin production in normal endometrial stromal cells (ESC) by malignant endometrial epithelial cells. We found that cyclooxygenase (COX)-2 mRNA and protein levels and prostaglandin (PG)E$_2$ production in ESC were significantly increased by Ishikawa malignant endometrial epithelial cell conditioned medium (MECM). By using transient transfection assays, we found that the −360/−218-bp region of the COX-2 promoter gene was critical for MECM induction of promoter activity. This MECM-responsive region contained a variant nuclear factor (NF)-κB site at −222 to −213 that, when mutated, completely abolished COX-2 promoter activation by MECM. Employing electrophoretic mobility shift assays, we further demonstrated that binding of NF-κB p65 to this NF-κB-binding site is, in part, responsible for the COX-2 promoter activation by MECM. To investigate further the potential effects of MECM on COX-2 mRNA stability, ESC were treated with MECM in the absence or presence of actinomycin D, a general transcription inhibitor. We found that MECM significantly increased COX-2 mRNA stability. Intriguingly, we found that PGE$_2$ was one of the major factors in MECM, which was responsible for up-regulating COX-2 expression in ESC. ECC-1 and HEC-1A malignant endometrial epithelial cell lines also produced significantly increased quantities of PGE$_2$. In conclusion, malignant endometrial epithelial cells secrete PGE$_2$ that induces COX-2 expression in normal endometrial stromal cells in a paracrine fashion through activation of transcription and stabilization of COX-2 mRNA.

Supported by a plethora of experimental evidence, prostaglandin (PG)$^1$ production emerged as a highly promising therapeutic target not only in the treatment of many inflammatory diseases but also several types of human cancers. The proposal that PGs contribute to carcinogenesis is supported further by compelling evidence that inhibitors of cyclooxygenase (COX) activity (and thereby of PGs formation) protect against colon, mammary, esophageal, and lung cancer in humans (1). The increased amounts of PGs in tumors reflect enhanced synthesis, which occurs by COX-catalyzed metabolism of arachidonic acid. PGs are synthesized from arachidonic acid by two different isoforms of COX, referred to as COX-1 and COX-2. They share ~60% identity at the amino acid level and have similar enzymatic activities, but although they catalyze the same reaction, these two isoforms have been suggested to have distinct biological functions (2–5). COX-1 is constitutively expressed in most mammalian tissues and is thought to carry out housekeeping functions such as cytoprotection of the gastric mucosa, regulation of renal blood flow, and control of platelet aggregation. In contrast, COX-2 mRNA and protein are normally undetectable in most tissues but can be rapidly induced in response to proinflammatory or mitogenic stimuli, which included various cytokines, growth factors, oncogenes, endotoxins, and chemicals (6–12). Enhanced expression of COX-2, but not COX-1, has been found in colon, pancreatic, and gastric cancer tissues (13–15). Previous studies (16–19) have shown that overexpression of COX-2 reduces the rate of apoptosis, increases the invasiveness of malignant cells, and promotes angiogenesis. Therefore, it is believed that increased production of PGs (especially PGE$_2$) in tumors is a result of enhanced COX-2 gene expression (13).

We and others (20–23)$^2$ have examined the expression and the distribution of the COX-2 by immunohistochemistry in human normal endometrium and endometrial cancer. Specific staining for COX-2 could be found only in the surface and glandular epithelium in normal endometrium but not in the endometrial stroma. On the other hand, not only the COX-2 expression in the surface and glandular epithelia of the endometrial cancer was increased as compared with normal endometrium, an increase in the COX-2 immunostaining in the stroma of the endometrial cancer relative to that of normal epithelial cell conditioned medium; Act D, actinomycin D; NF-κB, nuclear factor-κB; C/EBP, CCAAT/enhancer binding protein; RT, reverse transcriptase; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; C/EBP, CCAAT/enhancer-binding protein.

$^1$ M. Tamura, H. Sasano, and S. E. Bulun, unpublished observations.
endometrium was also noted. Similar results have been described for colon carcinoma, esophageal carcinoma, and malignant melanoma (24–26). These results were suggestive of a cross-talk between malignant epithelial cells and surrounding stromal cells to favor COX-2 expression in the endometrial tumors. To test this hypothesis, the present investigation was designed to examine the direct effect of conditioned medium of a cancerous endometrial cell line (Ishikawa cells) on COX-2 expression in normal stromal cells of the human endometrium. We hypothesized that malignant epithelial cells secreting factor(s) that act in a paracrine fashion might be responsible for increased COX-2 expression in the stromal cells. In addition, we attempted to characterize the critical cis-acting elements that mediate induction the human COX-2 gene expression in normal endometrial stromal cells by malignant endometrial epithelial cell conditioned medium.

**EXPERIMENTAL PROCEDURES**

**Materials**—Actinomycin D (Act D, general transcription inhibitor), indomethacin (non-selective COX-1 and -2 inhibitor), and PGE$_2$ were purchased from Sigma. Nuclear factor (NF)-κB consensus double-stranded oligonucleotide was purchased from Promega (Madison, WI). Antibodies against NF-κB p50, NF-κB p65, NF-κB p52, RelB, c-Rel, CCAT/enhancer-binding protein (C/EBP)α, C/EBPβ, and C/EBPδ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other materials used in the study are indicated in the appropriate context below.

**Cell Culture**—Human normal endometrial tissues were obtained at the time of surgery from reproductive aged women ($n=12$) who were undergoing hysterectomy for advanced cervical dysplasia after obtaining informed consent following a protocol approved by the Office for Protection of Research Subjects of the University of Illinois, Chicago. These patients did not receive hormonal treatments and not take anti-inflammatory drugs (especially COX inhibitors) before surgery. Six specimens were in the proliferative phase, whereas the other six were secretory. No differences in experimental results were noted with respect to the cycle phase. Normal endometrial stromal cells (ESC) were cultured using a protocol reported previously (27). The cells were studied at passage 4 and cultured using a protocol reported previously (27). The cells were studied at passage 4–6. Confluent ESC were serum-deprived for 16 h in serum-free, phenol red-free Dulbecco’s modified Eagle’s medium/Ham’s F-12 (DMEM/F-12) before subjected to the following two treatments: (i) serum-free, phenol red-free DMEM/F-12 as the base-line control, BECM, or MECM for 8 h. Total RNA was isolated from ESC using the RNeasy mini kit (Qiagen, Valencia, CA), following the protocol suggested by the manufacturer. The integrity of the RNA was confirmed by agarose gel electrophoresis. For RT-PCR analysis of COX-2 mRNA, First-Strand Synthesis System (Invitrogen, Carlsbad, CA) was used to synthesize the first strand cDNA as instructed by the supplier. Briefly, 5 μg of total RNA isolated from ESC was treated with DNase I (1 unit/μl). One μl of this was reserved for PCR amplification with primers specific for glyceroldehyde-3-phosphate dehydrogenase (GAPDH), providing a control for equal starting amounts of total RNA in samples and PCR efficiency. The remainder of the DNase-treated RNA was directly reverse-transcribed. One μl of the reverse transcriptase reaction mix was used for PCR with oligonucleotide pairs specific for COX-2 and GAPDH. The nucleotide sequences of the primer pairs employed and PCR conditions were reported previously (27). The PCR cycle numbers were 38 for COX-2 and 30 for GAPDH. PCR performed with the original RNA sample after DNase I digestion (see above) did not yield any products, confirming that amplified products were dependent on the presence of template generated by reverse transcription and not the result of contamination with extraneous DNA. Aliquots of the reaction products were analyzed by electrophoresis in an agarose gel and ethidium bromide staining. Intensity of PCR products was quantified using the Quasias Software One 3.0 Analysis Software (Bio-Rad). The insert plasmid pGL3-Promoter (Clontech) designed to examine the direct effect of conditioned medium of malignant epithelial cell conditioned medium (BECM) was prepared following a similar procedure described above. We also generated the conditioned medium from ECC-1 human malignant endometrial epithelial cell line (ECC-1CM), HEC-1A human malignant endometrial epithelial cell line (HEC-1CM), HEC-1A human malignant endometrial epithelial cell line (HEC-1CM), T47D human malignant mammary epithelial cell line (T47DCM), human malignant mammary epithelial cell line (T47D), human malignant mammary epithelial cell line (MCF-7CM) and LNCaP human prostate epithelial cell line (LNCaP) following a similar procedure described above. For assaying the concentration dependence, MECM was concentrated 5- or 10-fold in an Ultrazell FF-60 Ultrafiltration Device using 5,000 molecular weight cut-off membranes (Millipore, Bedford, MA).

**Semi-quantitative RT-PCR Amplification**—ESC were cultured in 100-mm dishes until confluent in the growth medium as described above and switched to serum-free, phenol red-free media for 16 h. These cells were then incubated under various conditions, i.e. control or MECM for 8 h. Total RNA was isolated from ESC using the RNeasy mini kit (Qiagen, Valencia, CA), following the protocol suggested by the manufacturer. The integrity of the RNA was confirmed by agarose gel electrophoresis. For RT-PCR analysis of COX-2 mRNA, First-Strand Synthesis System (Invitrogen, Carlsbad, CA) was used to synthesize the first strand cDNA as instructed by the supplier. Briefly, 5 μg of total RNA isolated from ESC was treated with DNase I (1 unit/μl). One μl of this was reserved for PCR amplification with primers specific for glyceroldehyde-3-phosphate dehydrogenase (GAPDH), providing a control for equal starting amounts of total RNA in samples and PCR efficiency. The remainder of the DNase-treated RNA was directly reverse-transcribed. One μl of the reverse transcriptase reaction mix was used for PCR with oligonucleotide pairs specific for COX-2 and GAPDH. The nucleotide sequences of the primer pairs employed and PCR conditions were reported previously (27). The PCR cycle numbers were 38 for COX-2 and 30 for GAPDH. PCR performed with the original RNA sample after DNase I digestion (see above) did not yield any products, confirming that amplified products were dependent on the presence of template generated by reverse transcription and not the result of contamination with extraneous DNA. Aliquots of the reaction products were analyzed by electrophoresis in an agarose gel and ethidium bromide staining. Intensity of PCR products was quantified using the Quasias Software One 3.0 Analysis Software (Bio-Rad). The insert plasmid pGL3-Promoter (Clontech) designed to examine the direct effect of conditioned medium of malignant epithelial cell conditioned medium (BECM) was prepared following a similar procedure described above. We also generated the conditioned medium from ECC-1 human malignant endometrial epithelial cell line (ECC-1CM), HEC-1A human malignant endometrial epithelial cell line (HEC-1CM), HEC-1A human malignant endometrial epithelial cell line (HEC-1CM), T47D human malignant mammary epithelial cell line (T47DCM), human malignant mammary epithelial cell line (T47D), human malignant mammary epithelial cell line (MCF-7CM) and LNCaP human prostate epithelial cell line (LNCaP) following a similar procedure described above. For assaying the concentration dependence, MECM was concentrated 5- or 10-fold in an Ultrazell FF-60 Ultrafiltration Device using 5,000 molecular weight cut-off membranes (Millipore, Bedford, MA).
quence of all constructs were verified by direct sequencing using the ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA).

Site-directed Mutagenesis—Mutant construct, pCOX2(RBM), with a mutation at the NF-kB site was constructed as described previously (28). Briefly, the sequence was changed from GGGGACTACCC to GGG-GACTACCC, the lowercase nucleotides indicate the mutations. The mutations and the orientation of insert were confirmed by direct sequencing. Plasmid used in the transfection experiment was purified using an EndoFree Plasmid Isolation Kit (Qiagen, Valencia, CA), and purity was verified by spectrophotometry and agarose gel electrophoresis.

Transient Transfections and Luciferase Assays—The day before transfection, ESC were plated into 6-well tissue culture plates at a density of 1.3 × 10^5 cells per well, reaches confluency by the time of transfection. Transfections were performed using the LipofectAMINE PLUS reagent (Invitrogen), following the protocol provided by the manufacturer. Each transfection was done using 0.4 μg of firefly luciferase reporter construct DNA that contains serial deletion and site-specific mutants of COX-2 promoter gene and 1 ng of an internal control Renilla luciferase reporter plasmid pRL-TK (Promega, Madison, WI). Three hours after transfection, the transfection medium was removed by aspiration; 2 ml of DMEM/F-12 containing 0.1% fetal calf serum and antibiotics was added, and the plates were returned to the incubator for 16 h. Cells received serum-free DMEM/F-12 for an additional 16 h and were then switched to control or MECM for another 24 h. Then medium was removed, and wells were rinsed with phosphate-buffered saline to remove detached cells and residual growth medium. Then 250 μl of 1× passive lysis buffer, provided in the Dual-Luciferase Reporter Assay System (Promega, Madison, WI), was added to each well. Two μl of the supernatant was used for assay of luciferase activities. Luciferase activities were determined using the LUMAT LB9507 luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). Firefly luciferase activities were normalized based on the Renilla luciferase activity in each well. These measurements were performed in triplicate and repeated in three independent experiments.

ESC were cultured in 100-mm dishes until confluent in the growth medium as described above and switched to serum-free media for 16 h. These cells were then incubated with control or MECM for 16 h. Nuclear protein was extracted from whole cells using NE-PER nuclear and cytoplasmic extraction reagents (Pierce), following the protocol suggested by the manufacturer. Protein concentration was measured with a BCA Protein Assay Kit (Pierce), according to the protocol suggested by the manufacturer. Protein concentration was determined using the LUMAT LB9507 luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). Firefly luciferase activities were normalized based on the Renilla luciferase activity in each well. These measurements were performed in triplicate and repeated in three independent experiments.

Effect of MECM on COX-2 mRNA and Protein Levels in Normal ESC—We initially carried out experiments to evaluate the optimal conditions for determining the effects of MECM on COX-2 mRNA levels in ESC. The time course of COX-2 mRNA abundance as examined by RT-PCR showed an increase following treatment at 4 h and peaked at 8 h (data not shown). Based on this observation, the ESC were treated with control or MECM for 8 h (Fig. 1, A and B). To determine where PCR amplification for COX-2 mRNA was in the logarithmic phase, total RNA isolated from ESC treated with MECM was reverse-transcribed and was amplified under different cycle numbers. Single PCR products were obtained for COX-2. A linear relationship between PCR products and amplification cycles was observed for COX-2 treated with MECM in ESC (Fig. 1A). Consequently, 38 cycles for COX-2 were employed for quantification. Compared with the control, MECM treatment significantly increased the COX-2 mRNA level in ESC (Fig. 1B). PCR was also performed using an aliquot of the same RT products for the housekeeping gene GAPDH mRNA to control for the RT reaction, PCR efficiency, and equal starting amounts of total RNA. There was no apparent change in the GAPDH mRNA abundance with MECM treatment. It should be pointed out that COX-2 seems to be constitutively expressed in Ishikawa malignant epithelial cells in relatively high levels (Fig. 1B, 1st lane). Quantitative densitometry for three independent experiments confirmed these results. We also performed a time course experiment for COX-2 protein levels in ESC treated with MECM employing Western analysis. COX-2 levels started to increase at 4 h, and peak level was observed at the 8-h treatment time (Fig. 1C, part of data not shown). To examine the specificity of effects of MECM on ESC, we used BECM to treat ESC and compared with MECM by immunoblot analysis (Fig. 1C). Incubation with BECM did not increase COX-2 levels demonstrating that the stimulatory effect was specific for malignant endometrial epithelial cells. These results demonstrate that malignant endometrial epithelial cells in culture produce specific factor(s), which stimulate COX-2 expression.

MECM Caused an Increase in PGE2 Synthesis in ESC—To determine whether the induction of COX-2 mRNA and protein levels was correlated with comparable changes in PGE2 production, PGE2 concentrations were measured in culture media of ESC after MECM treatment. The effect of MECM on the PGE2 synthesis in ESC is shown in Fig. 2. PGE2 synthesis in ESC was measured after incubation in control or MECM. Incubation of ESC with MECM for 24 h caused a significant increase in the PGE2 concentration in the medium by 4.2-fold compared with baseline PGE2 levels in ESC itself or by 3.2-fold compared with incubation in control medium.

Activation of the COX-2 Promoter by MECM Requires a Critical Regulatory Region Containing an NF-kB-binding Site—Deletion and site-specific mutants of COX-2 promoter-driven luciferase reporter gene constructs were transiently transfected to ESC and treated with MECM (24 h). As shown in Fig. 3A, an induction in promoter activity upon MECM treatment was observed only in the reporter construct containing the COX-2 promoter region −360/−218 bp, indicating that a critical regulatory region at −360/−218 bp was responsible for this induction. Sequence analysis of this region and the literature review (5) revealed the existence of an NF-kB-binding site at −222/−213 bp. Site-directed mutation of this NF-kB-binding site significantly reduced MECM-induced COX-2 promoter activity in ESC (Fig. 3A). Thus, the presence of this NF-kB element (−222/−213 bp) was required, at least in part, for MECM-mediated induction of COX-2 promoter in ESC. The concentration-dependent effect of MECM on COX-2 gene induction in ESC was evidenced by the transient transfection experiments using 5- and 10-fold concentrated MECM (Fig. 3B). Compared with a consistently observed 1.5-fold induction in −360/−56 bp construct by 1× MECM, treatments with 5 and 10× concentrated MECM elicited >2-fold inductions of COX-2 promoter activity in ESC.

Identification of Protein(s) That Bind to the NF-kB cis-Acting Element (−222/−213 bp)—EMSA was performed using nuclear proteins from ESC treated with control or MECM to
determine the protein/DNA binding activities at the NF-κB site. Nuclear extract from ESC incubated with control medium was composed of two faint but specific bands, upper and lower. Interestingly, nuclear extract prepared from MECM-treated cells showed strikingly more intense upper and lower complexes, suggesting increased protein/DNA binding activity at NF-κB site upon MECM treatment (Fig. 4A). Preincubation with a cold wild type −232/−205-bp probe completely abolished both shifted bands. Conversely, the cold −232/−205-bp probe containing a mutation in NF-κB (−222/−213-bp) element had no effect on the complex formation, confirming the specificity of the reaction. Furthermore, a generic probe containing a consensus NF-κB element also abolished both complexes.

To determine which proteins were responsible for the formation of the two inducible nuclear complexes, supershift experiments using antibody directed against members of the NF-κB/Rel family (p65, p50, p52, RelB, and c-Rel) were performed (Fig. 4B). Only the antibody against the NF-κB subunit p65 completely eliminated the formation of the lower complex. Antibodies against the p50, p52, RelB, or c-Rel, however, did not affect the formation of DNA-protein complexes. Comparable results were obtained using nuclear extracts derived from HeLa cells (data not shown). From these experiments, we concluded that p65 composed the lower complex. It should be noted that inability of any of the other members of the NF-κB family to compete or supershift the upper complex indicates involvement of additional transcription factor(s) in this complex formation.

**Effect of MECM on COX-2 mRNA Stability**—Because mRNA stabilization has been demonstrated as a major mechanism of regulation of COX-2 gene expression, we therefore investigated this possibility in MECM-mediated induction of COX-2 gene expression in ESC. To examine the stability of COX-2 mRNA in ESC, 10 μg/ml Act D was added with or without MECM (Fig. 5A). First, ESC in culture were treated with MECM for 8 h (maximum mRNA level). At this time point (control, 0 h), we distinguished four conditions. In the first two conditions, MECM was retained for another 4 h (upper portion of Fig. 5A and closed and open circles in Fig. 5B). Closed circles represent control cells with no additions. Open circles represent the addition of Act D to MECM. The COX-2 mRNA half-life value \( t_{1/2} \) was 5.7 h for MECM plus Act D treatment. On the other hand, when MECM was removed and replaced by the DMEM/F-12 serum-free control medium, COX-2 mRNA levels declined significantly (lower portion of Fig. 5A and open and closed inverted triangles in Fig. 5B). The absence or presence of Act D did not cause a significant difference in mRNA levels between these two new control medium conditions. The \( t_{1/2} \) for the new control medium treated with Act D was 3.0 h, and the difference of \( t_{1/2} \) between the treatment of MECM plus Act D (5.7 h) and the treatment of new control medium plus Act D (3.0 h) was significant \( (p<0.05) \). These experiments were performed on three different occasions with reproducible results. These results experiments are given at the bottom. Int, intensity. Results are expressed as the mean ± S.E. B, semi-quantitative RT-PCR for COX-2 and GAPDH in Ishikawa cells or ESC; shown is a representative of three independent experiments (top). Cells were treated with control (CON) or MECM for 8 h. Band sizes are as follows: COX-2, 305 bp; GAPDH, 593 bp. Summary data for three independent experiments are given at the bottom. COX-2 densitometry values corrected for GAPDH are expressed as a percentage in control ESC (mean ± S.E.). C, immunoblot analysis for COX-2 in ESC; shown is a representative of three independent experiments (top). Cells were treated with control (CON), BECM, or MECM for 8 h. COX-2 protein was detected at 72 kDa. Summary data for quantitative densitometry for the three experiments are given at the bottom. Mean ± S.E. values are depicted for protein abundance expressed as a percentage in control ESC. * \( p<0.05 \) versus control ESC.

![Fig. 1. Induction of COX-2 mRNA and protein by MECM in ESC.](http://www.jbc.org/)

**A** Cycles: 30, 34, 38, 42

| COX-2 | 305 bp |
|-------|--------|
| MECM  |        |

**B**

| COX-2 | 305 bp |
|-------|--------|
| GAPDH | 593 bp |

**C**

| COX-2 | 72 kDa |
|-------|--------|

**Summary data for three independent experiments** are given at the bottom. COX-2 densitometry values corrected for GAPDH are expressed as a percentage in control ESC (mean ± S.E.). C, immunoblot analysis for COX-2 in ESC; shown is a representative of three independent experiments (top). Cells were treated with control (CON), BECM, or MECM for 8 h. COX-2 protein was detected at 72 kDa. Summary data for quantitative densitometry for the three experiments are given at the bottom. Mean ± S.E. values are depicted for protein abundance expressed as a percentage in control ESC. * \( p<0.05 \) versus control ESC.
incubation with PGE2-deprived MECM (MECM prepared in the presence of indomethacin) did not increase COX-2 mRNA levels in Ishikawa cells. In addition, we showed previously (27) that treatment with indomethacin did not up-regulate the interleukin (IL)-1β-mediated COX-2 gene induction in many cell types prompted us to examine this possibility in ESC (32, 33). Compared with the IL-1β (1 ng/ml) treatment alone, co-incubation with exogenous PGE2 (40 pg/ml) significantly increased the COX-2 mRNA levels in ESC (Fig. 6C). The optimal concentration and time course of the IL-1β treatment used in the experiment were determined in a recent study (27) from our laboratory. IL-1β concentration in MECM was below the assay detection level by ELISA (data not shown).

To determine whether elevated PGE2 synthesis in Ishikawa malignant endometrial epithelial cells was typical of malignant endometrial epithelial cells or idiotypic for this cell line, PGE2 concentrations were measured in conditioned media of other malignant endometrial epithelial cell lines by ELISA. As shown in Fig. 6D, similar to the case of MECM, as compared with BECM, significantly elevated levels of PGE2 concentration were detected in conditioned media prepared from ECC-1 and HEC-1A. Interestingly, elevated levels of PGE2 concentration were also noted in conditioned media of malignant epithelial cells of mammary or prostatic origin. These results suggested that increased PGE2 production might be a common property of many malignant epithelial cells. In summary, our results suggest that modulation of COX-2 expression in stromal cells by malignant epithelial cells is achieved via a combination of paracrine/autocrine factors and/or signaling and that PGE2 is a key factor in this stimulatory mixture.

**DISCUSSION**

We showed here a potential paracrine interaction between malignant endometrial epithelial cells and adjacent stromal cells on endometrial cancer. This interaction favors increased prostaglandin synthesis in stromal cells and involves COX-2 and NF-κB. Interestingly, PGE2 can autoregulate its own synthesis through a positive feedback loop in endometrial cancer. Evidence for expression of all four PGE2 receptors (EP1, EP2, EP3, and EP4) in the endometrium (including stroma) further supports this hypothesis (34–36). A self-amplifying loop, based on increased PGE2 production leading to increased COX-2 expression and concomitant PGE2 production by ESC surrounding malignant epithelial cells, may be critical for the pathophysiology of the endometrial cancer growth. Recently, enhanced PGE2 synthesis has been shown to promote cell growth in some cancer models (1, 10). PGE2 can cause decreased programmed cell death in HCA-7 human colon cancer cells and increased growth and motility of the human colorectal carcinoma cell line, LS-174 (37, 38). In this study, we also determined that PGE2 production was commonly elevated in malignant epithelial cells irrespective of tissue origin and might contribute to the pathophysiology of tumor growth.

We have shown previously (27) that ESC express COX-2 in response to IL-1β stimulation and synthesize PGE2. The identification of the stimulatory effect of PGE2 on IL-1β-dependent COX-2 expression in ESC is likely to be of physiologic relevance. In fact, other investigators (6–8) showed that cytokines such as tumor necrosis factor-α and IL-1β increased the binding activity of NF-κB to the COX-2 promoter and up-regulated its activity in other systems. Interestingly, it was also proposed that NF-κB is important for oncogenic transformation, at least partly through its ability to block apoptosis (39–41). Because apoptosis is the primary mechanism of tumor cell killing by radiation and by chemotherapy, the speculation that the activation of NF-κB suppresses apoptotic potential generated interest in the role of NF-κB in cancer therapies. Indeed, suppression of NF-κB activation significantly enhances cell killing in culture in response to these treatments (42).

NF-κB is a dimeric DNA-binding protein composed of members of the NF-κB/Rel family of proteins including the mamalian forms, p65, p50, p52, RelB, and c-Rel (43, 44). NF-κB

\[^3\] K. M. Zeitoun and S. E. Bulun, unpublished observations.
proteins are capable of forming numerous homodimers and heterodimers with other family members, and this adds another level of complexity to the interaction of NF-κB with specific target genes. In this study, we found that NF-κB p65 subunit binds to the −222/−213-bp element in the CDX-2 promoter in response to MECM treatment. We continue to search for other partners in these DNA-protein complexes observed by EMSA. Supershift experiments showed that p50, p52, RelB, or c-Rel were not part of these complexes. Other investigators (45, 46) showed that members of NF-κB/Rel family interacted with other proteins, particularly members of the C/EBP family. In order to investigate the possibility, antibodies to various C/EBP proteins were tested in EMSAs. None of the antibodies against C/EBPα, C/EBPβ, and CEBPβ had any effect on the inducible complex formation (data not shown).

There are two NF-κB consensus sites in the promoter region of the human COX-2 gene (47): the NF-κB-5′ site (−447 to −438) and the NF-κB-3′ site (−222 to −213). NF-κB-5′ has been shown to have a role in the mechanism of COX-2 induction by tumor necrosis factor-α in a murine osteoblast cell line (6). NF-κB-3′ may play a role in facilitating the induction of COX-2 by lipopolysaccharide and phorbol ester in concert with the nuclear factor-interleukin-6 expression site and a cAMP-response element site in bovine aortic endothelial cells (12). We
discovered that the NF-κB-3’ is necessary for MECM-mediated COX-2 transcription. In addition, because the reporter construct containing the COX-2 promoter region –828/+56 was unresponsive to MECM, it appears that for the optimal induction of COX-2 by MECM, inhibitory site(s) are included between the –828 and –360-bp region of COX-2 promoter.

It now seems clear from published evidence (47–50) that the COX-2 gene is regulated through both 5’ (transcriptional) and 3’ (post-transcriptional) regulatory elements. We identified the critical cis-acting element, i.e. the NF-κB site in the COX-2 gene promoter required for the MECM-mediated COX-2 transcriptional increase. However, MECM did not affect the transcription of COX-2 reporter constructs so much. Early studies by Raz et al. (51) demonstrated that inducible COX-2 synthesis could be divided into early transcriptional and late post-transcriptional phases. High levels of encoded protein products from COX-2 genes are usually required for only a short period and must be expressed in a burst (50). The entire 3’–untranslated region (2.5 kb) of the human COX-2 gene is encoded by exon 10, which contains three canonical (AAUAAA) polyadenylation sequences and 22 copies of AUUUA “Shaw-
Kamen motifs (47–50). The latter sequences are believed to be associated with message instability, translational efficiency, and rapid turnover (52–54). Because COX-2 mRNA is highly unstable, and because MECM stabilizes COX-2 mRNA in the absence of transcription, we suggest that post-transcriptional mRNA stability is an important consequence of MECM action as well as the transcription step.

Thus, the three key molecules, PGE_2, COX-2, and NF-κB, are closely linked together with the common thread of oncogenesis. This observation promotes new insights into the paracrine interactions in cancer development and may lead to new therapeutic strategies capable of interrupting the oncogenic cascade at key points.

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