Eicosanoid Synthesis by Cultured Human Urothelial Cells: Potential Role in Bladder Cancer

Abraham Danon, Terry V. Zenser, David L. Thomasson, and Bernard B. Davis

Geriatric Research, Education, and Clinical Center, Veterans Administration Medical Center, St. Louis, Missouri 63125 [T. V. Z., B. B. D.]; Departments of Biochemistry [T. V. Z., D. L. T.] and Internal Medicine [T. V. Z., B. B. D.]; St. Louis University School of Medicine, St. Louis, Missouri 63104; and Clinical Pharmacology Unit, Ben-Curian University of the Negev, Beer-Sheva, Israel [A. D.]

ABSTRACT

Prostaglandin (PG) H synthase and eicosanoid products of arachidonic acid metabolism have been implicated in several steps in the carcinogenic process. This study assessed these parameters using primary cultures of human urothelial cells. To determine the possible presence of permeability barriers to agonist stimulation, incubations were performed with adherent cells in the presence or absence of thioglycollate pretreatment or with cell suspensions. No evidence for permeability barriers was observed. With adherent cells in the absence of thioglycollate, radioimmunoassayable PGE2 was stimulated by epinephrine < 12-O-tetradecanoylphorbo1-13-acetate = thrombin < bradykinin = A23187 < arachidonic acid. Tumor promoters but not non-tumor promoters stimulated PGE2 synthesis. 1-Oleoyl-2-acetylglycerol which like 12-O-tetradecanoylphorbo1-13-acetate activates protein kinase C also increased PGE2 synthesis. Cells prelabeled with 14C-jArachidonic acid were exposed to agonists and the profile of eicosanoids synthesized was assessed by high performance liquid chromatography. With bradykinin, the pattern of eicosanoids synthesized was 6-keto-PGE1\alpha (12% of total \( {\text{14C}} \) label), thromboxane B2 (0.4%), PGF2\alpha (1.7%), PGE2 (18%), PGD2 (1%), leukotrienes (0.4 to 1%), 12-hydroxy-5,8,10-heptadecatrienoic acid (3%), 15-hydroxy-5,8,11,13-eicosatetraenoic acid (4%), 12-hydroxy-5,8,10,14-eicosatetraenoic acid (0%) and 5-hydroxy-5,8,12,14-eicosatetraenoic acid (2%). Thus, human urothelial cells contain both prostaglandin H synthase and lipoxigenase pathways with the former being more prominent. These pathways may participate in urinary bladder carcinogenesis.

INTRODUCTION

Arachidonic acid metabolism may be involved in several steps in the carcinogenic process (e.g., initiation, promotion, differentiation, and metastases). Peroxidative metabolism of hydroperoxideicosanoids products of arachidonic acid can cause concomitant cooxidation of certain chemical carcinogens (e.g., aromatic amines). These carcinogens become activated and bind macromolecules including DNA. Thus, peroxidative activation of carcinogens by the hydroperoxidase activity of prostaglandin H synthase has been proposed to be involved in the initiation of carcinogenesis by certain chemicals (1, 2). Eicosanoid products of arachidonic acid metabolism by both prostaglandin H synthase and lipoxigenase pathways have been implicated in tumor promotion. This has been demonstrated by the capability of eicosanoid products to modify tumor yield and by inhibition of tumor promotion by putative inhibitors of these pathways (3–5). Tumor promoters such as TPA\(^{+}\) cause a variety of biochemical alterations including increased arachidonic acid metabolism (6, 7). In vivo and in vitro studies have shown that eicosanoid products of arachidonic acid can regulate the rate of proliferation and differentiation of certain mammalian cells (8, 9). In addition, numerous tumors demonstrate increased synthesis of eicosanoids (10). Eicosanoids may also alter host-tumor cell interactions that can affect host immunocompetence, indirectly facilitating tumor growth and spread (11).

Urothelial cells are capable of metabolizing-activating several classes of chemical carcinogens. This has been demonstrated with subcellular and intact cell preparations (12–17). In addition, transformation (18), unscheduled DNA synthesis (19), repair synthesis of DNA (20), and mutagenesis (21, 22) are induced in urothelial cells by bladder carcinogens. Two-stage carcinogenesis, similar to the initiation-promotion model originally described for mouse skin, has been demonstrated for the rat urinary bladder (23–25) and attempts are being made to demonstrate this in a human urothelial cell system (26).

Urothelial cancer is an industrially related cancer and a significant health problem in humans (27–29). The possible involvement of arachidonic acid metabolism in chemically induced bladder cancer in humans is not known. A great deal of information is available describing metabolism of arachidonic acid in nonhuman systems. Those results indicate tissue and species differences in arachidonic acid metabolism. Because most human malignancies of the urinary bladder are carcinomas derived from epithelial cells, it is important to determine the potential of urothelial tissue of human origin for eicosanoid synthesis. Large numbers of human urothelial cells can be obtained from small amounts of human ureter obtained from transplant surgery (30). Accordingly, these studies were initiated to characterize the pattern and regulation of arachidonic acid metabolism by human urothelial cells. Metabolism was characterized by use of a specific radioimmunoassay for PGE2 and by the HPLC elution profile of eicosanoid metabolites of 14C-jArachidonic acid.

MATERIALS AND METHODS

Preparation of Cell Cultures. Sections of human ureters (1 cm or larger) were obtained from the Department of Transplant Surgery, VA Medical Center, St. Louis, MO. Using aseptic procedures, the tissue was placed in a 100-mm plastic Petri dish containing a covering amount of Ham’s Nutrient Mixture F12+. An aliquot was placed in a 100-mm plastic Petri dish containing a covering amount of Hanks’ balanced salt solution and processed as described by Reznikoff et al. (30). A longitudinal cut with scissors was made to expose the lamina propria, which was stripped from the underlying stroma. Freshly detached cells were centrifuged for 5 min at 1000 x \( g \) and resuspended in a small volume of Ham’s Nutrient Mixture F12+. An aliquot was counted with a model Fm Coulter Counter, and viable cells were quantitated by the Pronase-cetrimide method (31). Plates receiving 10\(^4\) cells in 3 ml of media were incubated at 37°C in 5% CO\(_2\)-95% humidified air and fed biweekly.

To provide a substrate to enhance attachment and growth of urothelial cells, plastic tissue culture plates were coated with rat tail tendon...
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Collagen (Sigma Chemical Co., St. Louis, MO) or with 2% gelatin (Fisher Scientific Co.) as described previously (30, 32). The data indicated that there were no differences in morphology or metabolism between urothelial cells grown on gelatin and those grown on collagen substrate (not shown).

Experiments were conducted with 80-90% confluent cultures (3-4 × 10^5 cells) by aspirating growth medium and washing with 2-3 ml of phosphate-buffered saline. To assess the possible presence of permeability barriers to agonist stimulation (33, 34), cells were prepared for analysis by three separate methods. Adherent cells were analyzed with or without a 2-h pretreatment with 0.01 M thiglycolate (33). Cells were also suspended by treatment for 15 min with 0.1% EDTA. Regardless of the method of pretreatment, cells were equilibrated with 1 ml of serum-free medium for 30 min at 37°C in 5% CO2-95% air. A final 30-min incubation was carried out with serum-free medium in the presence or absence of test agents. Test agents used were arachidonic acid purchased from Nu-Chek Prep., Inc., Elysian, MN; calcium ionophore A23187 was from CalBiochem, La Jolla, CA; and bradykinin triacetate, epinephrine reagent, thrombin (2000 NIH units/ml protein), EDTA (disodium salt), indomethacin, platelet-activating factor, epidermal growth factor, anti-inflammatory hormone (Grade VI), sodium thioglycolate (Grade V), mezerein, and TPA and the other phospholipids were from Sigma. Final media were examined for the amount of PGE2 synthesized by radioimmunoassay and, when prelabeled with [3H]-arachidonic acid, for the total eicosanoid synthesis by HPLC.

Preparation of Culture Medium. Ham's Nutrient Mixture F12 powder (GIBCO, Grand Island, NY) was reconstituted for use. This medium was supplemented with 1% fetal calf serum and the following (final concentration in parentheses): insulin (10 μg/ml), hydrocortisone (1 μg/ml), and transferrin (5 μg/ml) which were obtained from Sigma; nonessential amino acids (0.1 mM), l-glutamine (2.0 mM), streptomycin (100 μg/ml), and penicillin (100 U/ml) which were purchased from GIBCO; and dextrose (2.7 mg/ml) from Fisher Scientific (30). Supplemented medium was designated F12+.

Ultrastructural Studies. To prepare urothelial culture cells for transmission electron microscopy, plates with attached cells were rinsed with phosphate-buffered saline and fixed for 3 h with 2% glutaraldehyde in 0.1 M Sorensen's buffer at room temperature. After the cells were fixed, they were rinsed several times with Sorensen's buffer. Caducydate buffer (0.1 M sodium cacodylate containing 3.5% sucrose and 1.0 mM calcium) was used for the final rinse. These fixed cells were postfixed for 1 h with 1% osmium tetroxide buffered with cacodylate buffer. Following dehydration in graded alcohols, cells were infiltrated with a graded mixture of enacrine and 40% propylene oxide and embedded in Araldite. Sections 60-90 nm and doubly stained with uranyl acetate and lead citrate. Cells were studied with a Phillips 200 electron microscope.

Alkaline Phosphatase Staining. This was performed according to standard procedures (35). After the cells were fixed using cold 10% formalin-90% methanol, they were incubated for 30 min with Naphthol AS-MX phosphate (Sigma) in the dark. Fast Blue BB (Sigma) was used as the diazonium salt. Mayer's hematoxylin solution was used as a counterstain. The stained cultures were then examined microscopically. Similarly treated fibroblasts were used as negative controls.

Labeling of Urothelial Cells with [3H]-Arachidonic Acid and Assessment of Eicosanoid Products. Cultures (17-21 days and approximately 80% confluent) were rinsed twice with serum-free medium containing 0.5 μCi [3H]-arachidonic acid (Amersham, Arlington Heights, IL; specific activity, 58 mCi/mmol). Following a 1-h incubation, cells were rinsed twice and allowed to equilibrate for 30 min. Cells were then incubated in fresh media with test agents for 30 min. After EDTA was added (final concentration, 1 mM) to retard degradation of the leukotrienes (36), media were frozen at −20°C. Before chromatography, a mixture of tritiated authentic standards (New England Nuclear, Boston, MA) as indicated in Fig. 4 was added. These standards were made up in 50 mM butylated hydroxyanisole in benzene:ethanol (1:1).

HPLC analysis of eicosanoids was carried out using a Beckman 112 solvent delivery module, a Beckman 421 controller, and a Waters WISP 710B automatic sample injection system. Separation of eicosanoids was affected by reversed phase chromatography, using an Altex ultra-phase-ODS 5-μm (250 × 4.6 mm) column with Rheodyne 2-μm precolumn filter and a Brownlee 5-μm Spheri-5 reverse phase precolumn (30 × 4.6 mm). Three different solvent systems were used at a flow rate of 1 ml/min (modified from system in Ref. 37). Solvent system A, made of 25% acetonitrile, 74.7% water, 0.2% benzene, and 0.1% acetic acid, was used initially to separate the prostaglandins and thromboxane B2 at 39 min, solvent system B, composed of 65% methanol, 34.9% water, and 0.1% acetic acid buffered with ammonium hydroxide to pH 5.2, eluted the hydroxyeicosatetraenoic acids and leukotrienes. From 31 to 115 min, solvent system C, consisting of 90% methanol, 9.98% water, and 0.02% acetic acid, eluted arachidonic acid. The column was washed with 30 ml of 3% EDTA in water at the beginning of each day to improve peak shape and resolution of the leukotrienes (38). Detection was achieved with a Flo-One Beta radioimmunoassay flow detector (Radioimmunostained and Chemical Co., Inc., Tampa, FL). The tritium channel had its discriminator set at 1–35 with 125 cpm as the background subtract and 10 cpm as the threshold settings. For the [14C]-metabolites, the carbon channel had its discriminator set at 35–100 with 75 and 10 cpm as the background subtract and threshold settings, respectively. A dual-label quench-correlated curve allowed determination of the absolute amount of radioactivity. Identification of metabolites was made by comparison with tritiated authentic standards that eluted simultaneously. Data were expressed as percentage of total radioactivity recovered.

Radioimmunoassay of Prostaglandins E2. The PGE2 content of the media was measured by double-antibody radioimmunoassay (39). Rabbit antiserum to PGE2 was obtained from Regis Chemical Co., Morton Grove, IL. Tritium-labeled PGE2 was supplied by New England Nuclear, and goat antiserum to rabbit γ-globulins was from Antibodies Inc., Davis, CA. Each condition was evaluated in two or three separate experiments. In each experiment, conditions were evaluated in at least triplicate plates. The medium from each plate was analyzed in duplicate and the value of duplicate determinations was averaged and considered as N = 1. Data were expressed as mean ± SE of ng PGE2 per 10^5 cells. Statistical differences between data were evaluated by Student's t test for unpaired values.

RESULTS

Cell Identification. Cultured cells were identified as epithelial by their morphological and histochemical characteristics. Cultures grew as continuous sheets of tightly adherent cells. Using phase contrast microscopy, cells observed in dense areas were cuboidal, while those growing out from the explants exhibited a more polygonal appearance and contained 1 to 4 prominent nucleoli. Transmission electron microscopy showed stratified cells containing tight junctions and desmosomes. Cells exhibited strongly positive staining for alkaline phosphatase.

PGE2 Synthesis. Cultured human urothelial cells prepared by three different methods were examined for their ability to synthesize PGE2 (Fig. 1). Those manipulations were made in an effort to overcome a possible permeability barrier at the apical surface of the transitional cells (33, 34). Regardless of how the cells were prepared for analysis, agonist stimulation followed the same general order of responsiveness. That is, TPA < bradykinin = A23187 < arachidonic acid. Both control and stimulated values for PGE2 synthesis were similar for adherent cells treated with thioglycolate and untreated. In contrast, resuspended cells had higher control PGE2 synthesis and a smaller response to agonists. Epinephrine and thrombin were also shown to enhance PGE2 synthesis in adherent cells.

Epinephrine was the weakest agonist tested and thrombin had activity similar to that of TPA. On the other hand, adherent cell PGE2 synthesis was not increased by antidiuretic hormone...
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Fig. 1. Profile of agonist stimulation of PGE2 synthesis. The following agonists were used: 150 nM arachidonic acid (AA); A23187 (0.5 μg/ml); bradykinin (BK) (1 μg/ml); 0.1 μM TPA; 0.1 mM epinephrine (Epi); and thrombin (1 unit/ml). Columns, mean of 6 experiments; bars, SE.

Table 1 Stimulation of human urothelial cell PGE2 synthesis by tumor and non-tumor promoters

<table>
<thead>
<tr>
<th>Condition</th>
<th>Concentration (μM)</th>
<th>PGE2 synthesis (ng/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.1</td>
<td>0.7 ± 0.4*</td>
</tr>
<tr>
<td>12-O-Tetradecanoylphorbol-13-acetate</td>
<td>0.1</td>
<td>5.0 ± 0.4*</td>
</tr>
<tr>
<td>+ Indomethacin</td>
<td>10</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Phorbol-12,13-di- butyrate</td>
<td>0.1</td>
<td>3.8 ± 0.2*</td>
</tr>
<tr>
<td>Phorbol-12,13-didecanoate</td>
<td>0.1</td>
<td>5.3 ± 1.9*</td>
</tr>
<tr>
<td>4a-Phorbol-12,13-didecanoate</td>
<td>0.4</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Mezerein</td>
<td>0.3</td>
<td>4.3 ± 0.3*</td>
</tr>
<tr>
<td>1-Oleoyl-2-acetylglycerol</td>
<td>200</td>
<td>3.5 ± 0.1*</td>
</tr>
</tbody>
</table>

* Mean ± SE.

Fig. 2. Time course for uptake of arachidonic acid by urothelial cells. [1-14C]-Arachidonic acid, 0.5 μCi, was added to cell cultures, and radioactivity was measured in the media at the times indicated.

The major lipoygenase products observed. The synthesis of 12-HETE was at the limit of detection.

DISCUSSION

This is the first report to demonstrate prostaglandin H synthase activity in human urothelial cells. Factors that regulate eicosanoid synthesis and determine the profile of eicosanoid products synthesized by human urothelial cells were also assessed. A variety of test agents were shown to increase synthesis of PGE2. In the presence of those test agents, other prostaglandin H synthase and lipoxigenase products of arachidonic acid were also demonstrated. Specific antagonists (indomethacin and nordihydroguaiaretic acid) of these pathways inhibited synthesis of these products (40, 41). Mechanical manipulation of rabbit bladder epithelium has been shown to increase prostaglandin synthesis (42). This may be the reason for the increased PGE2 synthesis observed in the resuspended control cells. Eicosanoids the synthesis of which was not verified by this study include thromboxane, the leukotrienes, and 12-HETE. HHT synthase was observed and may represent thromboxane synthase activity. However, in most instances, when thromboxane synthesis is observed, similar amounts of both HHT and TXB2 are reported (43). In a recent study with primary cultures of mixed renal cells, a large amount of HHT synthesis was observed with no detectable TXB2. HHT was subsequently shown to be derived from the breakdown of prostaglandin endoperoxides and not by way of thromboxane synthase (44). This distinction was not assessed in the present study.

Epidermal growth factor (45), platelet-activating factor (46), and antiinflammatory agents (47) which stimulate eicosanoid synthesis in other cells were not stimulatory. Thus, certain stimuli including hormonal, pharmacological, and mechanical can induce human urothelial cells to synthesize a wide range of eicosanoid products known to affect the carcinogenic process.
Prostaglandin synthesis is one of the pleiotropic biochemical responses to tumor promoters (6, 7, 48). With human urothelial cells, PGE₂ synthesis was observed with tumor-promoting, but not non-tumor-promoting phorbol esters. Mezerein, a related diterpene that is a weak complete promoter but a strong second stage promoter (49), also stimulated urothelial cell prostaglandin synthesis. Phorbol esters have been shown to increase synthesis of prostaglandin H synthase but not lipoxygenase products (40, 50). Certain actions of these tumor promoters are thought to be mediated by activation of protein kinase C. In fact, both TPA and mezerein can substitute for diacylglycerol generation. This was probably because this activity was not the focus of the studies. For example, when intact rabbit bladder was studied, only radioimmunoassayable PGE₂ was assessed (60). In addition, dog and rabbit microsomes were shown to make almost exclusively PGE₂ with lipoxygenase products not observed (17, 60). This was because the assay conditions were optimized for PGE₂ synthesis (e.g., high concentration of glutathione) and because lipoxygenases are soluble rather than microsomal enzymes. The present study prelabeled the urothelial cell arachidonic acid pool and thus was able to demonstrate both 15-HETE and 5-HETE lipoxygenase activities. The lipoxygenase responsible for 5-HETE synthesis, an essential component of the leukotriene pathway (61), was at the limit of detection in urothelial cells.

The physiological or pathophysiological roles of the various eicosanoids shown to be synthesized by urothelial cells are not known. Eicosanoids may play a role in the tone and contraction of the urinary tract smooth muscle, as suggested by the effects of exogenous prostaglandins on the one hand and of prostaglandin synthesis inhibitors on the other (62–65). Inotropic effects of leukotrienes have been reported with isolated urinary bladders of rat and guinea pig (66). Prostaglandins are thought to be released during stimulation of excitatory nerves to the detrusor (67, 68) or when the bladder is distended (69–71). Thus, eicosanoids that are synthesized by urothelial cells may contribute to bladder tone and contraction and they could be involved in metabolic or physiological functions of the urothelium itself.

Eicosanoids may play a role in bladder carcinogenesis. Prostaglandin H synthase activation of aromatic amines has been proposed to be involved in the initiation of bladder cancer. This is supported by in vitro (described above) and in vivo experiments. In the latter, aspirin was coadministered with the bladder carcinogen FANFT for 12 weeks to reduce the incidence of both bladder lesions and tumors (58, 72). A more recent study has used the two-stage bladder carcinogenesis model to specifically assess effects of aspirin on the initiation and promotion stages of FANFT-induced bladder cancer. Aspirin was shown to affect both initiation and promotion stages (73). In other two-stage models of carcinogenesis, such as the mouse skin model, inhibition of prostaglandin synthesis has been found to be related to inhibition of promotion by a variety of agents, in particular, the phorbol esters (5). This inhibition can be overcome by addition of specific prostaglandins (3, 4). Human urothelial cells contain TPA receptors (26). These receptors may participate in TPA-mediated increases in eicosanoid synthesis. Eicosanoids often exert opposing influences on physio-
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logical and pathological parameters. The hypothesis that different eicosanoids may exert opposing influences on tumor growth and metastasis (74) may serve to explain some of the varied reports (75).

ACKNOWLEDGMENTS

The authors appreciate the expert technical assistance of Mark Palmer in conducting these experiments and of Sandy Melliare in preparation of the manuscript.

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