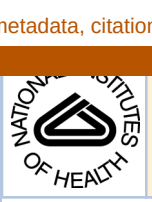

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Re-establishment of Gap Junctional Intercellular Communication (GJIC) between Human Endometrial Carcinomas by Prostaglandin E_2

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Abstract

Reduced intercellular communication via gap junctions is correlated with carcinogenesis. Gap junctional intercellular communication (GJIC), between normal human endometrial epithelial cells is enhanced when endometrial stromal cells were present in culture. This enhancement of GJIC between normal epithelial cells also occurs when they are cultured in medium conditioned by stromal cells. This observation indicated that a soluble compound (or compounds) produced and secreted by stromal cells mediates GJIC in epithelial cells. Previous studies have shown that endometrial stromal cells release prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) under physiological conditions. When we evaluated the response of normal endometrial epithelial cells to various concentrations of PGE_2, we found enhanced GJIC with 1 nM PGE_2. This is a smaller increase in GJIC than that induced by medium conditioned by stromal cells. When the extracellular concentration of PGE_2 was measured after incubation with stromal cells, it was found to be similar to the concentrations showing maximal GJIC between the normal epithelial cells. When indomethecin was used to inhibit prostaglandin synthesis by stromal cells, GJIC was reduced but not eliminated between normal endometrial epithelial cells. These observations suggest that although PGE_2 secreted by stromal cells is an important mediator of GJIC between the epithelial cells, it is not the sole mediator. Transformed endometrial epithelial cells did not demonstrate GJIC even in the presence of stromal cells. However, we were able to re-establish GJIC in transformed epithelial cells when we added PGE_2 to the cells. Our findings show that PGE_2 may serve as an intercellular mediator between stromal and epithelial cells that regulates GJIC in normal and malignant epithelial cells. This suggests that maintenance of GJIC by preserving or replacing PGE_2 secretion by endometrial stromal cells may have the potential to suppress carcinogenesis in endometrial epithelial cells.

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Conflicts of Interest Statement

The authors declare that there are no conflicts of interest.

Keywords

Endometrium; Prostaglandin; Gap Junctional Intercellular Communication; Epithelial-Stromal Interaction; Human; Cancer

Introduction

With about 47,000 new cases per year, endometrial carcinoma is the invasive cancer of the female genital tract with the highest incidence in women in the United States (American Cancer Society, 2012). Even though these cancers are commonly detected early in their natural history and therapeutic measures effectively treat, and even cure, a large fraction of these cancers, still about 8,000 American women die of this disease each year (American Cancer Society, 2012). While the incidence and death rate from endometrial cancer varies over time the approximate proportion of women succumbing to this disease has not changed greatly. This continuing toll of endometrial cancer deaths despite the current armamentarium of surgical, radiologic and chemotherapeutic treatments points out the need for further investigations of endometrial cancer that may reveal new therapeutic options. We recognize that estrogen exposure not counteracted by progesterone is the primary etiologic risk factor for developing this disease but we do not know conclusively the mechanism of pathogenesis by which endometrial cancer is caused by estrogen.

One of the hallmarks of endometrial cancer that is used as one of the criteria for its diagnosis is the relationship between endometrial glands and the intervening stroma. In the normal endometrium endometrial glands are separated by ample quantities of loose stroma predominantly populated by endometrial stromal cells. The transition to endometrial cancer is typically accompanied by the loss of most of the stroma between glands yielding back-to-back apposition of malignant glands. The common type of endometrial cancer is preceded by a form of hyperplasia thought to have a high risk of progression to cancer ("Endometrial Intraepithelial Neoplasia" (EIN)), and this reduction of stroma also is seen at this stage of the natural history of endometrial cancer (Mazur, 2005). An objective analysis of endometrial hyperplasias by computerized imaging techniques distinguished EIN, based largely on reduction of stromal cells between glands. Diagnosis of EIN by morphometric criteria proved to be a better predictor of progression to cancer than traditional classifications of hyperplasia (Baak, et al., 2005). The decreased proportion of stromal cells between glands in EIN suggests that mechanisms regulating homeostatic stromal-epithelial cell interactions are defective in endometrial cancer and its pre-invasive precursor.

Over a number of years our laboratory has studied the regulation of homeostatic stromal-epithelial cell interactions in human endometrium using human endometrial stromal and epithelial cells in co-cultures. We showed that endometrial epithelial cells grown on "Matrigel" would generate well-formed glands with polarized cells and a central lumen (Rinehart, et al., 1988). When colonies of stromal and epithelial cells were co-cultured on Matrigel and grew to contact each other they formed a basement membrane-like structure, with dense extracellular fibrillar protein networks and hemi-desmosomes at the basal surface of epithelial cells at the interface (Hopfer et al., 1994). Stromal cells embedded in Matrigel reduce the proliferation of epithelial cells as measured by ^{3}H-thymidine uptake, cell counting, and labeling of DNA with BrdU *in situ* (Arnold et al., 2001). This inhibitory effect is not observed when the stromal cells are grown on plastic, suggesting that the interaction between stromal cells and Matrigel influences paracrine factors produced by stromal cells. When normal human foreskin fibroblasts (NHF-1 cells) or medium conditioned by them were used in place of endometrial stromal cells in parallel studies they had no influence on endometrial epithelial cell growth. This eliminates the possibility that these results were due

to depletion of the medium by the stromal or NHF-1 cells or that these effects were not specific to endometrial stromal cells.

This co-culture system has been validated further by demonstrating the ability of epithelial cells to respond to hormonal stimulation. In endometrial tissue reconstructed in this manner, appropriate hormonal responses to estrogen and progesterone regulating epithelial cell proliferation and differentiation of these cells depends on the presence of stromal cells together with epithelial cells (Arnold et al., 2001). In the presence of progesterone, normal endometrial epithelial cells were shown to increase their secretion of glycodelin when co-cultured with stromal cells. We found that stromal cells mediate the proliferative effect of estrogens (or anti-proliferative effect of progestins) on endometrial epithelial cells by their secretion of paracrine growth factors. This interaction could be reproduced with medium conditioned by stromal cells in place of direct co-culture. Moreover, we found that only stromal cells grown on extracellular matrix (ECM) could mediate the estrogen regulation of epithelial cell proliferation (Arnold et al., 2002). The studies using conditioned medium point out the role of paracrine growth factors secreted by stromal cells as regulators of epithelial cell proliferation.

We subsequently immortalized a primary stromal cell population by transducing a human telomerase reverse transcriptase subunit (hTERT) (Barbier et al., 2005). This cell line, named SHT290, has been shown to substitute for normal primary stromal cells in the co-culture system, and can mediate the hormonally regulated proliferative response in the same manner. We used this system to recreate the progestagenic effects of the hormone replacement therapy drug Tibolone in endometrium *in vivo*, and showed that it results from the metabolism of the drug by co-cultures of endometrial cells (Barbier et al., 2008). This pattern of metabolism is not demonstrated by epithelial cells cultured in the absence of stromal cells; Tibolone was also estrogenic in mono-cultures. This is further evidence that endometrium reconstructed in these co-cultures reproduces the normal endometrium *in vivo* better than endometrial mono-cultures.

Another aspect of endometrial epithelial-stromal interaction that we studied is gap junctions, the semi-permeable transmembrane pores formed between adjacent cells that permit the exchange of molecules smaller than one kilodalton (KDa) [Larsen and Risinger, 1985; Spray, 1985; Revel et al., 1985]. Gap junctions are formed by hemichannels composed of six protein subunits referred to as connexins in both adjacent cells which align and assemble into a channel between the cells called a connexon [Beyer, 1990]. This aspect of cell interaction is important because several groups of investigators have shown a correlation between reduced functioning of gap junctions, known as gap junctional intercellular communication (GJIC), and carcinogenesis and metastatic potential, primarily in cells of epithelial origin [Cronier, L., et al., 2009; Fitzgerald et al., 1994; Klann et al., 1989; Leithe, et al., 2006; Mesnil, et al., 2005; Nicholson et al., 1988; Trosko, 2003; Vinken, et al., 2009;]. Consistent with this, our earlier studies on gap junctions showed an inverse correlation between connexin 43 (Cx 43) protein expression in endometrial cells and the degree of progression in grade of endometrial epithelial carcinoma *in vitro* and *in vivo* [Schlemmer et al., 1999]. Conversely, studies with normal endometrial epithelial cells showed that co-culture with normal endometrial stromal cells increases Cx 43 expression in the epithelial cells. Subsequent studies showed that GJIC was induced in normal epithelial cells when they were co-cultured with stromal cells; this effect could not be reproduced when malignant epithelial cells were cultured with stromal cells [Schlemmer and Kaufman, 2000]. Furthermore, ultrastructural studies showed that interactions between stromal and epithelial cells in the normal human endometrium caused an increase in the size and number of gap junctions in epithelial cells [Roberts et al., 1988].

In this report we consider how human endometrial stromal cells mediate their effects on GJIC between endometrial epithelial cells and whether this effect could be mediated by medium conditioned by stromal cells. Further, based on reports that exogenous application of prostaglandin E_1 [Radu et al., 1982] increased GJIC between mammalian cells we evaluated whether prostaglandins could replace stromal cells or stromal cell-conditioned medium on GJIC in endometrial epithelial cells and whether prostaglandins could influence GJIC between endometrial cancer cells.

Material and Methods

Materials

Indomethecin and prostaglandins E_1, E_2, and $F_{2\alpha}$ were purchased from Sigma-Aldrich (St. Louis, MO).

Endometrial Tissue Isolation

Samples of uterine tissue were obtained from patients at the University of North Carolina Hospitals. Fragments of endometrial tissue are collected and placed in ice cold F12 media with antibiotics, minced into 1 mm pieces, and enzymatically digested with collagenase I and III (Sigma-Aldrich., St. Louis, MO) overnight at 4°C. Endometrial epithelial and stromal cells were separated by differential centrifugation at 4°C using 1000g for 3 min to isolate epithelial cells and 2500g for 5 min to isolate stromal cells.

Tissue Culture

Endometrial epithelial cell lines used for these studies were FEEC (Fetal Endometrial Epithelial Cells; immortalized with SV40 large T antigen), HEC-1A (Stage 1A endometrial carcinoma, [Kuramoto et al., 1972]), and RL-95-2 (Grade II endometrial carcinoma, [Way et al., 1983]). Normal endometrial stromal cells were obtained as described above and grown from frozen stocks maintained in our laboratory. Endometrial epithelial and stromal cells were cultured on 100 mm plastic dishes (Thermo Fisher Scientific, Pittsburgh, PA) coated with a 1:5 dilution of phenol-red free Matrigel (Collaborative Biomedical Products Division, BD Biosciences Discovery, Bedford, MA). The growth medium consisted of phenol-red free M199/F12 (1:1) with 2 μg/ml insulin. Antibiotic/antimycotic (Life Technologies, Gibco BRL Division, Gaithersburg, MD), Mitoplus (Collaborative Biomedical Products, Bedford, MA), bovine pituitary extract (Collaborative Biomedical Products, Bedford, MA), and insulin-transferrin-selenium (Boehringer-Mannheim, Indianapolis, IN) were added according to manufacturers' instructions. Phenol red was omitted to help reduce potential effects of estrogen agonists [Bindal et al., 1988]. The FEEC, HEC-1A, and RL-95-2 cell lines were chosen to represent progressive stages in the evolution of endometrial cancer. Cells were harvested while cultures were in exponential growth phase by removal of growth medium and treatment of cells with 2 ml of Dispase (Collaborative Biomedical Products, Bedford, MA) per 100 mm plastic dish for 10 min at 37°C. Subsequently the cells were washed with HBSS containing 10 mM EDTA, washed again with HBSS, and then replanted on single- well LAB-TEK glass microscope slides (NUNC Division, Thermo Fisher Scientific, Pittsburgh, PA) that had been coated with a 1:5 dilution of phenol-red free Matrigel for microinjection experiments.

Microinjection

This procedure was performed in a manner similar to Ruch and Klaunig with minor modifications [Ruch and Klaunig, 1988]. Glass micropipettes were made with a Model P-87 micropipette puller (Sutter Instrument Co., Novato, CA) and backfilled with 5% (w/v) Lucifer Yellow CH (Sigma-Aldrich Co., St. Louis, MO) in 0.1 M LiCl. Microinjections

were performed on exponentially growing cells using a Model 5242 air pressure microinjector (Eppendorf North America Inc., Hauppauge, N.Y.) under differential interference contrast (DIC) microscopy. Photographs were taken of epifluorescent light generated from the donor and dye coupled cells 5 min after microinjection using a Zeiss Inverted Microscope (Carl Zeiss Co., USA, Peabody, MA). For co-cultured epithelial and stromal cells, the donor and recipient cells were distinguished from each other as described previously [Schlemmer and Kaufman, 2000].

Statistical Methods

The student's t-test was used to compare results from the microinjection experiments.

PGE_2 ELISA

An ELISA analysis for PGE_2 was performed with a PGE_2 immunoassay kit (R&D Systems Inc., Minneapolis, MN) according to manufacturer's directions.

Results

Figure 1 illustrates results of dye transfer experiments. Panels 1A and 1B illustrate cultures of normal epithelial cells, cultured alone in conventional medium. Panels 1C and 1D illustrate epithelial cells that were cultured using medium that was conditioned by stromal cells. Conditioning of medium was done by culturing young stromal cells in the medium for 48 hours, and then diluting it 1:1 with unconditioned medium. Panels 1E and 1F illustrate epithelial cells that were cultured in the presence of stromal cells. The photos on the left side (panels 1A, 1C, and 1E) were taken using differential interference contrast microscopy to identify the location of the cells analyzed, and photos on the right side (panels 1A, 1C, and 1E) were taken using epifluorescence to demonstrate cells containing Lucifer yellow. When epithelial cells were cultured alone, dye did not spread to adjacent cells, which indicates a lack of GJIC, (panel 1B). In contrast, there was clear evidence of dye spreading to adjacent cells when the epithelial cells are exposed to media conditioned by stromal cells (panel 1D) or co-cultured with stromal cells (panel 1F). Panels 1A, 1B, 1E, and 1F were previously published [9], and are shown here only for the purpose of comparison to panels 1C and 1D. The number of cells showing dye transfer was determined for replicate experiments and the results were used as a measurement of GJIC capacity under the given experimental conditions (Table 1). These results indicate that GJIC between endometrial epithelial cells is induced by endometrial stromal cells, but the stromal cells do not have to be physically present to exert their effect on GJIC between the epithelial cells. This indicates that induction of GJIC in endometrial epithelial cells is mediated by one or more diffusible extracellular factors secreted by stromal cells. Data in Table 1 showing GJIC between epithelial cells cultured alone and with stromal cells was previously published [Schlemmer and Kaufman, 2000] and is reprinted here for the purpose of comparison with new findings.

Because prostaglandins are known to be secreted by endometrial cells and because they were known to increase GJIC between epithelial cells in other tissues, we evaluated certain aspects of prostaglandin metabolism in these endometrial cell cultures. To determine whether stromal cell cultures secreted PGE_2 into the culture medium, we performed a competitive ELISA using growth medium recovered after incubation with near-confluent stromal cells for 48 hours. The results showed a PGE_2 concentration of 182±23 ρM. Next, we attempted to determine whether addition of PGE_2 to culture medium could substitute for stromal cells or stromal cell conditioned medium as an inducer of GJIC in endometrial epithelial cells. Normal epithelial cells were cultured for 24 hours in growth medium to which 1 μM, 1 nM, or 1 ρM PGE_2 had been added and then GJIC was assayed. As compared with epithelial cells cultured alone without PGE_2, GJIC was inhibited by 1 μM

PGE_2, stimulated by 1 nM PGE_2, and not changed significantly by 1 ρM PGE_2 (Figure 2). The results for GJIC in epithelial cells in coculture with stromal cells are shown for purposes of comparison, and are the same as epithelial cells exposed to medium conditioned by stromal cells. Further evidence for the involvement of PGE_2 in controlling GJIC between normal epithelial cells and stromal cells was demonstrated by pre-treatment of a coculture of normal epithelial cells and stromal cells with 1 μM of indomethecin 24 hours before microinjection. These experiments showed there was 85.5% inhibition of GJIC as compared to epithelial cells cultured alone (Figure 2).

Figure 3 illustrates dye transfer following microinjection as an indication of GJIC in transformed endometrial epithelial cells. Results show GJIC where transformed epithelial cells were cultured without PGE_2 (panels 3a, 3c, and 3e) or with the concentrations of PGE_2 shown to produce the maximal GJIC effect in each of the transformed epithelial cell lines PGE_2 (panels 3b, 3d, and 3f). All of the photos were taken under epifluorescent illumination five minutes after microinjection. FEEC cells cultured alone failed to transfer dye between cells (panel 3a) while after treatment with 10 μM PGE_2 the injected FEEC cell transferred dye to four cells (panel 3b). HEC-1A cells cultured without PGE_2 did not show GJIC (panel 3c) but after treatment with 100 nM PGE_2 the injected HEC-1A cell transferred dye to four cells (panel 3d). Similarly, untreated RL-95-2 cells did not transfer dye (panel 3e) whereas RL-95-2 cells treated with 100 nM PGE_2 transferred dye to four adjacent cells (panel 3f). These inductive effects of PGE_2 on GJIC in FEEC, HEC-1A, and RL-95-2 cells were specific in so far as treatments with 1μM PGE_1 or 1μM $PGF_{2\alpha}$ did not re-establish GJIC (data not shown).

The results of studies comparing the concentration of PGE_2 to the induced effect on GJIC in transformed endometrial epithelial cells are illustrated in Figure 4. These studies assess the GJIC induced in response to one-log increments in concentration of PGE_2 when added to cultures of FEEC, HEC-1A, and RL-95-2 cells. Results were compared to control experiments without PGE_2. All observations showed increases in GJIC that were statistically significant except at 10 μM PGE_2 with the HEC-1A cells where the increase did not achieve statistical significance. The results show that GJIC can be induced in each of the three cell lines by treatments with PGE_2. The maximal induction of GJIC between HEC-1A cells and between RL-95-2 cells was observed with 0.1 μM PGE_2, while it required 10 μM PGE_2 to achieve the maximal induction of GJIC in FEEC cells.

Discussion

Previous investigations have shown that intercellular communications through gap junctions is decreased in malignancies (Cronier, L., et al., 2009; Leithe, et al., 2006; Mesnil, et al., 2005; Vinken, et al., 2009; Yamasaki, et al., 1995). In previous studies we have demonstrated an abnormality of connexin expression in endometrial cancers (Schlemmer, et al., 1999). We followed this by demonstrating that intercellular communication via gap junctions was absent in endometrial epithelial cells or endometrial cancer cells when assessed in cell culture (Schlemmer and Kaufman, 2000). We showed, however, that co-culture of the normal epithelial cells with stromal cells would restore intercellular communication between the endometrial epithelial cells. Therefore, interaction between stromal and epithelial cells was essential for this normal epithelial cell function. The results of this study extend our previous observations concerning the activity of GJIC in human endometrial epithelial cells. The current study shows that the stromal cells need not be physically present for GJIC to be induced. Medium in which normal endometrial stromal cells had been grown (conditioned medium) was able to substitute for stromal cells in causing an increase GJIC between normal epithelial cells. The induction appears to be mediated by one or more soluble and diffusible factors made by the stromal cells and

secreted into their culture medium in vitro and presumably into their immediate environment in vivo. Our studies *in vitro* also indicate that PGE_2 is one such mediator. We have shown that the stromal cells secrete PGE_2 into the conditioned medium that can induce GJIC. We also have shown that treatment of cultures of normal endometrial epithelial cells with 1 nM PGE_2 can induce GJIC. Conversely, we have shown that if PGE_2 production is inhibited in cultures containing stromal cells, GJIC is blocked in the epithelial cells. While treatment of normal endometrial epithelial cell cultures with 1 nanomolar (nM) PGE_2 can induce GJIC, treatments with much higher (1 micromolar (μM)) or much lower (1 picomolar (ρM)) concentrations of PGE_2 inhibited GJIC or had no effect. Even at its most effective concentration, treatment of epithelial cell cultures with PGE_2 restored only part of the induction of GJIC produced by stromal cells. This indicates that other factors produced by stromal cells contribute to the induction of GJIC.

Several other investigators have done studies *in vitro* and *in vivo* attempting to resolve the role of PGE_2 in tumorigenesis. Studies done with human colorectal tumor tissue have documented an increase in cyclooxygenase 2 (COX-2) expression [Sano et al., 1995], and suggested that increased PGE_2 production is a factor contributing to the induction of colorectal cancer. Consistent with this, studies using transgenic mice that overexpress the human COX-2 gene in their mammary glands showed tumorigenesis and metastasis in a tissue-specific manner [Lui et al., 2001]. However, increased production of PGE_2 was not sufficient for mammary cancer development; this effect of increased PGE_2 only occurred in the mice after three to four rounds of weaning and pregnancy. Other investigators have shown that homologous disruption of the genes coding for COX-1 or -2 expression reduced polyp formation in *Min*/+ mice by approximately 80% [Chulada et al., 2000]. These investigators suggested that other factors are required in addition to PGE_2 for carcinogenesis, and that PGE_2 is involved with malignant progression. Our results indicate that a sufficient and localized concentration of PGE_2 may block GJIC between normal epithelial cells (Figure 2) at a concentration of 1 μM. It is conceivable that this concentration could be attained by inflammation associated with colon carcinoma *in situ* due to the release of PGE_2 by macrophages responding to intestinal microorganism invasion.

Consequently, suboptimal concentrations of PGE_2, e.g. 1 ρM, may not be sufficient to maintain epithelial cell differentiation and sustain GJIC; the absence of normal epithelial differentiation and loss of GJIC may contribute to carcinogenesis. Conversely, the presence of excessive concentrations of PGE_2, e.g., 1 μM (Figure 2), may act to inhibit GJIC, which would also result in carcinogenesis. Measurements done with stromal cell conditioned media using an ELISA kit showed the PGE_2 concentration to be in the picomolar range, and maximal GJIC was observed when a similar concentration was applied to normal epithelial cells in vitro (Figure 2).

Besides its effect on normal endometrial epithelial cells, our results also show that PGE_2 can cause GJIC to be re-established in the three transformed endometrial epithelial cells lines examined. The concentration of PGE_2 required for reestablishing GJIC varied for the three cell lines. It required a 100-fold greater concentration of PGE_2 to re-establish GJIC in the FEEC cells as compared to HEC-1A and RL-95-2 cells. The RL-95-2 cells showed a greater response than the HEC-1A cells in terms of the number of cells induced to communicate at the same PGE_2 concentrations. HEC-1A cells showed a broader concentration/response effect than FEEC or RL-95-2 cells, but also show a reduced number of cells that re-establish GJIC.

Previous observations and those that we report here suggest features of the mechanism by which GJIC is controlled between endometrial epithelial cells by factors secreted by stromal cells. Endometrial stromal cells produce PGE_2 and $PGF_{2\alpha}$ under physiological conditions,

and the amount of PGE_2 produced is much greater than $PGF_{2\alpha}$ [Gal et al., 1982]. Other studies have shown that the enzymes for metabolism of prostaglandins are almost exclusively located in the endometrial epithelial cells and not in the stromal cells [Casey et al., 1980]. Presumably, stromal cells synthesize and secrete PGE_2 into the extracellular milieu and it then diffuses into the epithelial cells where it is metabolized and induces GJIC and a number of other cellular functions. This stromal cell induction of GJIC in epithelial cells is essential to the normal functioning of the epithelium. It is thought that normal GJIC function might suppress progression of carcinogenesis whereas the loss of GJIC between the epithelial cells appears to be a feature of developing malignancies. Consistent with this conjecture is the previous observation that showed normalization of growth control in transformed cells by transfections that increased the amount of connexin proteins and enhanced GJIC [Mehta et al., 1991; Rose et al., 1993].

Other studies done with PGE_2 have sought to understand the role of PGE_2 regarding labor induction [Adamo, et al., 2001] in a murine model and expression of connexin 43, cyclooxygenases, and $G_{s\alpha}$ proteins in the human uterus during pregnancy and labor using myometrial tissue [Cheng et al., 2001]. The studies described in this report only involve interactions between uterine stromal and epithelial cells; myometrial tissue was discarded during tissue processing to isolate primary stromal and epithelial cells. Additionally, studies done with osteocyte-like MLO-4 cells have documented an increase in Cx 43 expression and a small but significant increase in GJIC between these cells occurs in the presence of PGE_2. This effect was inhibited by treatment with indomethecin and inducible by the stimulatory effect of fluid flow, which causes mechanical strain to the cells [Cook et al., 2000]. However, other studies relating Cx 43 expression in oral-derived human osteoblasts with PGE_2 exposure showed no change in Cx 43 expression [Sparey et al., 1999]. In addition, these studies documented that the levels of Cx 43 expression varied significantly from one patient to another and also from which oral bone source the cells were isolated. Overall, our data and the studies of other investigators using different organ systems seem to indicate that connexin expression, GJIC, and the effects of PGE_2, if any, appear to be tissue specific.

Conclusions

Endometrial stromal cells use PGE_2 as a paracrine factor to facilitate differentiation (as exemplified by GJIC function) and to inhibit cell proliferation in endometrial epithelial cells. The evolution of endometrial cancer is characterized by a loss of endometrial stroma from between endometrial epithelial glands. We have shown previously that epithelial endometrial cancer cells secrete products that inhibit endometrial stromal and normal epithelial cell growth (Albright and Kaufman, 1995; Albright et al., 1995) and that stromal cells in the absence of hormonal stimulation secrete paracrine products that inhibit endometrial epithelial cell growth (Watson et al., 1995). It is likely that paracrine factors secreted by malignant endometrial epithelial cells cause the observed reduction in the ratio of stromal to epithelial cells seen in endometrial cancer. Consequently the differentiating effects and reduction of cell proliferation that stromal cells normally produce in epithelial cells is lost or greatly reduced during the evolution of endometrial cancers. Because the endometrial epithelial cells in evolving cancers are not constrained by stromal cell paracrine factors because the stromal cells are decreased in number or are absent, cancer development would not require the malignant cells to develop abnormal signaling and regulatory pathways to nullify the inhibitory effects of the stromal cells. Conceivably, endometrial cancer cells retain their responsiveness to stromal-derived paracrine factors that affect GJIC, cell proliferation and other cell functions. Our studies test this concept. While malignant endometrial epithelial cells lose responsiveness to stromal cell regulation by PGE_2 as a consequence of transformation, the loss of responsiveness to PGE_2 is not complete because a 100-fold or greater increase in PGE_2 can overcome this deficiency. This is significant

because it suggests a possible new therapeutic approach for treatment of incipient or overt endometrial cancer. Perhaps, prostaglandin-like compounds or compounds that cause increased PGE_2 production in the stromal cells could be of benefit in treatment of endometrial carcinomas or might suppress progression of premalignant lesions of the endometrium. Other approaches to cancer therapy via effects on gap junctions and GJIC have been discussed recently (Kandouz and Batist, 2010).

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Abbreviations

Cx	connexin
DIC	differential interference contrast
ELISA	enzyme linked immunosorbent assay
GJIC	gap junctional intercellular communication
IL-1	interleukin 1
KDa	kilodalton
LiCl	lithium chloride
Min	Multiple intestinal neoplasia
PGE_1	prostaglandin E_1
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$

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Highlights

- Endometrial epithelial and stromal cells in co-culture reproduce tissue functions in vivo
- Stromal cells in co-culture activate GJIC in normal endometrial epithelial cells
- Conditioned medium can replace stromal cells in co-cultures for activating GJIC
- Stromal cell conditioned medium does not restore GJIC in epithelial cancer cells
- Prostaglandin E_2 in medium induced GJIC activity in normal epithelial cells
- Prostaglandin E_2 restored some GJIC activity in epithelial cancer cells

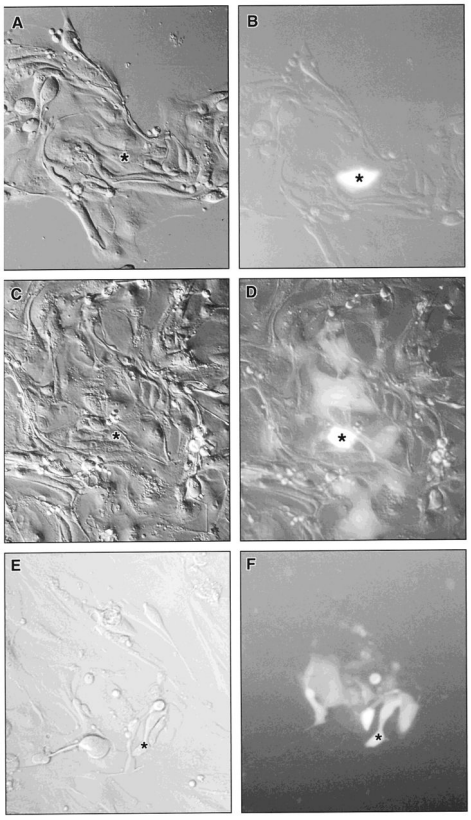

Figure 1.

Lucifer yellow dye coupling assay of normal epithelial cells cultured alone, cultured with medium conditioned, and co-cultured with stromal cells. The black asterisk marks the donor cell that was injected with Lucifer yellow. **1A**. Differential interference contrast (DIC) photo of normal epithelial cells[a]. **1B**. Epifluorescence photo of cells shown in **1A** five minutes after microinjection with Lucifer yellow. **1C**. DIC photo of normal epithelial cells cultured with medium conditioned by stromal cells[a]. **1D**. Epifluorescence photo of cells shown in **1C** five minutes after microinjection with Lucifer yellow. **1E**. DIC photo of normal epithelial cells co-cultured with stromal cells[a]. **1F**. Epifluorescence photo of cells shown in **1E** five minutes after microinjection with Lucifer yellow. [a]These photos were published previously [Schlemmer and Kaufman, 2000].

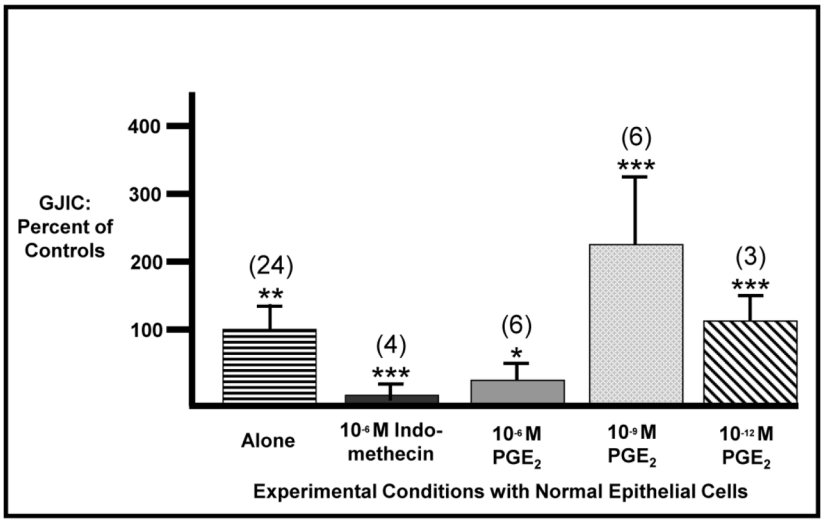

Figure 2.

The effect of the concentration of PGE_2 on GJIC between normal endometrial epithelial cells. The percentage of epithelial cells showing GJIC (using epithelial cells alone as 100%) is shown by the height of the bar graphs under the experimental condition is shown in parentheses above the asterisks. The statistical probability (*p*) is shown by the number of asterisks above each standard deviation upper bound, and are expressed as: * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. The number of individual experiments (n) for each condition is shown in parentheses above the asterisks.

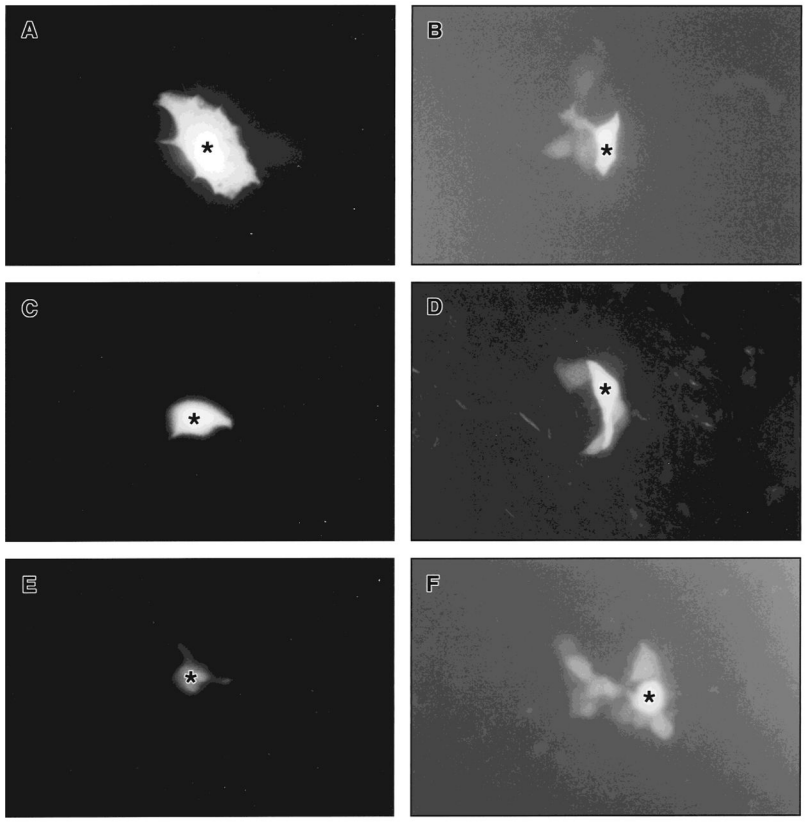

Figure 3.

Lucifer yellow microinjection of FEEC, HEC-1A, and RL-95-2 transformed epithelial cells alone and with the concentration of PGE_2 that produced the maximum amount of GJIC. The black asterisk marks the dye donor cell. The photos were taken five minutes after microinjection with Lucifer yellow. **3A**. Epifluorescence photo of FEEC cells alone. **3B**. Epifluorescent photo of FEEC cells with 10 μM PGE_2. **3C**. Epifluorescence photo of HEC-1A cells alone. **3D**. Epifluorescent photo of HEC-1A cells with 100 nM PGE_2. **3E**. Epifluorescence photo of RL-95-2 cells alone [a].**3F**. Epifluorescent photo of RL-95-2 cells with 100 nM PGE_2[a]. [a]These photos were published previously [Schlemmer and Kaufman, 2000].

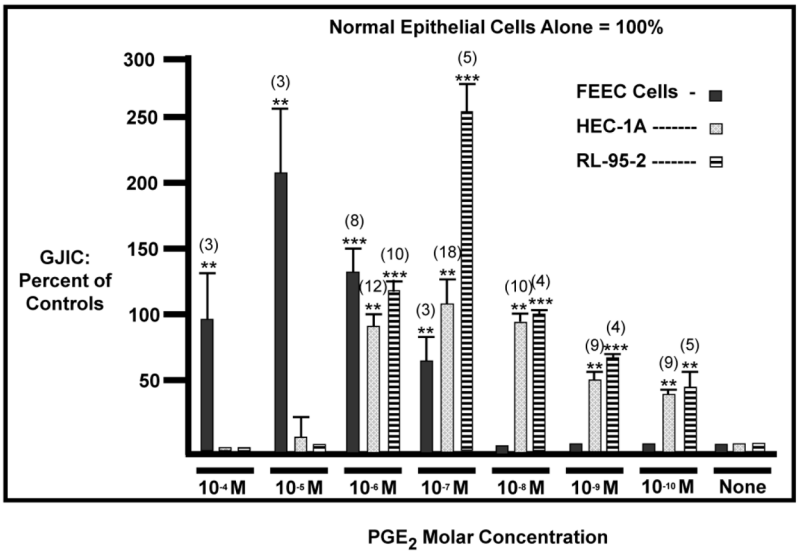

Figure 4.

Concentration/response of PGE_2 on GJIC between transformed epithelial cells. The percentage of transformed epithelial cells showing GJIC (using normal epithelial cells alone as 100%) is shown by the height of the bar graphs and the PGE_2 concentrations below each set of bars. The results from the FEEC cells are shown as white bars, HEC-1A as gray bars, and RL-95-2 as black bars. The standard error of the mean is shown by the T-shaped symbols on top of each bar. The statistical probability (p) is shown by the number of asterisks above each standard deviation upper bound, and are expressed as: * p <0.05; ** p <0.01; and *** p < 0.001. The number of individual experiments (n) for each condition is shown in parentheses above the asterisks.

TABLE 1

Epithelial Cells Cultured	Average Number of Cells Showing GJIC (± SD)	n	P[a]
Alone	1.0 (1.0)	24	0.0025[b,e]
With Stromal Cells Present	3.2 (2.2)	25	0.00044[c,e]
With Stromal-Cell-Conditioned Medium	2.5 (1.9)	6	0.0094[d]

[a] Statistical significance determined by Student's t test

[b] Epithelial cells cultured alone vs. malignant epithelial cells cultured alone

[c] Epithelial cells cultured alone vs. epithelial cells co-cultured with stromal cells

[d] Epithelial cells cultured with stromal-cell-conditioned medium vs. epithelial cells cultured without stromal cell conditioned medium

[e] These data were published previously [Schlemmer and Kaufman, 2000].