A Three-Dimensional and Temporo-Spatial Model to Study Invasiveness of Cancer Cells by Heregulin and Prostaglandin E2

Liana Adam, Abhijit Mazumdar, Tushar Sharma, Terence R. Jones, and Rakesh Kumar

Abstract

To study the temporal expression of motile structures and protease activity during colon cancer cell invasion by heregulin-β1 (HRG) and prostaglandin E2 (PGE2), we have developed a three-dimensional spatial model system. HRG and PGE2 each induced the formation of well-organized, three-dimensional structures with empty spaces in the center and stimulated the expression of urokinase plasminogen activator (uPA) with differential localization of membrane-bound uPA at the focal adhesion points and leading edges of the motile cells. A specific cyclooxygenase-2 inhibitor blocked the formation of these luminal glandular structures induced by PGE2 but did not block those induced by HRG. A specific antagonist of uPA receptor completely blocked the formation of these luminal glandular structures induced by PGE2 and HRG. These findings suggest that HRG-mediated increased invasiveness of colon cancer cells is augmented at least in part by induction of PGE2 and uPA, and this augmentation may involve the formation of three-dimensional invasive structures via the uPA pathway. In addition, the three-dimensional model system presented here may have a wider application to screen the effects of therapeutic compounds and biomolecules on different spatial aspects of colon cancer biology, including cell growth, motility, invasion, survival, and apoptosis.

Introduction

Accumulating evidence suggests that colonic tumorigenesis is partially regulated by the growth factor-inducible form of COX-2, an enzyme responsible for the conversion of arachidonic acid to PGs. PGs appear to play a variety of roles in the gastrointestinal tract, including participation in physiological processes such as cell motility and in pathological processes such as neoplasia. The primary PG generated in colon cancer tissue appears to be PGE2. The COX-2 isozyme is inducible and found in very low levels in normal tissues but in greatly increased levels in inflamed tissues. In addition, increased levels of PGs, COX-2 protein, and COX-2 mRNA have been reported in colorectal adenomas and carcinomas, but not in adjacent normal-appearing mucosa, suggesting a potential link between intestinal COX-2 activity and tumorigenesis.

COX-2 expression in colorectal tumorigenesis and showed dramatic reductions in the number and size of intestinal polyps by a specific COX-2 inhibitor. Furthermore, ectopic overexpression of COX-2 can also lead to inhibition of apoptosis in colon cells and increased metastatic potential of colon cancer. Alterations in cytoskeleton reorganization in head and neck cancers have been shown to be related to PGE2-mediated modulation of cell adherence.

Recent studies suggest that in addition to the COX-2 pathway, the invasiveness of colon cancer can be also influenced by growth factors such as HRG and serine proteases such as uPA. For example, HRG, which binds to the HER-3 and HER-4 receptor, regulates the progression of cancer cells to a more invasive phenotype. Recently, we confirmed that in the absence of HER-2 (also known as c-erbB2 or c-neu) overexpression, HRG activation of breast and colon cancer cells promotes the development of a more invasive phenotype in these cells. The mechanisms and pathways by which HRG influences the biology of colon cancer cells remain elusive.

One of the important steps for tumor progression and invasion is the destruction of the ECM that separates the epithelial and stromal compartments by serine proteinases, thus facilitating the migration and invasiveness of cancer cells. The uPA system-mediated proteolysis contributes to the dissolution of connective tissues surrounding the cancer cells and of the perivascular basement membrane. Evidence suggests that the progression of colon cancer cells also involves the uPA, which has its own high-affinity cell surface receptor, uPAR, that greatly enhances the action of uPA on plasminogen.

The expression of uPA and uPAR have been demonstrated in essentially every solid tumor type examined to date. Expression is not restricted to the tumor cells themselves; several tumor-associated cell types including macrophages, mast cells, endothelial cells, natural killer cells, and fibroblasts are all capable of uPA and uPAR expression. The pattern of expression in these cells differs, depending on the type of tumor. For example, tumor cells that exclusively express uPA (but not uPAR), such as some forms of colon cancer, can recruit uPA made by surrounding stromal cells.

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Our previous studies showed that HRG stimulates the secretion of PGE2 and in vitro invasiveness of human colon cancer cells. It is not known, however, whether uPA plays a role in the actions of HRG and PGE2 in colon cancer cells. The purpose of this study was to establish whether HRG-mediated stimulation of colon cancer invasiveness could be influenced by PGE2 and uPA. To demonstrate this, we used a three-dimensional model in which we could modulate the
architectural remodeling of FET colon cells by blocking different levels in the proposed HRG-induced uPA activation pathway. Using a simplified in vitro model, this study demonstrated for the first time that both HRG and PGE2 augmentation involve the formation of distinct three-dimensional glandular structures. Because HRG is a paracrine growth factor, these findings raise the possibility that HRG- and PGE2-mediated up-regulation of uPA in tumor cells may enhance the ability of colon tumor cells to degrade the extracellular milieu and thereby invade normal tissue.

Materials and Methods

Cell Culture and Reagents. FET human colon carcinoma cells (15, 23) were maintained in DMEM:F12 (1:1) supplemented with 10% FCS. Anti-epidermal growth factor receptor antibody and HRG were purchased from Neomarkers, Inc. (Fremont, CA). The β-catenin polyclonal antibody (24) was a gift from P. McCrea (The University of Texas M. D. Anderson Cancer Center, Houston, TX). The anti-uPA monoclonal antibody and recombinant low molecular weight uPA were purchased from American Diagnostica. The COX-2 inhibitor NS398 was purchased from Sigma.

Boyden Chamber Assay. The effect of HRG on the invasiveness of FET colon cells was determined by the Boyden chamber assay (14). Briefly, the bottom of the porous 8 μm filter was coated with a thick layer of Matrigel/DMEM:F12 (1:2) that serves as a chemoattractant for the cells that are plated on the upper side of the filter. After 12 h, the cells that pass through the pores and reattach on the lower surface of the filter were quantitated. The filters were fixed, stained with propidium iodide (0.1%, w/v), mounted on a slide, and analyzed by confocal microscopy. The cells that invaded the Matrigel were considered invasive, and results were expressed as the percentage of total cells/ microscopic field (×40 magnification) from both sides of filter plus those en passage that were inside the pores.

Formation of Lumen-containing Three-dimensional Structures. Cells were allowed to grow on a thick layer of Matrigel. Briefly, 100 μl of Matrigel/DMEM:F12 (diluted 1:2) were added to each well of a Lab-Tek eight-chamber slide (Molecular Probes) and allowed to gel for 30 min at 37°C. FET cells were trypsinized, washed sequentially in DMEM:F12 supplemented with 10% serum and DMEM:F12 without serum, and resuspended into single cells, and 10,000 cells were resuspended in 400 μl serum-free DMEM:F12/ well. After 10 min, HRG (10–100 ng/ml) or PGE2 (10–100 ng/ml) was added, and slides were monitored for several days for morphological changes. A concentration of 30 ng/ml HRG and 50 ng/ml PGE2 was able to induce formation of cellular clusters after 48 h for HRG and after 8 h for PGE2. In some cases, we used Matrigel/DMEM:F12 (diluted 1:4)-coated Lab-Tek chambers to analyze changes in cellular shapes reminiscent of a motile phenotype. All morphological changes were analyzed after immunostaining and confocal microscopy analysis.

Northern Analysis. Total cytoplasmic RNA was analyzed by Northern blot analysis using cDNA probes against uPA and uPAR (American Type Culture Collection, Manassas, VA).

Growth Assay. Cell proliferation was measured by the MTT dye (Sigma) uptake method, as described previously (25). About 7000 cells/0.5 ml culture medium were seeded into each well of a 24-well plate. After 24 h, appropriate cultures were supplemented with HRG or PGE2. For each time point, the medium was removed from triplicate wells, MTT (5 mg/ml PBS) was added, and the plate was wrapped in aluminum foil and kept at 37°C for 4 h. The dye solution was aspirated, and the dye taken up by the cells was extracted in 1 ml of isopropanol:1N HCl (96:4) for determination of absorbance at 570 nm.

Immunostaining and Confocal Analysis. For immunofluorescence staining, cells were grown on uncoated or Matrigel-coated Lab-Tek chamber slides. After appropriate treatments, cells were fixed for 10 min in a 1:2 mixture of cold methanol and ethanol and incubated with the indicated primary antibodies followed by the respective FITC- or rhodamine-conjugated secondary antibodies (Molecular Probes). Confocal imaging, three-dimensional analysis, and intensity quantification were performed using a Zeiss LSM and personal computer-based LSM510 Version 2.01 software (14, 15).

Fig. 1. HRG and PGE2 induce cytoskeleton modifications in colon carcinoma cells. FET cells cultured on (A–C) plastic or (D–F) Matrigel were processed for indirect immunofluorescence microscopy. Representative examples of rhodamine-phalloidin staining (A–C) show the distribution of F-actin; representative examples of FITC-conjugated antimouse secondary antibody staining (D–F) indicate the localization of β-tubulin. G, the Boyden chamber assay showing the effects of HRG (30 μg/ml, 12 h) and PGE2 (50 μg/ml, 12 h) on the invasion of FET cells. H, MTT assay showing the effects of HRG (30 μg/ml, 48 h) and PGE2 (50 μg/ml, 48 h) on the growth of FET cells.
Results and Discussion

**HRG and PGE$_2$ Induce Cytoskeleton Modifications.** We have shown previously that treatment of colon cancer cells with HRG leads to increased production of PGE$_2$ (15). Here we compared the effects of HRG and PGE$_2$ on the motility of a noninvasive FET colon cancer cell line. Incubation of colon cells grown on an uncoated surface with HRG or PGE$_2$ triggered cytoskeleton modifications in the FET cells that were visualized by F-actin staining (Fig. 1, A–C); these modifications were reminiscent of those in cells acquiring a more motile phenotype. HRG and PGE$_2$ treatments induced membrane ruffling (arrows) and changes in cell shape. In contrast to the uncoated slide, when cells were grown on Matrigel, the effect of PGE$_2$ was different from that of HRG, as assessed by staining with β-tubulin antibody (Fig. 1, D–F). HRG treatment induced the formation of oblong cell clusters (Fig. 1E); the PGE$_2$ treatment induced the formation of intracellular pseudoglandular structures containing large empty spaces (Fig. 1F). Consistent with the invasive nature of PGE$_2$-treated cells grown in Matrigel, PGE$_2$ promoted the invasion of FET cells using Boyden chamber assays (Fig. 1G) but did not have any effect on cell growth as measured by the MTT assay after 48 h of treatments (Fig. 1H). In contrast, HRG induced cell invasion as well as cell proliferation (Fig. 1G and H, respectively).

**Three-dimensional Structures Induced by PGE$_2$ Differ from Those Induced by HRG.** Treatment with PGE$_2$ of cells plated on Matrigel induced the formation of three-dimensional cellular structures that were visible by phase-contrast microscopy after 12 h of treatment. The treatment of FET cells with HRG induced the formation of three-dimensional structures that were visible after 48 h of treatment (Fig. 2A). The results of phase-contrast microscopy studies suggested that the structures induced by PGE$_2$ were morphologically different from those induced by HRG and characterized by different time kinetics. Using specific markers such as β-catenin for intercellular junctions or propidium iodide for nuclear DNA, we analyzed these structures by confocal scanning microscopy followed by three-dimensional reconstructions.
HRG and PGE₂ Differentially Modulate the Expression Level and Temporal Distribution of uPA. Modification of the invasive potential of a cell is generally accompanied by rearrangement of the cytoskeleton and changes in the pericellular proteolytic activity, where the uPA/uPAR system is playing an important role. Recently, we showed that HRG up-regulates the expression and functions of uPA/uPAR in breast cancer cells (26). In the present study, we analyzed whether either HRG or PGE₂ up-regulates the expression of uPA and uPAR mRNA in FET cells (26). Northern blot analysis demonstrated that HRG (30 ng/ml), but not PGE₂ (50 μg/ml), up-regulated the level of uPAR mRNA to levels higher than those seen in untreated control cells (Fig. 3A). Both HRG and PGE₂ up-regulated the levels of uPA mRNA, although they did so to different extents (Fig. 3A).

Because spatially localized degradation of the ECM is important for tissue remodeling and directed motility and invasion, we used confocal microscopy and three-dimensional reconstruction methods to analyze the temporal distribution of uPA bound to the plasma membrane of the cells. First, we examined the effect of HRG (30 μg/ml, 12 h) and PGE₂ (50 ng/ml, 12 h) on the distribution of uPA in FET cells plated on a thin layer of Matrigel (Fig. 3B). Both HRG and PGE₂ treatments substantially altered the cell shapes compared with no treatment in the control cells. These morphological modifications were accompanied by a dramatic redistribution of uPA, both at the level of focal adhesion points and, in the case of PGE₂ treatment, at the site of dome-like structures (Fig. 3B, lower and upper, respectively). More interestingly, the uPA redistribution occurred most frequently at the leading edges of the cells, followed in incidence by redistribution at the focal adhesion-like complexes in the case of cells treated with HRG. A low, uniform, peripheral localization of uPA could be observed in the case of the untreated FET cells (Fig. 3B). The epidermal growth factor receptor was costained to identify the cell membranes, including the free edges of FET cells, because costaining with β-catenin would identify mainly the intercellular junctions.

To evaluate the status of uPA distribution in the three-dimensional clusters induced after 48 h of PGE₂ and HRG treatment, cells were costained for β-catenin and uPA proteins and analyzed using confocal microscopy and three-dimensional reconstruction (Fig. 4). We also quantified the intensities of the pixels in each channel on the predefined horizontal sections, where the values are plotted on (a) a red histogram for the Alexa546 fluorochrome channel that corresponds to β-catenin and (b) a green histogram for the FITC channel that corresponds to uPA. Superimposition of the red and green peaks suggests the colocalization of proteins β-catenin and uPA (Fig. 4). In the structures induced by HRG, uPA was distributed mainly on the internal membrane facing the empty space of the structure and occasionally on cell membrane projections, which, as an example, are represented by the middle peak showing high pixel intensities in both the red and green channels. This was different from the uPA expression pattern in the PGE₂-induced structures, in which uPA was located on the cell membrane facing the outside surface of the cellular walls (Fig. 4). The levels of membrane-bound uPA were also different. Thus, the staining intensity of uPA in HRG-treated cells was two to three times higher than that in the PGE₂-treated cells. However, PGE₂ treatment was accompanied by an increase in the level of uPA staining compared with that found in the untreated control cells. In brief, these results suggested that the temporal uPA distribution and intensities in three-dimensional structures were differentially influenced by HRG versus PGE₂. However, both HRG and PGE₂ increased the level of membrane-bound uPA above the levels found in untreated control cells.

uPAR/uPA Interaction Is Necessary for the Formation of Lumen-containing Three-dimensional Structures. To understand how the three-dimensional structures are formed, we analyzed uPA
Fig. 4. Quantification of membrane-bound uPA in FET cells plated on Matrigel. Cells were costained for β-catenin and uPA and analyzed by confocal microscopy, followed by quantification of pixel intensities in each channel on predefined confocal Z sections. Numerical values are plotted on a red histogram for the channel corresponding to β-catenin and on a green histogram for the channel corresponding to uPA. Three different sections are shown for untreated cells (CON), HRG (30 ng/ml, 48 h)-treated cells, or PGE2 (50 ng/ml, 48 h)-treated cells.
suggest that PGE₂ synthesis may be necessary for the formation of initiation of lumen formation (Fig. 5).

They represent sites where the cellular junctions are disconnected, and the resulting free edges of the cells are flipped to allow the appearance of “hot spots” (arrows) on the cell-cluster surface that are not in direct contact with the Matrigel; these hot spots are characterized by uPA-bound and β-catenin-positive membrane projections. They show late morphogenetic cell modifications (after 48 h of treatment) with lumens (arrow) inside the three-dimensional structures and blockage by interference with the uPA/uPAR pathway. D. Boyden chamber invasion assay. Consistent with the effects of inhibitors in the three-dimensional assay, Å36 blocked the stimulation of FET cell invasion by both PGE₂ and HRG.

Fig. 5. Effects of specific inhibitors on the formation of three-dimensional structures by HRG and PGE₂. FET cells plated on Matrigel were treated with various combinations of PGE₂, HRG, NS398, and Å36 and processed for indirect immunofluorescence microscopy. A and B, whole-image projection show early morphogenetic cell modifications in PGE₂-treated (A) and HRG-treated (B) colorectal carcinoma cells. These early modifications correspond to a 6-h PGE₂ treatment and a 12-h HRG treatment. FITC-conjugated antimouse antibody staining shows the distribution of uPA; Alexa546-conjugated antirabbit secondary antibody indicates the localization of β-catenin. C, confocal images show late morphogenetic cell modifications (after 48 h of treatment) with lumens (arrow) inside the three-dimensional structures and blockage by interference with the uPA/uPAR pathway.

To understand the potential contribution of COX-2 and uPA pathways in the cellular remodeling leading to the formation of luminal three-dimensional structures (Fig. 5, arrows), we examined the effects of a specific COX-2 inhibitor, NS398 (15), and a specific uPAR antagonist, Å36, which acts as a competitive inhibitor (IC₅₀ = 5 nM) of uPAR on colon cancer RKO cells (27). Interestingly, cotreatment of cultures with both HRG and Å36 blocked the formation of lumens containing three-dimensional clusters (Fig. 5C). Cotreatment of cells with HRG and COX-2 inhibitor NS398 also blocked the formation of a lumen inside the three-dimensional clusters as well as the HRG effect on uPA distribution, despite a slight increase in uPA staining at the level of the focal adhesion complexes (Fig. 5C). Treatment of the cells with NS398 did not affect the formation of PGE₂-induced three-dimensional structures (Fig. 5C). Treatment of the cells with NS398 blocked the HRG-triggered increase in the invasiveness of FET cells (Fig. 5D). Similarly, treatment with Å36 inhibited the HRG- and PGE₂-triggered invasiveness of FET cells (Fig. 5D). These results suggest that PGE₂ synthesis may be necessary for the formation of HRG-induced cell structures on Matrigel and that both PGE₂ and HRG may use the uPA pathway for the formation of luminal three-dimensional structures. These events may be closely linked to the invasiveness of colon cancer cells. Colon cell lines are known to develop a distinct crypt-like architecture when cocultured with fetal rat mesenchyme cells (28) or implanted under the kidney capsules of Swiss nu/nu (nude) mice and allowed to grow for 10 days (29). These models, although very laborious, emphasized the need for a very active cellular stroma that originates from the animal host and leads to different types of glandular structures. Substitution of the stroma with exogenously added HRG in our model enabled us to study, in a more accessible way, distinct multicell architectural modifications, as well as dynamics of different constituents such as cytoskeletal proteins or proteolytic enzymes.

In summary, the results presented here suggest that HRG affects colonic tissue remodeling, at least, in part, by PGE₂ up-regulation. In this model, HRG can induce a more invasive phenotype, probably due to its effects on the uPA/uPAR pathway as well as up-regulation of PGE₂, which can, in turn, also feed into the uPA pathway. Although both HRG and PGE₂ can trigger a series of distinct morphogenetic alterations, PGE₂ was more potent than HRG. It is possible that over a period of time, the HRG-induced PGE₂ may attain a significantly higher level in the conditioned medium. In addition, there may be other unidentified effects of HRG that could partially attenuate PGE₂-stimulated remodeling, and HRG-treated cells may have a greater amount of bound uPA than PGE₂-treated cells. Additional studies are required to address these and other possibilities. There is a regulatory interplay between the stroma-secreted ECM proteins and the epithelial cells that harbor various surface receptors. This interplay may be critical for a dynamic control of cellular movement and cytoskeleton organization. HRG and PGE₂ heavily modulate these events in the...
colon system. The results of our study suggest a model of colon cancer progression in which the binding of HRG to its HER-3 and HER-4 receptors on adjacent tumor epithelial cells induces a series of events, including secretion of PGE₂ and up-regulation of uPA. This new pathway may be integrated into a broad model of how cells function, die, migrate, or differentiate under the influence of HRG and PGE₂ signals. In addition, our three-dimensional model system presented here may potentially be used to screen the effects of therapeutic compounds and biomolecules on different spatial aspects of colon biology, including cell growth, motility, invasion, survival, and apoptosis.

References


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